ROOT MORPHOLOGICAL AND PHYSIOLOGICAL BASES TO UNDERSTAND GENOTYPIC CONTROL OF MINERAL ACQUISITION IN RICE GRAINS

A Dissertation

by

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ABSTRACT

Rice (*Oryza sativa* L.) supports half of the human population. However, predominant rice consumption leads to malnutrition due to mineral deficiencies. The research goal was to support identification of genes responsible for the uptake/accumulation of potassium (K), iron (Fe), zinc (Zn) and molybdenum (Mo), thus promoting the breeding for rice with high grain concentrations of these elements. Prior studies identified rice genotypes with high grain-K, -Fe, -Zn or -Mo concentrations that were hypothesized to be due to differences in root traits. The research objective was to identify root traits associated with these elements. These traits could be bases for identifying genes. The first study determined if these genotypes showed similar accumulation patterns in leaves as in grains, which would hint at influences of the roots and enable identifying distinct root traits and possible genes in vegetative growth stages. The second study determined if root traits of high grain-Mo genotypes displayed an acid-tolerance mechanism as these genotypes originated from Malaysia where acidic soils strongly adsorb Mo making it unavailable for plants. The third study identified root trait differences of high grain-K, -Fe, -Zn and -Mo genotypes in hydroponics media, while the fourth determined root trait differences in these genotypes in sand-culture media including a 1-Naphthalene Acetic Acid (NAA) seed treatment for perturbation.

The first study identified several high grain-Mo genotypes with similar Mo accumulation patterns in V4 to V6 stage-leaves as in grains, suggestive of a root influence. The second study established that gross morphological and physiological root traits of a high grain-Mo genotype were not part of an acid-tolerance mechanism. Neither the third nor fourth study identified root traits related to shoot K, Fe, Zn or Mo concentration, however positive associations of seedling vigor traits with several beneficial elements, including K, and negative associations with numerous toxic elements were established. Lack of correlation with root traits suggests other mechanisms (e.g. active uptake transporters) instead control the observed grain accumulation differences. Based on the fourth study, either direct effects of NAA on element uptake/transfer or indirect effects on soil pH and redox potential altered tissue Fe and Zn levels.

DEDICATION

I would like to dedicate this book to my very best friend and husband, Sree, for his patience and love.

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NOMENCLATURE

As	Arsenic
Ca	Calcium
Cd	Cadmium
Cu	Copper
Fe	Iron
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
K	Potassium
Mn	Manganese
Mo	Molybdenum
Mg	Magnesium
Na	Sodium
NAA	Naphthalene Acetic Acid
Ni	Nickel
PGR	Plant Growth Regulator
P	Phosphorus
Rb	Rubidium
S	Sulfur
Sr	Strontium
Zn	Zinc

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

GENERAL

Rice (*Oryza sativa* L.) is the only grain crop grown nearly exclusively to feed human beings (Dethloff, 2002). It is a staple food and the chief source of nutrition in developing countries of South America, Asia and Africa (Chang, 2002). The major cause of mineral nutrient malnutrition is the predominant consumption of plant-based food with inadequate levels of mineral nutrients (Welch and Graham, 2004). The consumption of excessive amounts of detrimental minerals can adversely affect human health. In addition, the acquisition of beneficial nutrients and the exclusion of detrimental mineral elements are critical for rice crop health. The first step towards mineral element acquisition in plants is the efficient absorption/uptake of minerals from the soil by roots (Fageria and Stone, 2006). Root physiological activities modify physical (soil temperature), chemical (soil pH, redox potential, root exudates, allelochemicals, soil nutrient concentrations) and biological (microbial associations) properties of the rhizosphere, which greatly impact the availability, solubility and mobility of minerals in the soil (Clarkson, 1985; Fageria and Stone, 2006). Root morphology (root length, root hairs, root biomass) influences mineral absorption/uptake from soil into the roots (Atkinson, 1990; Chen *et al.*, 2009; Clark, 1990). Following absorption/uptake into the root cells, minerals

undergo long distance transport from roots to leaves through xylem and phloem (Mengel and Kirkby, 2001). Mineral movement is regulated by transpirational pull, electrochemical potential gradient or by gene-controlled ion pumps and channels (Baxter, 2009; Mengel and Kirkby, 2001). Minerals are utilized as nutrients in various physiological and biochemical reactions towards plant growth and development, or are sequestered in various plant tissues. Subsequent mineral translocation from xylem to phloem results in varying accumulation patterns different from the uptake patterns.

Plant physiology with emphasis on root system

Rice roots consist of three types of roots (Moldenhauer and Gibbons, 2002): radicle or seminal roots (from cotyledon), mesocotyl roots (from mesocotyl region, occur rarely) and nodal or adventitious or crown roots. Crown roots are formed following submergence and are stimulated in flooded conditions (Mergemann and Sauter, 2000). The rice shoot consists of developmental shoot units or phytomers. Each phytomer possesses a leaf, a node to which the leaf is attached, a tiller node and two rings of crown nodes, one above the leaf node (“upper” root) and the other below the tiller node (“lower” root). Typically, the root and shoot growth are synchronized such that when the leaf blade emerges from the N^{th} phytomer, crown roots (from both “upper” and “lower” nodes) emerge from the N-three phytomer (Hoshikawa, 1989) . The same principle also applies for lateral roots (Fujii, 1961). First-order

lateral roots emerge when the leaf emerges from the third phytomer, second-order lateral roots emerges when the leaf emerges from the fourth phytomer, and so on. Roots grow thicker, longer and wider as they mature (Counce *et al.*, 2000). The total root length increases with time and undergoes branching and re-branching up to the sixth order with progressive reduction of root diameter. Root hair formation is positively influenced in aerobic conditions and negatively influenced in flooded conditions (Tarafder, 1997). Under flooded conditions, root growth is rapid during vegetative stages and root length is at a maximum during the panicle initiation stage (Beyrouty *et al.*, 1988). Following panicle initiation, a highly interwoven, fibrous root mat forms at the soil surface and is thought to be oxygen scavenging/respiratory in function (Alberda, 1953). Root weight increases with increase in length and number of roots. Root color changes with age and is milky white in color, which successively turns yellow, pale brown, brown and dark brown (Yoshida, 1981).

Rice roots in flooded conditions undergo morphological and physiological adaptation by forming aerenchymal spaces (air passage) connecting the leaves to roots that facilitate oxygen transport from above ground shoots (Counce *et al.*, 2002; Kirk, 1994). These structures are formed through programmed cell death of radial files in the root cortex, making way for functional, large intercellular spaces (Kawai *et al.*, 1998). Aerenchyma is also present in nodes and internodes and aids in oxygen transport when leaves die out (Counce *et al.*, 2002). Under flooded conditions, there are three main reactions involving roots

that alter soil pH (Kirk, 1994). Firstly, oxygen from roots converts ferrous ion to ferric ion forming ferric oxide plaque around the roots and releasing protons into the soil. Secondly, roots tend to take up more cations than anions and this difference is balanced by releasing protons into the soil. Thirdly, high pressures of carbon dioxide arise in anaerobic soil and in roots. Roots tend to either take up or release carbon dioxide and thus, alter pH.

Additional effects involving root release of compounds indirectly influence the soil environment around the root, thus influencing rice root uptake of minerals. Oxygen diffusing from roots is capable of oxidizing pigments like α – naphthylamine (Matsunaka, 1960). The root's ability to oxidize α -naphthylamine is correlated with respiratory rate (Ota, 1970). Rice roots exude organic acids and carbohydrates that along with autolysed root parts trigger methane production in flooded conditions (Aulakh *et al.*, 2001; Bacilio-Jiménez *et al.*, 2003; Mitra *et al.*, 2005). In aerated soil, root exudations can increase water infiltration and water-holding capacity (Fageria and Stone, 2006; Gupta *et al.*, 1977). Thus, exudations can result in cooler soils and can act as a buffer preventing sudden temperature fluctuations. Allelochemicals like phenolic acids, coumarins, aliphatic acids, terpenoids, lactones, tannins, flavonoids, alkaloids, cyanogenic glycosides, and glucosinolates sometimes cause autotoxicity, which can lead to significant yield reductions in monoculture systems (Fageria and Baligar, 2003). Roots also exude compounds that can act to chelate specific minerals, thus altering their potential for root uptake (Fageria and Baligar, 2003).

Plant physiology with emphasis on mineral acquisition

Genotypic variations in mineral uptake occur primarily due to variations in mineral uptake mechanisms from soil solution into roots (Gerloff and Gabelman, 1983). Except for carbon, hydrogen, and oxygen, plants obtain minerals from the soil solution. Although minerals exist both in organic and inorganic forms in soil solution, plants usually take up the inorganic form (ionic state) (Glass, 1990). Potassium (K), iron (Fe), and zinc (Zn) exist in four basic forms, namely solution, adsorbed or exchangeable, organic complexed, and in primary or secondary minerals (Norman *et al.*, 2002). Molybdenum (Mo) also exists as four forms, namely in solution, adsorbed or exchangeable, organic complexed, or as oxides (Reddy *et al.*, 1997). All these forms exist in equilibrium with the solution form. Minerals reach the root surface primarily through diffusion (e.g., K) and mass flow (through transpirational pull) (Epstein and Bloom, 2004).The availability of minerals in the soil solution differs in aerated and flooded conditions (Patrick *et al.*, 1985). Flooded soils undergo chemical reduction resulting in decreased redox potential ranging from +0.2 and -0.3 V, and typically increased pH, which increases the solubility and availability of K^+ and Fe^{2+} in soil (De Datta, 1987). Through reduction reactions, Fe^{2+} is brought into the solution (Moore, 1989; Patrick *et al.*, 1985). The Fe^{2+} displaces exchangeable K^+ ions from the soil exchange complex into the soil solution. The Zn^{2+} concentrations may increase or decrease after flooding depending on soil pH (Norman *et al.*, 2002). With every one unit increase in pH, Zn availability decreases by 100 -fold (Patrick *et*

al., 1985). Molybdenum becomes more available with increase in pH (Mengel and Kirkby, 2001). As pH falls, Mo is strongly adsorbed by soil particles, thus making it unavailable to plants.

Apart from mass flow and diffusion, minerals reach roots through root interception (growing of roots into the site of mineral location) (Oliver and Barber, 1966). Absorption of minerals through root interception is greatly influenced by root morphology (Atkinson, 1990). Increased root number, root length, root hair, root diameter, root weight and increased root-to-shoot ratio enhance mineral absorption (Atkinson, 1990; Chen *et al.*, 2009; Clark, 1990; Zheng *et al.*, 2000). In addition, root physiological processes alter physical, chemical and biological aspects of soil and thus impact mineral solubility, availability and mobility in soil (Fageria and Stone, 2006). Soil physical properties like temperature influence the availability of water-soluble K^+ concentrations in the soil (Ponnamperuma, 1977); chemical properties such as soil pH and temperature impact the availability of minerals like Zn (Patrick *et al.*, 1985). Root respiration and oxygen diffusion modify rhizosphere redox potential, which influences mineral absorption at the root soil interphase (Bloom *et al.*, 1992). Root exudates are rich in minerals and thereby stimulate and harbor symbiotic microorganisms that affect the mobilization of other minerals (Bacilio-Jimenez *et al.*, 2001; Bacilio-Jiménez *et al.*, 2003). Production of allelochemicals like phenolic acid is considered as a defense mechanism in response to low soil fertility (low nitrogen). Low soil fertility triggers the synthesis of allelochemicals

that are secreted as root exudates. Some of these can help solubilize and mobilize minerals in the soil (Fageria and Baligar, 2003).

Absorption of minerals varies with crop stage (Norman *et al.*, 2002). Potassium uptake is rapid during the tillering stage (Slaton *et al.*, 2004). Maximum K uptake occurs from 1 to 5 weeks after flooding, before panicle differentiation (Slaton *et al.*, 2004). Maximum absorption of Fe occurs 10 days after tillering (Grist, 1965).

Uptake mechanisms of minerals in rice

Potassium (K) uptake

Two distinct K transporters, namely “high affinity” and “low affinity” transporters, mediate K influx into the root cells (Epstein and Bloom, 2004). “High affinity” transporters include KT/HAK/KUP (K+/H+ symporters), HKT/Trk (K^+/ Na^+ co-transporters) and CHX (cation-H+ exchangers (Gierth and Mäser, 2007). The K is taken up by root epidermal and cortical cells (Karley and White, 2009). Members of CPA2 (Cation Proton Antiporter) like AtCHX17/AtCHX20 (CHX: Cation H^+ exchangers), AtNHX5/AtNHX5 (NHX: Na^+ H^+ exchangers) and AtCCX4 (CCX: Calcium Cation exchangers) mediate K^+ influx into the root cells (primarily held in vacuoles) (Gierth and Mäser, 2007; Maser *et al.*, 2002; Morris *et al.*, 2008). The K^+ is symplastically transferred through endodermal cells into the stele (Karley and White, 2009). Release of K^+ from vacuoles is accomplished by TPK (Tandem-Pore K+ Channels) or KCO (K^+ Channel Outward Rectifier)

(Lebaudy *et al.*, 2007). From the stele, K is loaded into the xylem which is thought to be done by the Shaker-type channel, SKOR (Shaker type K^+ Outward Rectifier Channel), and in the steler parenchyma in *Arabidopsis* (Johansson *et al.*, 2006; Karley and White, 2009). From xylem, the K^+ is removed by parenchyma cells and symplastically distributed to mesophyll cells (Karley and White, 2009). The K^+ transport in shoots occurs largely by transpirational pull. Although not much is known about phloem loading or unloading in rice, in *Arabidopsis*, AtAKT2/AKT3 (protein transporters) in phloem companion cells load K^+ into phloem allowing transport to the seed (Deeken *et al.*, 2002).

Iron (Fe) uptake

Iron uptake occurs either through strategy I or II (Charlson and Shoemaker, 2006) . Strategy I (for e.g., *Arabidopsis*) involves reduction of Fe^{3+} to Fe^{2+} by FRO (Ferric Reductase Oxidase) and uptake of Fe^{2+} ions (Mukherjee *et al.*, 2006). During this process, dicots and non-graminaceous plants undergo morphological modifications like enhanced lateral root development and increase in the rate of physiological processes like acidification of the rhizosphere (Ghandilyan *et al.*, 2006). Strategy II physiology involves synthesis and secretion of phytosiderophores (PS; low molecular weight, non-protein amino acids that form soluble complexes with minerals) that chelate Fe^{3+} from soils. Rice uses strategy II, but it possesses the $Fe2^+$ transporter, *Os*IRT1 in the epidermis, exodermis and cortex of roots (Bughio *et al.*, 2002; Ishimaru *et al.*,

2006). Thus, rice plants can absorb PS-Fe^{3+} complexes and Fe^{2+} ions directly from soil. The PS synthetic pathway components and genes are shown in page 117, Fig. 1 (Römheld and Marschner, 1990; Shojima *et al.*, 1990). Rice is reported to have NA (nicotinamine) and DMA (deoxymugenic acid) and genes *Os*NAS (nicotinamine synthase) and *Os*NAAT (nicotinamine aminotransferease) in roots (Fushiya *et al.*, 1982; Inoue *et al.*, 2008; Kobayashi *et al.*, 2001). The gene *IDS3* catalyzing DMA to MA in barley is not reported in rice, but a homologue *Os*YS1 (Yellow Stripe 1) is reported for the same. It is essential for Fe^{3+} transport in phloem (Inoue *et al.*, 2009; Nakanishi *et al.*, 2000; Negishi *et al.*, 2002).

Proteins that load Fe (thought to be Fe^{2+}) into xylem are not yet identified (White and Broadley, 2009). The *Arabidopsis* FRD3 (Ferric Reductase Defective3) protein present in the root pericycle loads Fe into the xylem and transports it from roots to shoots (Durrett *et al.*, 2007). In rice, ZIP (ZIP: ZRT/IRT-like Protein1, Zinc-Regulated Transporter/Iron-Regulated Transporter) family members are responsible for Fe uptake into shoot cells (Ishimaru *et al.*, 2005). The NRAMP3 (Natural Resistance-Associated Macrophage Protein3) and NRAMP4 are responsible for Fe^{2+} transport out of the vacuole (Grotz and Guerinot, 2006). In *Arabidopsis*, the loading of Fe^{2+} - nicotinamine (Fe^{2+}-NA) complex from phloem into developing seeds is done by YSL (Yellow Stripe Like) and OPT (Oligo Peptide Transporter) proteins (Koike *et al.*, 2004; Stacey *et al.*, 2008). In *Arabidopsis*, VIT1 (Vacuolar Iron Transporter 1) plays an important role

in Fe homeostasis sequestering Fe in vacuoles (Kim *et al.*, 2006). Although it is found in low levels in all parts of the plant, it is more highly expressed in cotyledons of seed embryo. In rice grains, iron is mainly associated with the aleurone layer and hence, VIT1 may play a similar role in rice as well.

Zinc (Zn) uptake

The first step towards Zn uptake involves desorption or dissolution of Zn from soil solid to soil solution (Arnold *et al.*, 2010). Once in the soil solution, Zn may either adsorb onto root surfaces (if soil grown) or onto iron oxide plaque (if grown under flooded conditions), or form a complex with PS (DMA in rice). Other than adsorption or forming a PS complex, Zn may directly diffuse through soil solution into the roots (Arnold *et al.*, 2010). Roots take in either Zn^{2+} ions or PS-Zn^{2+} complexes giving rise to Strategy I and Strategy II plants, the same as for Fe. The Zn^{2+} uptake into the roots in Strategy I plants like *Arabidopsis* and barley (*Hordeum vulgare*) is mediated by transporter proteins like AtZIP1; uptake of the Zn^{2+}-phytosiderophore complex in strategy II plants is done by YSL proteins (Ishimaru *et al.*, 2005; Suzuki *et al.*, 2006). The Zn^{2+} moves from roots to xylem mostly through symplasm and occasionally through apoplasm (Broadley *et al.*, 2007). Although not documented in rice, Zn^{2+} is transported into the vacuole by a Zn^{2+}/H^{+} antiport mechanism and is transported out of the vacuole by NRAMPs in *Thalaspi caerulescens* and *Arabidopsis halleri* (Broadley *et al.*, 2007; Thomine *et al.*, 2003). In rice and *A. thalliana*, ZIP and YSL proteins

mediate Zn^{2+} movement from xylem to phloem and redistribution within the plant (Ishimaru *et al.*, 2005; Waters and Grusak, 2008).

Molybdenum (Mo) uptake

Plants take up Mo as molybdate anions (MoO_4^-) (Mengel and Kirkby, 2001). In *Arabidopsis*, Mo content at the whole-plant level is regulated by the mitochondrial molybdenum transporter *MOT1* (Wang *et al.*, 2008). It is a high-affinity molybdate transporter belonging to the sulphate transporter superfamily. Although it is strongly expressed in root cells and localized in mitochondrial membranes, it is found throughout the plant and is believed to be crucial for efficient uptake of molybdate ions into the cells (Römheld, 2001). Once inside the cells, molybdenum is inert and is not biologically active until it forms complexes with a pterin compound forming a molybdenum cofactor (moco) (Hansch and Mendel, 2009). This pterin compound is unique and is called molybdopterin (MPT) or metal-containing pterin. (Yan *et al.*, 2007). Apart from moco or MPT, Mo can also complex with anthocyanin or malic acid (Gupta and Chauduri, 1978). A moco-carrier-protein (MCP) is documented in *Chlamydomonas,* but no homologues have been found in higher plants (Salt *et al.*, 2008). Moco is either used by Mo-enzymes for biological activities or used as a protection mechanism against oxidation of MPT (Gupta and Chauduri, 1978).The form or the process by which Mo efflux occurs out of the cell is not known yet.

The main goal of this research was to support the identification of genes that control uptake and accumulation of four elements- K, Fe, Zn and Mo, in rice grains. Prior studies identified genotypes with extreme grain-K, Fe, Zn and Mo concentrations which were hypothesized to occur due to differences in root morphological and physiological traits. The objective of this research was to evaluate if root morphological and physiological traits influenced uptake and accumulation of these elements in genotypes selected for producing grains with extremely high concentrations of K, Fe, Zn or Mo. This holistic approach connecting physiology, morphology, and genotype will improve the knowledge of mineral uptake by rice grains and of nutrient use efficiency of plants. Physiological processes are strongly interlinked with genotype and are constrained by morphology (Counce *et al.*, 2002). The increased knowledge of genes and their functions involved in determining mineral content in rice grains will help identify mechanisms for alleviating mineral malnutrition in humans and animals and advancing rice plant nutrition.

The increased knowledge of genes, their functions, and how they operate in concert to determine mineral concentrations in rice grains will help identify mechanisms for alleviating mineral malnutrition in humans and animals and advancing rice plant nutrition.

1. Grain versus leaf element concentrations – The hypothesis was that these genotypes showed similar element accumulation patterns in their leaves as that in the grains which was later carried into grains. The objective of this

study was to investigate if differences in grain-K, -Fe, -Zn or -Mo accumulation patterns in these select genotypes were also present in their leaves at any particular range of vegetative growth stages. This would suggest the root's influence if similar accumulation patterns of these elements existed in different above-ground plant organs. Also, it would help identify root trait differences, and the genes and alleles associated with these element concentration differences, through screening at a vegetative stage instead of requiring that plants be grown to grain maturity before analyzing for element concentration differences.

2. Root traits of high grain-Mo genotypes and acid tolerance – Most of the genotypes selected for high grain-Mo originated from Malaysia, or nearby Brunei, characterized by acidic soils where Mo is strongly adsorbed to soil and not readily available to plants. The objective of this study was to identify if a representative Malaysian genotype showed a root-localized acid-tolerance mechanism which thus enabled it to efficiently mine Mo under acidic conditions.

3. Root traits of high grain-K, -Fe, -Zn and -Mo genotypes grown in hydroponics – The grain accumulation differences in high grain-K, -Fe, -Zn and -Mo genotypes were hypothesized to be due to differences in root traits. The objective of this study was to analyze gross morphological and physiological traits of roots of these genotypes and to establish if root traits were associated with the grain accumulation of these elements.

4. Root traits of high grain-K, -Fe, -Zn and -Mo genotypes grown in sand culture coupled with NAA seed treatments – Similar to study 3, the differences in grain

accumulation patterns in these genotypes were hypothesized to be due to differences in gross morphological and physiological traits of roots. The NAA seed treatments were provided to perturb root development and thus help better identify relevant root trait differences. The objective was to analyze root traits of control and NAA-treated plants of these genotypes then to identify root trait associations with the concentrations of these elements among and between control and NAA-treated plants.

CHAPTER II

LEAF ELEMENT CONCENTRATIONS IN RICE CAN BE USED TO ACCELERATE IMPROVEMENT OF GRAIN NUTRITIONAL QUALITY AND GENE DISCOVERY

INTRODUCTION

Rice is currently the preferred source of caloric supply in many of the developing countries of South America, Asia and Africa (Chang, 2002), thus the improvement of the nutritional quality of rice is an important aspect of global food security. Rice is used as a model crop for genomics research because of synteny among cereal crops (Gale and Devos, 1998; Mayer *et al.*, 2011), thus the identification of genes, mechanisms and methods for the improvement of rice nutritional quality will also be of value in improving the nutritional quality of other cereal crops.

The elemental composition of the rice grain is influenced by several soil and plant variables (Epstein and Bloom, 2004). In soil, elements are present as adsorbed or exchangeable, within organic complexes (generally bound and unavailable), and as primary or secondary minerals (Norman *et al.*, 2002). The soil redox potential and pH have strong influences on solubility of elements (Fageria and Stone, 2006). Elements displace into the soil solution and reach roots through mass flow and diffusion. Once inside roots, elements undergo long-distance transport through xylem or phloem and move from roots to leaves

or other plant parts (Mengel and Kirkby, 2001). Element uptake is regulated by transpirational pull, electrochemical potential gradient or by genetically controlled ion pumps and channels (Baxter, 2009; Mengel and Kirkby, 2001). Plants utilize elements for various physiological and biochemical reactions towards plant growth and development, or sequester them in various plant tissues. Sequestration of elements in different plant parts depends largely on the plant growth and developmental stages and may be permanent or include redistribution to a different plant organ causing change in element composition (Birsin *et al.*, 2010; Hocking, 1994; Wu *et al.*, 2010). Since root uptake and root-to-shoot transfer are early steps necessary for grain accumulation of all elements drawn from the soil, one can anticipate instances where genetic differences in grain concentration of one or several elements might also show as concentration differences in other vegetative organs as well, such as leaves. For example, micrografting studies in *Arabidopsis thaliana* showed that root transport processes control whole shoot accumulation for elements such as Mo, Ca, Na, Cd and P (Bari *et al.*, 2006; Baxter *et al.*, 2009; Baxter *et al.*, 2008; Chao *et al.*, 2011; Chao *et al.*, 2012; Morrissey *et al.*, 2009). In cases where strong leaf- and grain-concentration associations are found to exist, screening of vegetative-stage leaves could be used as a surrogate for the much more laborious and resource-consuming process of screening grain, and so assist in meeting goals of benefiting research aimed at identifying mechanisms and

genes controlling element concentrations in grain and ultimately improving the nutritional quality of cereal crops.

The purpose of the present study was to identify a range of vegetative stages in which leaf element concentrations could expose element-uptake or leaf-accumulation differences that carry through to grain-accumulation differences, The first stage of a larger study aimed at identifying genes affecting rice grain element composition involved the evaluation of grain concentration of multiple elements among a set of 1640 diverse rice genotypes to identify those with extreme grain compositions (Pinson *et al.*, 2010). The current study used a subset of lines selected as having high grain concentrations of specific elements to investigate if the element composition in leaves during any of the vegetative growth stages was strongly associated with that of the grains. The specific objectives of this study were to 1) identify the youngest potential range of vegetative growth stages for comparing leaf and grain element concentrations, 2) determine the viability of the youngest potential range of growth stages for ability to indicate grain differences using cobalt (Co) and molybdenum (Mo), known to show root-to-whole shoot concentration associations in *Arabidopsis, (Baxter et al., 2008; Morrissey et al., 2009)* and 3) evaluate 15 additional elements to identify those for which vegetative-stage leaf concentrations are found to be indicative of grain concentrations.

MATERIALS AND METHODS

This outdoor-potted plant study was conducted in the summer of 2010 at the Texas A&M AgriLife Research Center (Beaumont Center), Texas, USA.

Plant materials

Thirty-nine rice genotypes with extreme grain element composition were selected based on a previous field experiment (Pinson *et al.*, 2010). The previous experiment consisted of four replications of 1640 rice (*Oryza sativa* and *O. glaberimma)* genotypes from the USDA rice core collection representing rice germplasm originating from 114 countries around the world (Pinson *et al.*, 2010; Yan *et al.*, 2010; Yan *et al.*, 2007) that were grown under both flooded and unflooded field conditions at the Beaumont Center. The 39 rice genotypes were identified as having exceptionally high grain concentration (Z-scores ≥ 2.5) of one or more particular elements. Genotypes identified from Z-scores as being exceptionally high in one particular element were often relatively high (i.e. Z-scores from 2.0 to 2.5) for some other elements as well. Apart from the thirty-nine genotypes, 'Lemont' (http://www.gramene.org/newsletters/varieties/Lemont.html), was added to the study to represent US rice germplasm. The variety, Lemont, is popular among rice researchers for the development of rice mapping populations, and all 39 genotypes have been crossed with Lemont to get F_2 segregating progenies with the main intention of identifying genes responsible for particular element

accumulation. To classify the 39 genotypes in the present study as high or not for each element, the data from the previous study of 1640 genotypes was evaluated anew, this time using a ranking scheme. Each flooded or unflooded replication in the prior study was ranked from 1 to 1640 for highest to lowest grain concentration of each of the 16 elements. Then, to account for the fact that various genotypes were missing data for one or two replications per field condition (flooded or unflooded), a weighted average ranking was calculated for each field condition. A genotype was considered to be “high” in concentration for a particular element when it had a weighted average ranking ≤ 200. Conversely, the two genotypes having the lowest rankings among these 40 genotypes were considered to be “low” in concentration for a particular element. Rankings were done separately for flooded versus unflooded conditions, which resulted in two different sets of genotypes representing “high” or “low” grain concentration for the same element in the present study. All 39 genotypes were selected for their high grain concentrations of one or more elements. The top five (if available) high genotypes and two low genotypes were used for initial evaluations including to determine if the proposed approach also complements in the low direction. Low genotypes were used for the first two objectives in identifying and evaluating the suitable growth stages, but were not used for the third objective and were not expected to show associated low mineral concentrations in their leaves.

Plant growth conditions

The set of 40 genotypes was planted repeatedly at 7 to 10 day intervals to provide plants with a wide range of vegetative growth stages from which to obtain leaf samples on a single sampling date. Plants were grown in square pots (10 cm x 10 cm x 12 cm) filled 10-cm deep with Beaumont field soil (page 118, Fig. 2). The soil is characterized as League clay, a fine, smectitic, hypothermic Oxyaquic Dystrudert (USDA, 1999). Each planting set consisted of a single pot per genotype containing five seeds covered by 2.5-cm of soil with 2.5-cm spacing between seeds. With imperfect germination, each set provided one to five plants per genotype for leaf sampling. These pots were housed in wooden boxes (1 m x 1 m) lined with black plastic sheeting. Plants were irrigated regularly from below by supplying water to the boxes. Water level was maintained at a 2.5-cm depth in the box throughout the experiment to keep the soil moist, but with sufficient aeration to prevent development of reduced soil redox and so mimic the unflooded field conditions used in the preliminary field experiments (Pinson *et al.*, 2010). Plants were fertilized weekly from above, while avoiding vegetation, with Peters Professional, 20:20:20 N:P:K (United Industries, Alpharetta, GA, USA) at a rate of 3.72g/L.

Leaf sampling

The main intent of the present study was to evaluate element concentrations in vegetative growth stages (page 119, Fig. 3), prior to

translocation of nutrients from leaves to reproductive organs and grains. On the sampling date, fully or partially emerged flag leaves were observed on less than one-fifth of the plants in the eldest set. Plants less than two-weeks old were not sampled as they did not provide sufficient leaf material for analysis. And indeed, most but not all of the 3-week old plants had developed sufficient leaf material for sampling and analysis, In this manner, leaves from plants ranging from V2 to V10 growth stages, i.e. plants with 2 to 10 leaves on the main culm, as defined by Counce et al. (2000) (Counce *et al.*, 2000) were available for sampling on a single sampling date using seven different planting sets. The date of planting, number of plants in each pot, and the growth stage of each plant in terms of leaf number (Counce *et al.*, 2000) were recorded at the time of leaf sampling. The growth stage of each plant was recorded as the number of fully opened leaves on the main culm followed by a decimal representing the percentage of the newest leaf emerged (e.g. V3.2 indicates three fully opened leaves plus approximately 20% of the fourth leaf emerged). Approximately 5-cm lengths of leaf tips were sampled from the most recently fully expanded leaf of the main culm resulting in at least 50 mg dry weight of plant tissue for ICP-MS (Inductively Coupled Plasma Mass Spectrometry) elemental analysis (Salt *et al.*, 2008). All leaves were sampled by hand, thus avoiding potential contamination by metal shavings from use of metal scissors or knives. Samples (and fingertips) were rinsed by dipping them into reverse-osmosis water and then placing them into 2-ml polypropylene micro-centrifuge tubes which were capped, placed on ice,

and shipped overnight to the ICP-MS laboratory at Purdue University (West Lafayette, Indiana, USA) where they were freeze dried, ground, and analyzed for 17 elements, namely As, Ca, Cd, Co, Cu, Fe, K, Mn, Mo, Mg, Ni, P, Rb, S, Sr, Zn, and Na as described by (Danku *et al.*, 2012).

Data analysis

For each element, Lemont, the five genotypes selected as high, and the two genotypes selected as low were graphed for their leaf element concentrations against vegetative stages, analyzing the genotypes selected for extreme grain phenotype from flooded field conditions separately from the genotypes selected under unflooded field conditions. This was done to determine if any genotypes consistently showed high and low leaf element concentrations across a range of growth stages. For better understanding of the element patterns across growth stages when addressing Objective 1, best-fit curves were made using hyperbolic fits. Once suitable growth stages were identified, the concentrations across the suitable growth stages were averaged to get overall leaf mineral concentrations for each genotype. When addressing the third objective, leaf data for all plants of each genotype collected between the V4 to V6 leaf stages were combined to provide an overall mean and standard error per genotype to use for comparing the leaf-concentrations between genotypes. All of the lines in this study had been selected for high grain concentration of one or a few specific elements. For each specific element, this

resulted in the retention of 2 to 8 genotypes selected for high grain-concentration that could be compared to all the other genotypes that had not been ranked high for that specific element. In this manner, we compared 2 to 8 “selected” versus a set of 24 to 34 “unselected” genotypes for each element. The unselected genotypes did not contain any genotype selected for either flooded or unflooded conditions. For each element, the leaf data of the “selected” genotype sets were compared both as a group and individually to the unselected genotypes using t-tests (IBM-SPSS Inc., Armonk, New York, USA). Because different though often overlapping sets of genotypes were selected based on flooded versus unflooded field conditions for each element, two sets of selected genotypes (flooded and unflooded) were compared with all other (unselected) genotypes (page 148, Table 1). Because these genotypes have been crossed with Lemont to make F_2 progenies and screen for genes and/or mechanisms responsible for a particular element accumulation, t-tests were also run between unselected genotypes and Lemont for each element.

RESULTS AND DISCUSSION

Identification of the youngest potential range of vegetative growth stages for comparing leaf and grain element concentrations

Our first objective was to identify the youngest range of vegetative growth stages potentially suitable for comparing genotypes for element uptake. A series of graphs examining the concentration of each individual element over

sequential plant growth stages (V2 to V10) was created. The graph for each element included a total of eight genotypes: five which were previously selected as accumulating high concentrations of that particular element in their grain, two other genotypes which had low grain-concentrations of that element among the 40 genotypes used in the study, and the US check variety, 'Lemont'. These graphs revealed that for several elements (Co, Fe, K, Mg, Mo, Ni, P, Rb, Sr, and Zn) some of the high-concentration genotypes and at times, low-concentration genotypes showed a decline in their leaf-element concentrations between V2 to V4 growth stages which gradually leveled off beyond V4. For example, as shown in page 120, Fig. 4, for two out of the five high grain-Zn genotypes (GSORs 310769 and 310197) and for one low grain-Zn genotype (GSOR 311693) leaf Zn concentrations showed a notable decline as the plants advanced from the V2 to the V4 growth stages, then gradually leveled off after the V4 growth stage. Seeds serve as an important source of nutrition for seedlings (Tyler and Zohlen, 1998). Hence, the decline observed in leaf element concentrations in the early vegetative stages could possibly be due to the fact that these plants were initially more concentrated for particular elements not due to enhanced uptake, but rather due to receiving enhanced levels of that element from their seeds. While such vegetative differences may associate well with grain element concentration, they comprise a more difficult and less direct measure of grain concentration than actual measurement of grain concentration. Since the purpose of the present study was to identify vegetative stages in which leaf

element concentrations could identify element uptake or leaf accumulation differences that would carry through to grain accumulation differences, these early biases partially caused by grain element concentrations do not fit the present goal. Thus, plants younger than the V4 stage were deemed unsuitable for our purpose of evaluating vegetative leaf element concentrations. Several genotypes did not produce more than 8 leaves and the earliest flag leaf observed in this study was formed by leaf number seven (V7). The flag or terminal leaf of a tiller is not representative of vegetative-stage leaves because the developing reproductive organ has a relatively strong influence on the flag leaf including demand for stored assimilates and nutrients and altered regulation of photosynthetic activity (Gifford and Evans, 1981; Wu *et al.*, 2010). This narrowed the range of plant stages potentially suitable for predicting grain element concentrations from leaf element concentrations to V4 through V6.

Determination of viability of V4-V6 growth stages for comparison purposes through evaluation of select elements

Our second objective was to test if the V4-V6 growth stages can be used for comparing grain and leaf element concentrations. Since studies on *A. thaliana* showed organ-to-organ associations for elements Mo and Co (Baxter *et al.*, 2012; Baxter *et al.*, 2008; Morrissey *et al.*, 2009), we used these two elements as test cases to examine the trends among high and low genotypes for these elements from V4 to V6 growth stages. We found that the majority of the

genotypes selected for high grain-Co and Mo from unflooded/ flooded plots also showed high leaf-Co (3 of 5) and Mo (3 of 5) concentrations between V4 and V6 growth stages (page 121, Figs. 5a and 5b). We also noticed that all genotypes selected for low grain-Co and Mo also showed low leaf-Co and Mo concentrations in this range of growth stages thus indicating that the association can hold in the low direction as well. For example, in flooded conditions GSORs 311130, 311041, and 310769 selected for high grain-Co exhibited high leaf-Co concentrations and GSORs 311693 and 310266 selected for low grain-Co exhibited low leaf-Co concentrations (page 121, Fig. 5a). Also, GSORs 311643, 310356, and 310355 selected for high grain-Mo, and GSORs 311689 and 310197 selected for low grain-Mo showed consistently high leaf-Mo and low leaf-Mo concentrations, respectively (page 121, Fig. 5b). In this manner, the V4 to V6 growth stage range was deemed useful, and was used in further studies that examined the rest of the elements.

Evaluation of additional 15 elements for which leaves of V4 to V6 growth stages can be used to screen for grain concentrations

Our third objective was to determine how well leaf-element concentrations associated with the previously observed concentrations of 16 elements in the grain. Strong association between leaf and grain concentrations would suggest that the V4-V6 growth stages could be used for preliminary screening of diverse populations, and/or for pre-flowering identification of segregating cross-progeny

containing alleles or mechanisms contributing to desirable grain concentrations of a particular element.

For elements Cd, Co, Mo, and Sr, the genotypes selected based on grain harvested from flooded field plots, as a group, showed higher ($\alpha = 0.05$) leaf-element concentrations compared to all other genotypes (i.e., those not selected for high grain concentration of those particular elements, also excluding 'Lemont') (page 148, Table 1) and for elements Cd, Mo, Rb and S, the genotypes selected based on grain harvested from unflooded field plots, as a group, showed higher leaf-element concentrations as well. Stated another way, selection for high grain concentration of Cd, Co, Mo, Rb, Sr and S under flooded and/or unflooded conditions from among a set of 1640 diverse rice genotypes resulted in the identification of multiple genotypes that also exhibited enhanced concentrations of these same elements in their V4-V6 leaves. The association observed between grain and leaf concentrations within this smaller set of 40 genotypes suggests that the converse is also likely, that selections made among a diverse set of germplasm based on concentrations of these elements in leaves would be expected to identify a subset of rice genotypes with higher probability of having high grain concentration of the selected element(s). In this manner, a large number of genotypes could be screened using less time and other resources than that required to grow all replicated plants to grain maturity. Note that for elements Mo and Cd, both the flooded and unflooded selections, as groups, showed 2 to 3 times more leaf-Mo or leaf-Cd than the

unselected genotypes. Unfortunately, this study can comment only on flooded grain-Sr selections in that selections for Sr had not been done under unflooded conditions in the previous trials.

Though both the flooded and unflooded grain-Cd selections identified multiple lines whose leaf-Cd concentrations were higher than the unselected group (page 148, Table 1), the leaf-to-grain associations were stronger and more consistent for grain selections made under flooded than unflooded conditions. All five of the individual genotypes selected for high grain-Cd under flooded conditions had average leaf concentrations more than 2x the unselected average, albeit low sample number and high variance between plants per genotype caused only two of them (GSORs 310993 and 310364) to be statistically significantly different from the unselected group (page 148, Table 1). The consistency of the trend for grain-selected genotypes to also have high leaf-Cd suggests that leaf concentrations can indeed be used to identify a subset of diverse genotypes having increased probability of exhibiting high grain-Cd under flooded conditions. The Core genotypes found to have high grain-Cd under unflooded conditions included the five genotypes selected specifically for high grain-Cd under flooded conditions, plus four additional lines. The unflooded Cd selections that also exhibited high leaf-Cd were those that had shown high grain-Cd under flooded conditions as well. Reduced agreement between the leaf and grain element concentration patterns could be due to a lack of reliability between the leaf and grain selection conditions or due to reduced validity among the

unflooded grain selections. The fact that most of the flooded grain selections were also unflooded grain selections, but not vice versa, suggests that selections under flooded field conditions might indeed be more accurate than unflooded selections. Though many soil factors can influence element availability for root uptake, three that have strong influence and can display large spatial variance in unflooded field conditions relative to flooded conditions are soil redox potential, soil pH and puddling. The presence of low spots in unflooded fields causes local variation in redox potential, pH and moisture content, which all trend toward uniformity under flooded conditions. The relatively high variation in soil redox potential, pH, moisture and other factors in the unflooded field can present as increased variation in element availability among replicates and ultimately in their grain concentrations.

Among the elements showing high leaf-to-grain association for both flooded and unflooded grain selections, Mo presents a particularly interesting story. Molybdenum was one element that showed a strongly consistent pattern of maintaining ranks. For example, genotypes GSORs 311643, 310356 and 310354 showed high leaf-Mo concentrations and the low grain-Mo genotypes GSOR 311689, 310197 showed low leaf-Mo concentrations (page 121, Fig. 5b). It is worthwhile to note that all but one of the highest grain-Mo genotypes (all but GSOR 310823) originate from Malaysia or the nearby island of Brunei (page 148, Table 1). The similar geographic origin of these five consistently high grain-Mo genotypes suggested they may share the same Mo-accumulation

mechanism(s) and allele(s). Of the seven high grain-Mo genotypes, five lines exhibited high leaf-Mo, suggesting that high grain-Mo from flooded fields is often, but not always, associated with enhanced leaf-Mo. Conversely, selection for high leaf-Mo would often, but not always, identify from among a diverse set of genotypes those expected to have high grain-Mo. Curiously, one of the five high grain-Mo lines from the Malaysia/Brunei area did not exhibit high leaf-Mo. As seen similarly for Cd, among the nine genotypes that showed high grain-Mo under unflooded conditions, the only selections that also showed high leaf-Mo were those that had also exhibited high grain-Mo under flooded conditions. The Malaysia/Brunei origin of many of the high-Mo genotypes is of further interest because the Malaysian soils are characterized as acidic soils in which Mo is strongly bound to soil particles and not available to plants (De Datta, 1987). We hypothesize that these genotypes are showing an acid tolerance mechanism that results in the accumulation of high levels of Mo in their grains, possibly due to enhanced mining abilities which may be due to a root-localized trait.

Grain accumulation of any particular element results from a series of uptake and transport steps, each potentially capable of limiting or determining the final grain concentration of any particular element, with only some steps or processes also being associated with increased accumulation of that element in the leaves. Therefore, we were not surprised to find that some, but not all, high grain-element genotypes also exhibited high leaf-element concentrations. In the case of Mo, GSOR 310354 had exhibited high grain-Mo in all flooded and

unflooded replications of the previous field study that led it to be included in the present study (Pinson *et al.*, 2010), yet it did not exhibit high leaf-Mo. It appears that one can likely use leaf concentrations to identify plants containing the mechanism/gene(s) underlying the high grain-Mo of GSORs 310355, 310366, 311643, 131735, and 310823, but not that of 310354. This means that for characterization of segregating cross-progeny, whether for breeding or gene-mapping purposes, one could potentially select for the mechanism/allele(s) contained in many of the high-Mo genotypes, but not that of GSOR 310354. The association observed here between leaf-Mo and grain-Mo for several of the high-Mo genotypes should be verified, however, among a subset of F_2 or later-generation cross progeny before using leaf data as a surrogate for grain evaluations in future breeding and genetics studies.

For all elements except Cu and Ni, at least one of the genotypes selected based on grain phenotype also exhibited high-elemental leaf phenotype (page 148, Table 1). Even for those elements where the selected genotypes as a group did not display high leaf concentration, individual genotypes were often found to exhibit higher leaf concentration (page 148, Table 1). For those specific genotypes where leaf concentrations did associate with grain concentrations, leaf data may prove useful for making breeding selections among cross-progeny, or for characterizing progeny in mapping populations to identify the alleles and/or mechanisms underlying the increased leaf and grain concentrations. For example, GSOR 311689, which produced grain high in Mg

under both flooded and unflooded conditions, was the only genotype selected for high grain-Mg that also exhibited high leaf-Mg (page 148, Table 1). It is quite probable that different genotypes contain different genetics and/or mechanisms affecting grain concentrations of specific elements, some of which would lead also to enhanced element accumulation in leaves, and some of which would not. While it would not be recommended that one use leaf data for diversity screening to identify lines high in grain-Mg, it might yet prove possible to use leaf data to identify genes responsible for high grain-Mg among cross-progeny of GSOR 311689.

Not all elements are desirable. Breeding programs target the development of rice genotypes having lower concentrations of potentially toxic trace elements along with higher concentrations of beneficial elements in their grains. The genotypes noted in page 148, Table 1 to have higher levels of leaf-As, -Cd or -Co concentrations, or lower levels of leaf-P, -K, or -Fe than the unselected genotypes, are not desirable for breeding purposes but could still be useful for the identification of alleles one would want to select against. Sodium is generally an undesirable element to have present in high concentrations in plant tissues. Sodium is typically not determined in elemental analyses of seeds because of low levels, and was not measured in the grains in the previous field trials (Pinson *et al.*, 2010); Na was, however, measured in the leaves in the current study. In evaluating genotypes for salinity tolerance, one measure that is often used is the Na/K ratio in leaf tissue, for which a low value would support

possible sodium exclusion and salinity tolerance. One of the high grain-K genotypes, GSOR 310354, showed relatively high leaf-Na (page 122, Fig. 6). Studies on Na and K partitioning in wheat showed that high leaf-Na was due to high Na loading into the xylem for shoot transfer (Davenport *et al.*, 2005; Nabipour *et al.*, 2007). A study in rice identified that high leaf-Na accumulation was linearly associated with transpiration (Das, 2004; Ren *et al.*, 2005). In a study examining natural variation in element concentrations in *Arabidopsis* shoots, two accessions with high Na levels in shoots also appeared, somewhat surprisingly, to closely co-segregate with increased NaCl tolerance rather than increased sensitivity (Rus *et al.*, 2006). Both accessions possessed a weak allele resulting in reduced expression of the sodium transporter, At*HKT1*, in roots. The leaf Na levels of 310354 observed in the present study would suggest that mechanisms beyond transpiration influence the leaf Na levels in rice.

One or more genotypes selected for elements Ca, Cd, Co, P, K, Fe, Mg, Mo, Rb, Sr, S, and Zn ranked high with respect to grain element concentrations under both flooded and unflooded growth conditions, indicating that, in these genotypes, grain element accumulation was not dependent on the soil redox potential. For other elements, As, Cu, Ni, and Mn, the flooded and unflooded conditions identified different genotypes as having high grain element concentrations, indicating that mechanisms explaining high grain accumulation of these elements interact more with soil chemistry, and suggesting that grain element accumulation was strongly dependent on the soil redox potential. It is

interesting to note that for the element As , one genotype (GSOR 310769), when grown under unflooded conditions, ranked high in grain-As concentration (in the top 200 of 1640 lines) but when grown under flooded conditions, produced grains notably lower than average for As concentration (page 148, Table 1), supporting previous studies that As exists in different forms under different soil conditions, including different redox potentials, and so is taken up by the plant through different mechanisms (Norton *et al.*, 2012)

Soil redox potential is a major source of environmental variation influencing grain accumulation of several elements. The variations in soil redox potential and other soil chemical factors that are associated with the higher spatial heterogeneity of an unflooded vs. flooded field likely explain at least in part our ability to distinguish more significant associations between leaf concentrations and those in grain selected under flooded conditions as opposed to unflooded conditions. However, the availability of minerals also differs in flooded and unflooded conditions (Patrick *et al.*, 1985). Flooded soil is abundant in P, S and reduced forms of Fe, Mn and Cu, while unflooded soil is abundant in K, Ca, Mg, Zn, and oxidized forms of Fe and Mn (De Datta, 1987). However, in this current unflooded study, only a couple of genotypes selected for high grain-K under unflooded conditions showed high leaf-K concentrations and only one genotype selected for high grain Ca, Mg, Zn, Fe or Mn showed corresponding high leaf-Ca, Mg, Zn, Fe or Mn concentrations, respectively. These observations suggest that field variability led to increased within-genotype

variability and might have decreased the detectability of genotype differences in element accumulation, although physiological factors are also likely to contribute to a lack of association between leaf and grain levels of some elements. Another source of variation is analytical. Some genotypes selected for low trace element grain concentrations showed no associations. A contributing factor that might have limited the ability to identify some genotype-level associations for these elements may be a lower signal-to-noise ratio in the instrumental analysis of these low-concentration elements. In a similar study, analytical variation was a major contributing factor when assaying levels of some elements(Lahner *et al.*, 2003).

Screening of vegetative-stage leaf element concentrations to estimate grain element concentrations should be useful for other cereal and non-cereal crops for which the method might help offer an efficient means for selecting among numerous germplasm accessions, for example, and/or identification of genes influencing seed element concentrations s they segregate within mapping populations. The method is based on screening the element concentrations of vegetative-stage leaves and can thus accelerate efforts to breeding for improved nutritional value of crops. Rice is used as a model crop for genomics research because of synteny among cereal crops (Gale and Devos, 1998; Mayer *et al.*, 2011), thus the identification of genes, mechanisms and methods for the improvement of rice grain nutritional quality will also be of value in improving the nutritional quality of other cereal crops.

CONCLUSIONS

For the elements, Cd, Co, Mo, Rb, Sr and S, the group of genotypes selected for high grain concentration of the element exhibited excellent agreement between grain and leaf element concentrations; thus it may be possible to use leaf element concentrations from V4- to V6-stage leaves to screen diverse germplasm to identify accessions likely to have high grain element concentration. However, diverse screening may not capture all mechanisms affecting a particular element's accumulation. For many of the elements, including As, Ca, Cd, Co, Fe, Mg, Mn, Mo, P, K, Rb, Sr, S and Zn where some, but not all of the individual selected genotypes exhibited high grain element concentrations in their leaves, the screening of leaf element concentrations may prove useful for identifying genes affecting element accumulation that are segregating among F_2 or later progenies derived from these specific genotypes. When possible, focusing future studies on vegetative growth stages would accelerate breeding efforts aimed at improving rice grain nutritional value, thus saving time and resources in the crop improvement process.

CHAPTER III

DOES THE HIGH Mo PHENOTYPE OF SEVERAL MALAYSIAN RICE (*ORYZA SATIVA* L.) GENOTYPES REPRESENT AN ACID-TOLERANCE MECHANISM: EXAMINATION OF ROOT MORPHOLOGICAL RESPONSES

INTRODUCTION

Growth media properties such as pH influence relative growth rate of roots resulting in changes in morphological traits like root length, root area, and root biomass (both fresh and dry) (Fageria and Stone, 2006; França *et al.*, 2006). Root morphological traits in turn influence absorption and uptake of elements (Beebe *et al.*, 2006; Chen *et al.*, 2009; Ding *et al.*, 2012; França *et al.*, 2006). In combination, growth media and root traits may influence plant elemental concentrations such as molybdenum (Mo) which is of particular interest in this study. Molybdenum is an essential micronutrient required in very low amounts (0.1 to1 μg g^{-1} dry weight) in plants (Hansch and Mendel, 2009). It acts as a co-factor necessary for activation of certain enzymes that carry out redox reactions and is required for various physiological, biochemical and metabolic processes. Mo plays an important role in growth and physiology (Bala and Hossain, 2008; Moraes *et al.*, 2009). It is a constituent of nitrate reductase, an enzyme essential for nitrogen assimilation. With an increase in nitrogen uptake, there is often an increase in plant height, tillering, and shoot biomass (Bala and Hossain, 2008; Gupta, 1997). Molybdenum is involved in protein

synthesis and is required for chloroplast development. It has been shown to increase the net rate of photosynthesis (Gupta, 1997; Moraes *et al.*, 2009). Molybdenum absorption and uptake is influenced by the concentration of Mo in the nutrient media (Kannan and Ramani, 1978). At higher pH, Mo exists as highly mobile molybdate $(MoO_4)^{2-}$ anions in solution (Mengel and Kirkby, 2001). Once inside the plant, MoO_4^{2-} anions are transported in both xylem and phloem (Marschner, 1995; Zimmer and Mendel, 1999). It is a highly mobile anion and moves from root to shoot and also from shoot to root (Baxter *et al.*, 2008; Kannan and Ramani, 1978). High/low levels of Mo in the solution alter the uptake and accumulation of other elements like nitrogen and sulfur resulting in distinct leaf and root mineral compositions and reduction of dry matter content of plants (Brune and Dietz, 1995; Ide *et al.*, 2011; Moore and Patrick, 1991; Moraes *et al.*, 2009). The solubility, availability and activity of MoO_4^{2-} anions decrease with decreases in pH (Kannan and Ramani, 1978; Moore and Patrick, 1991). At very acidic pH, Mo is strongly adsorbed to soil making it unavailable for uptake by plants (Mengel and Kirkby, 2001).

There is interest in identifying genes responsible for molybdenum (Mo) uptake/accumulation so that breeding programs can develop rice genotypes with improved Mo shoot concentrations which may ensure good plant health and crop productivity. In *Arabidopsis*, *MOT1* (Molybdenum Transporter) localized in plasma membrane and mitochondria, is found to be associated with Mo uptake (Baxter *et al.*, 2008; Tomatsu *et al.*, 2007). It is highly expressed in roots though

it is present everywhere in the plant. Shoot-Mo concentrations were correlated with root uptake suggesting that root uptake drove shoot-Mo concentrations (Baxter *et al.*, 2008). In *Arabidopsis*, concentration of Mo was found correlated between roots and shoots, but also between these vegetative organs and seed (Baxter *et al.*, 2012). Little is known about Mo uptake in rice, but the Arabidopsis findings suggest that root-based control of grain Mo concentrations is plausible.

In 2007 and 2008, prior flooded and unflooded field studies on 1640 rice genotypes from 114 countries identified genotypes with extreme grain-Mo concentrations (Pinson *et al.*, 2010). Three out of the five highest grain-Mo genotypes originated from Malaysia or nearby Brunei, which are known to have acid soils as low as pH 4.7 (De Datta, 1987). These high grain-Mo genotypes were later found to also have high leaf-Mo in vegetative-stage leaves (Chittoori et al., submitted), suggesting efficient Mo mining can occur in early development and that root-based control of grain Mo concentrations is plausible.

The objectives of the current study were 1) to analyze and compare root traits of two high grain-Mo genotypes GSOR 310356 (Malaysia) and GSOR 310823 (Iraq) with a low grain-Mo genotype, Lemont (standard US genotype), grown at three pH regimes, and 2) to determine if the high grain-Mo genotype GSOR 310356 from Malaysia exhibited acid tolerance. This would test the hypothesis that GSOR 310356 contained an acid tolerance mechanism which increased root development and in turn enhanced Mo uptake. If true, then I further hypothesized that GSOR 310356 would show distinct root traits at pH 4.7

when compared to the other two genotypes. Root traits determined from this study would then be potentially related to Mo uptake and transport, which could be verified in F_2 studies. If proven to be contributors toward high grain-Mo, the root traits would provide a faster, more direct, and less-environmentally variable phenotype with which to identify genes essential to high grain-Mo. As mentioned earlier, high grain-Mo genotypes, GSOR 310356 and GSOR 310823 exhibited high-leaf Mo in a study conducted exclusively on vegetative growth stages (Chittoori et al., submitted). The similar Mo-accumulation patterns observed in different plant organs (seeds and leaves) suggests significant influence by root uptake and root-to-shoot transfer of Mo starting in young seedlings and carrying over to affect grain-Mo. Since the previous study documented that differences in leaf-Mo were detectible in leaves of young seedlings, the current root and seedling study was conducted on vegetative growth stages from two to four weeks after germination (Wagner, 2001).

Hydroponics were used for this study as root biomass can be obtained accurately unlike soil culture where there is significant loss and damage of roots while uprooting roots from the soil or while washing soil from the roots (Neumann *et al.*, 2009). Hydroponics provide better control of the rooting environment, such as pH (Ellis and Swaney, 1938) which was critical in this study where genotypes were grown in three pH regimes.

MATERIALS AND METHODS

Plant growth conditions

This hydroponic experiment was conducted in 2011 at the Texas A&M AgriLife Research Center at Beaumont, Texas, USA, in a growth chamber maintained at 30/25 °C (day/night) and 10-h photoperiod (daylength from 0800 h to 1800 h) illuminated using white fluorescent lamps (55 µmol m^{-2} s^{-1}) and incandescent bulbs (40 W) to give 200 photosynthetic photon flux density (PPFD) (µmol m^{-2} s^{-1}). This experiment had a factorial design with three genotypes, GSOR 310356 (high grain-Mo from Malaysia), GSOR 310823 (high grain-Mo from Iraq) and Lemont (low grain-Mo from US, serving as control) and three pH regimes of 4.7, 5.4, and 6.1. This experiment was repeated three times and each replication consisted of six plants of each genotype grown at each pH. Plants were placed at different locations in each replication though not randomized.

Seed sterilization

Seeds of GSOR 310356, GSOR 310823 and Lemont were sterilized using hydrogen peroxide (10%) for 10 minutes followed by ethyl alcohol (70%) for a minute (Iyer-Pascuzzi *et al.*, 2010) (page 123, Fig. 7) and then thoroughly rinsed with reverse osmosis (R.O.) water.

Netted foam discs

Root traits would be analyzed by taking digital images of roots fully immersed in water and later thresholding these images in MATLAB software (Iyer-Pascuzzi *et al.*, 2010). This required plants to freely float on water on some support, black in color so that root images could be thresholded against a black background. In a preliminary trial, plants were grown in sand culture. However, getting the roots through the mesh support at the time of root imaging proved impossible without damaging the roots. We then devised a system wherein roots were allowed to grow through the mesh of the plant-support system beginning at germination. Commercially available hydroponic plant-supports, usually in the form of netted baskets made of plastic, were either white or assorted colors both of which merged with the color of the roots while thresholding thus biasing root traits. Also, they had big netting-holes which would not provide adequate mechanical support for the rice plants, unless it was filled with some inert light-weight pebbles. However, pebbles of the right size could not be found except in bright unsuitable colors that could have potentially discolored and stained the nutrient solution over time and changed the elemental concentration of the medium, a factor that is very critical in this study. Because of these disadvantages, commercial plant floats were not used. Instead, black netted foam discs with netting-holes appropriately sized for rice seeds and roots were custom designed for this study which provided both mechanical support and met image thresholding requirements as they were

black in color. To make these discs, black foam insulation tubes of 2.5-cm outer diameter and 2-cm inner diameter were sliced to 1-cm thick rings. Two of these rings were stitched together using a Micro Stitch Gun (Avery Dennison, Pasadena, California) with black nylon netting placed between the two rings (page 124, Fig. 8).

Seed-germination

Reverse osmosis (R.O.) water was selected for this experiment based on preliminary trials where seeds responded with better germination percentages in R.O water than in distilled water and nano-pure water. Germination trials on filter paper inside petri-dishes resulted in seedlings with agravitropic or deformed roots. To ensure natural root morphology and architecture, seeds were germinated over sand culture. Washed sea-sand (Fisher Scientific, Pittsburgh, PA) was used to fill a clear plastic box (40 cm long x 27 cm wide x 23 cm deep) to a depth of approximately 2.5-cm and was moistened with just enough R.O. water to wet the sand; excess water was blotted away using paper towels to prevent puddling. Netted foam discs were uniformly spread over the sand (page 125, Fig. 9).Inside each disc, sand was added until its upper surface was level with the netting. This allowed the sand to wick water up to each seed, providing uniform moisture at the center of each disc. One sterilized seed per disc was placed with forceps at the center of the netting, covered with sand to a 0.5 cm depth and then moistened with approximately 5 ml R.O. water per disc with a

hand-held squirt bottle. The box was covered with Reynolds 900 Clear Wrap (Alcoa Inc., New York, NY) to retain moisture and placed approximately 1.5 meters away from the lights in a growth chamber for two weeks for germination at 30/25 °C (day/night), at which time the seedlings of all genotypes were at the 1.5 ± 0.2 leaf stage (V 1.5).

Seedling preparation

To loosen the 2-week old seedlings for placement into hydroponics, the germination box was filled approximately 5-cm deep with R.O. water and gently agitated to loosen the roots from the sand while sustaining minimal injury (page 126, Fig. 10). Each seedling with disc intact was carefully uprooted, rinsed thoroughly with running water (R.O), and then, placed in its appropriate hydroponic vase. The disc kept the seedling afloat while the roots were immersed in hydroponic solution, unlike those grown on a stationary support where nutrients are refilled only on a weekly basis leaving parts of the root systems exposed to air which could trigger stress responses (Meyer *et al.*, 2009).

Hydroponic medium

A new hydroponic medium was developed for this study to ensure healthy plants devoid of low-pH-induced element toxicities/deficiencies even at the lowest pH. In hopes of keeping the pH constant, acid and basic buffers {namely

acetic acid-potassium acetate, acetic acid-MES [2-(N-morpholino) ethanesulfonic acid], PIPES (1, 4-piperazinediethanesulfonic acid)-potassium hydroxide, and potassium dihydrogen phosphate- potassium hydroxide} were tried at different concentrations and different pH levels. However, they all reacted with the medium turning the solution turbid/milky white and had to be eliminated. Preliminary trials included an array of pH regimes from pH 4 to 7. However, pH levels above 6.1 were Fe deficient and were not successful in maintaining healthy plants and hence, could not be used. The media developed for this study was based on a nutrient solution developed for salinity stress studies at the International Rice Research Institute (Manila, Philippines) (Adorada and Gregorio, 2009) and is cost, labor and time effective. This media mainly used commercially available fertilizer which is readily available in the market. It takes minimum skills to prepare as it does not require an array of expensive chemicals or the need to mix them in different proportions. The solution can be made fresh in less time than other hydroponic media and does not require prior preparation of stocks or the need to preserve them in an ultra-low freezer. The media comprised of 1g L^{-1} Peters Professional 20-20-20 granulated fertilizer (Everris International B. V., Geldermalsen, The Netherlands) supplemented with 0.1 g L^{-1} $FeSO_4$ as per Adorada and Gregorio (2009), 0.74 g L^{-1} $CaNO_3$ (Salisbury and Ross, 1978), and 0.005 g L^{-1} of $CaCl_2$ (Salisbury and Ross, 1978) brought to volume with R.O water. The pH was adjusted to 4.7, 5.4 or 6.1 using HCl or KOH. The hydroponic medium was replaced every two to

three days to maintain constant pH throughout the experiment and was made fresh at each time of replacement.

Hydroponic setup

The hydroponic system consisted of a Whisper® air pump (Tetra Inc., Blacksburg, Virginia) connected to eight-way plastic manifolds which in turn were connected to eight syringe needles of 25-gauge size and 0.5-mm outer diameter (Fisher Scientific, Pittsburgh, PA) (page 127, Fig. 11a). To these needles, 0.5-mm inner diameter airline tubings (Fisher Scientific, Pittsburgh, PA) were connected. Each of these tubings was immersed into a 25-cm high x 8-cm wide cylindrical clear glass vase. The tubing rested at the base of the vase and was weighted without obstructing the air-flow using a white plastic paper clip. The medium was aerated to provide oxygen to the roots, to ensure solution movement and to avoid stagnation of the solution. Each vase was wrapped with aluminum foil to prevent light from reaching the roots and nutrient solution. Each vase contained 1 L of hydroponic medium and received a seedling that floated on the netting of its foam disc. The remaining exposed surface of the nutrient solution was covered using a piece of black foam felt cut in the shape of a horse-shoe to contain the foam disc carrying the seedling (page 127, Fig. 11b). This black foam felt on the surface of the medium, along with aluminum foil wrapped around the vases, blocked light and prevented algae from growing in the medium (page 128, Fig. 12).

Parameters measured during growing season

Root imaging

Roots were imaged 1 week after transplanting (WAT) to the hydroponics growth medium. Images were captured inside a photo studio (page 129, Fig. 13) made by taping six pieces of black coroplast board (48-cm long x 36-cm wide) to form a closed chamber. The edges and crevices were sealed to block light, thus providing a dark environment that minimized glare from the glass vase while roots of floating plants were imaged. Imaging consisted of three components, 1) a laptop which was connected to 2) a turntable, and 3) a camera mounted on a tripod (Iyer-Pascuzzi *et al.*, 2010). Plants with discs intact were rinsed and placed in two L of R.O. water contained within an ungraduated glass cylinder approximately 52 cm high and 8.5 cm wide. The cylinder was placed on the turntable allowing use of the PhotoCapture 360 (Ortery Technologies Inc., Irvine, California) to image the roots after every 18° rotation giving a total of 20 images per plant. Following root imaging, seedlings were carefully placed back into the hydroponic system for another week of growth.

Root traits

The root images were adaptively thresholded (page 130, Fig. 14). (black and white image; background in black pixels and roots in white pixels) in MATLAB software to give binary images (Iyer-Pascuzzi *et al.*, 2010) which calculated 16 different root traits per image. These traits were then averaged

across images for a single average per plant. Traits measured included: total root length (total length of all the rootlets in the root system), root volume (total volume of the rootlets) , specific root length (total root length/root volume), maximum number of roots (MaxR, total number of rootlets in the root system), median number of roots (MedR, total number of rootlets with the median value), root bushiness (ratio of MaxR /MedR), root length distribution (ratio of total root length in the top $1/3^{rd}$ versus the bottom $2/3^{rd}$ of the root depth on a single plane) of that same image, root network area (total root area), root convex area (root area constituting the convex hull of the root architecture), solidity (network area/convex area), root surface area (surface area of all the rootlets), root perimeter, root radius, root width (width of the root architecture), root depth (depth of the root architecture), and root width to depth ratio.

Leaf photosynthesis

Photosynthesis was measured 2 WAT (4 WAG, ~V4 growth stage) using a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE) (page 131, Fig. 15). Measurements were taken between 1000 h and 1200 h on the most recent fully opened leaf of the main tiller on each plant. The ambient carbon dioxide concentration (400 µmoles $mole^{-1}$), temperature (30 °C), photosynthetic photon flux density or PPFD (1500 µmol m^{-2} s^{-1}), and leaf area (6 cm^2) were set prior to taking measurements.

Chlorophyll fluorescence

Photosynthetic efficiency (Fv/Fm) was determined using a fluorometer (Opti-Sciences, Tyngsboro, MA) on the same leaves used for photosynthesis measurements (page 132, Fig. 16). Leaves were dark-acclimated using dark-adaption cuvettes (Opti-Sciences, Tyngsboro, MA) 20 minutes prior to taking chlorophyll fluorescence measurements.

Leaf respiration

Respiration was measured between 2300 h and 0200 h using a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE) duly set for ambient carbon dioxide (400 µmoles $mole^{-1}$), temperature (25 °C), PPFD (0 µmol m^{-2} s^{-1}), and leaf area (6 cm^2). Measurements were again taken on the most recent fully opened leaf on the main tiller of each plant.

Parameters measured at the time of harvest

Shoot traits

Plants were harvested 15 DAT (29 DAG) and final plant height (from the base to the highest reaching leaf tip), number of leaves per main culm, and number of tillers were measured. Leaf color was determined by visually rating against a leaf color chart (IRRI-Phil Rice, Manila, Philippines) on a scale from 1 (light yellow) to 6 (dark green) (page 133, Fig. 17).

Shoot and root biomass

Shoot and roots were separated and fresh and dry weights (dried at 70°C for 48 h) were determined.

Element analysis

Root and shoot element concentrations were determined using inductively coupled plasma- mass spectrometry (ICP-MS) courtesy of David Salt, University of Aberdeen, UK (Salt *et al.*, 2008). The samples were digested over-night with nitric acid at room temperature followed by heat-digestion using a heat block before being passed through ICP-MS (Danku *et al.*, 2012).

Data analysis

Data were subjected to analysis of variance (ANOVA) and means were separated using Tukey's least significance difference (LSD) at $P \leq 0.05$ using SPSS (IBM-SPSS Inc., Armonk, New York).

RESULTS AND DISCUSSION

A proper hydroponic media that was capable of holding three different pH levels for a period of days was developed exclusively for this experiment. Because media was replaced every two or three days, all three genotypes grew healthily at all three pH regimes with leaf element concentrations (pages 169-171, Tables 2-4) indicating no nutritional deficiencies or toxicities. All genotypes

showed high shoot-Fe concentrations at pH 4.7 and 5.4 when compared to pH 6.1 but those Fe concentrations were not toxic. As a matter of fact, media had to be supplemented with 100 mg L^{-1} Fe because, prior to Fe supplements, plants developed interveinal chlorosis in leaves characteristic of Fe deficiency and died. P, Fe and As are less available to plants at low pH and all genotypes showed high concentrations of shoot-P, -Fe and -As concentrations at pH 4.7 and 5.4 (pages 169-171, Tables 2-4) when compared to pH 6.1. At low pH, Cu, Zn and Mn are more available to plants; however, all genotypes showed lower shoot-Cu, -Zn and -Mn concentrations at both pH 4.7 and 5.4 compared to pH 6.1. The lower shoot-Cu and -Zn concentrations may be attributed to competition of H^+ ions with Cu^{2+} and Zn^{2+} ions for binding sites (Campbell and Stokes, 1985). Competition of Fe^{2+} ions with Mn^{2+} ions for binding sites may have caused low shoot-Mn corresponding with the high shoot-Fe concentrations (Tanaka and Navasero, 1966) .

At pH 4.7, all three rice genotypes showed signs of injury and poor health in both shoots and roots. Among the shoot traits observed, all genotypes showed lower shoot fresh weights and lower rates of photosynthesis and leaf respiration at pH 4.7 when compared to 6.1 (page 172, Table 5). The Iraq, high-Mo genotype GSOR 310823 and Lemont especially showed less intense leaf greenness at pH 4.7 than 6.1. Among the various root traits, all three genotypes showed reduced root radius, root perimeter and specific root length at the lower pH, while GSOR 310823 and Lemont also showed reduced root fresh weight at

pH 4.7 than pH 6.1 (page 173, Table 6). Also, at pH 4.7, the Malaysian genotype GSOR 310356 showed less root width, while GSOR 310823 showed reduced root depth, and Lemont showed reduced root dry weight and root volume (page 174, Table 7). The two high grain-Mo genotypes, GSORs 310356 and 310823 both showed a reduction in median and maximum number of roots, root surface area, and root width-to-depth ratio at pH 4.7 when compared to pH 6.1 (page 175, Table 8).

Root-Mo concentrations could not be accurately measured because Mo strongly adsorbed to root surfaces of all genotypes at pH 4.7 and 5.4. As a result of these biased concentrations, all genotypes exhibited similar root-Mo concentrations at pH 4.7 in spite of the genotypic differences in root-Mo concentrations at pH 6.1 (page 134, Fig. 18).

The GSOR 310356 from Malaysia showed constitutively high shoot-Mo concentrations when compared to GSOR 310823 from Iraq and Lemont from the US under adequate nutrient supply in all three pH regimes (page 135, Fig. 19). Shoot-Mo concentrations of GSOR 310356 doubled at pH 6.1 when compared to pH 4.7 and 5.4 which may be due to increased availability, mobility and activity of MoO^{4-} ions at higher pH until neutral pH (Kannan and Ramani, 1978; Moore and Patrick, 1991). Though GSOR 310356 was efficient at mining Mo, it did not appear to possess acid tolerance per se. Molybdenum is a constituent of nitrogen assimilating enzymes and plants with improved nitrogen assimilation may show increased shoot fresh weight and plant height (Bala and Hossain,

2008; Moraes *et al.*, 2009). However, GSOR 310356 showed both reduced Mo and reduced shoot fresh weight at pH 4.7 when compared to pH 6.1 and produced lesser/similar shoot fresh weight as the other two acid-susceptible genotypes at pH 4.7 (page 136, Fig. 20). The plant height of GSOR 310356 in all pH regimes was shorter or similar to that of plants of the other two genotypes at pH 4.7 (page 137, Fig. 21). Thus, GSOR 310356 did not exhibit acid tolerance in terms of relative increased shoot growth at the low pH. Molybdenum has been shown to increase photosynthetic rate (Bala and Hossain, 2008; Gupta, 1997), while GSOR 310356 showed reduced shoot-Mo concentrations and a reduced rate of photosynthesis at pH 4.7 when compared to pH 6.1. The photosynthetic rates of GSOR 310356 at pH 4.7 were similar to rather than higher than the other two genotypes at pH 4.7 (page 138, Fig. 22). Mo uptake in *Arabidopsis* is associated with *MOT1* gene which is in the root regions just behind the root tips (Baxter *et al.*, 2008). While this suggests that shoot-Mo concentrations in *Arabidopsis* may be increased with more roots, in the present study, GSOR 310356 showed increased Mo at all pHs, but if anything, had reduced root number compared to the other two genotypes, and was especially reduced in root number at pH 4.7 (page 139, Fig. 23).

Principal Component Analysis was done using SPSS (IBM-SPSS Inc., Armonk, New York) to identify a reduced set of independent variables called principal components that explain the maximum variances in the hope of identifying sets of morphological and physiological traits that could be related to

the differences in shoot-Mo concentrations among genotypes and pHs. However, these analyses did not show any significant associations between the same.

CONCLUSIONS

The Malaysian rice genotype, GSOR 310356, showed the highest shoot-Mo concentrations of all genotypes and at all pH regimes suggesting a unique mechanism of Mo-mining different from that of GSOR 301823 (from Iraq) and Lemont (US). However, since no evidence of tolerance to acid conditions, in the presence of Mo sufficiency was found for this genotype, it is concluded that the enhanced Mo uptake of GSOR 310356 is not due to an acid-tolerance mechanism. Indeed, many of the shoot and root traits we observed differed from the other genotypes in an opposite manner from what might be expected if this genotype was indeed acid-tolerant.

CHAPTER IV

DO ROOT MORPHOLOGICAL AND PHYSIOLOGICAL TRAITS INFLUENCE SHOOT ELEMENTAL CONCENTRATIONS OF TWENTY FOUR DIVERSE RICE (*ORYZA SATIVA*) GENOTYPES GROWN IN HYDROPONIC CULTURE?

INTRODUCTION

This study addresses the hypothesis that rice (*Oryza sativa* L.) genotypes selected for extreme grain-K, -Fe, -Zn and -Mo concentrations in a previous field study differed in root morphological and physiological traits that influenced element absorption and uptake at a young vegetative stage in a manner consistent with an ultimate influence on grain element concentrations. The approach was to grow the rice plants in hydroponic culture and relate the root morphological, physiological and ionomic traits, with an emphasis on K, Fe, Zn, and Mo.

Hydroponic experiments have been used to quantify rice seedlings for root morphological and physiological traits with respect to acid/salinity tolerance, element absorption/uptake, element deficiency/efficiency/toxicity and synergistic/antagonistic uptake of elements (Adorada and Gregorio, 2009; Chen *et al.*, 2009; Famoso *et al.*, 2010; França *et al.*, 2006; Guo *et al.*, 2012; Kannan and Ramani, 1978; Wang *et al.*, 2008). This study is novel in using hydroponics to study root traits in relation to multiple elements and their ionomic patterns in rice roots and shoots. Hydroponic culture allows for the study of root morphology

and physiology in a more efficient way than with other media. Soil culture requires significant time and labor in harvesting and washing roots which also causes significant damage or loss of roots (Neumann *et al.*, 2009). Use of hydroponic cultures conserves time and labor in harvesting/washing and enables the collection of accurate root biomass. It provides a better control of the root environment such as pH and nutrients (Ellis and Swaney, 1938) which is very critical in ionomics studies. Also, media is more uniform from batch to batch when compared to soil. It eliminates soil problems like soil-borne pathogens causing diseases, soil-borne pests, salinity, poor soil structure and drainage (Jensen, 1999). Roots are visible in hydroponics and growth and development may be easily analyzed at any time (Hershey, 1994). However, use of hydroponics also has some disadvantages (Zinnen, 1988). Root rot may occur as pathogens spread more quickly in water than soil. Hence, proper sanitation and maintenance are required. Growing conditions such as temperature, light intensity, and photoperiod are critical (Zinnen, 1988).

Root gross morphology and physiology influence element uptake and thus element partitioning in plants (Atkinson, 1990; Chen *et al.*, 2009; Clark, 1990; Zheng *et al.*, 2000). Rice roots undergo morphological and physiological adaptations under flooded conditions and form aerenchymal spaces that allow oxygen transport from leaves to roots (Counce *et al.*, 2002; Kirk, 1994). Oxygen diffusing from roots causes oxidation of Fe^{2+} to Fe^{3+} releasing H^{+} ions resulting in localized acidification. This root-induced acidification influences root

morphological traits thus further influencing element absorption/uptake. Root physiological factors like root respiration alter soil temperature, pH and redox potential, thus influencing element availability, solubility and uptake (Fageria and Baligar, 2003; Yang *et al.*, 2004). Apart from these factors, chemical properties of growth media such as pH and redox potential also influence element availability, solubility and absorption by roots thus influencing plant element concentrations (Norman *et al.*, 2002). A relatively greater rate of photosynthesis can increase the quantity of photosynthates reaching the roots which can in turn increase root respiration. Conversely, higher root respiration supplies higher rates of elements to shoots, which in turn potentially contribute to photosynthetic productivity (Osaki *et al.*, 1997).

Prior flooded and unflooded field trials in 2007 and 2008 identified genotypes with extreme grain-K, -Fe, -Zn or -Mo concentrations. My hypothesis was that these genotypes had different root morphological and physiological traits that influenced element absorption and uptake which in turn, influenced grain element concentrations. I further hypothesized that these high grain-K, -Fe, -Zn and -Mo genotypes would reveal distinct root traits associated with a particular element uptake 4WAG (~ V4 growth stage) which may carry over to the grain-filling stage. The overall goal of this research was to identify genes that are responsible for the uptake, transport and accumulation of four elements of interest in this study – K, Fe, Zn and Mo. This will help breeders develop rice genotypes with improved grain concentrations of these elements. The distinct

root traits identified will be used as bases for identifying genes associated with these elements acting in early vegetative stages. The specific objectives of this study were 1) to analyze and compare root traits of 24 genotypes 4WAG (~V4 growth stage) which included 23 genotypes from around the world known for their phenotypic variation in grain-K, -Fe, -Zn and -Mo concentrations and 'Lemont', a standard US genotype and 2) to identify distinct root traits associated with the various shoot elemental concentrations.

MATERIALS AND METHODS

Plant environmental conditions and general setup

This hydroponic experiment was conducted in 2012 at the Texas A&M AgriLife Research Center at Beaumont, Texas, USA, under controlled conditions in a growth chamber set at 30/25 °C (day/night) temperature and 10-h photoperiod (daylength from 0800 h to 1800 h CST) illuminated using white fluorescent lamps (55 µmol m^{-2} s^{-1}) and incandescent bulbs (40 W) producing 200 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The experiment design was a randomized complete block consisting of 24 genotypes. The experiment was repeated three times with pot locations randomized between the repeats, and each repetition had two plants per genotype. Out of the 24 genotypes, Lemont represented US germplasm and 23 other genotypes represented germplasm from around the world with extreme grain-K, Fe, Zn or Mo concentrations.

Preparation of plants and hydroponic system

Pre-soak

Seeds were soaked in reverse osmosis water for better, faster and more uniform germination. I chose Reverse Osmosis (R.O) water for this experiment as seeds responded with better germination in R.O. water compared to distilled water or nano-pure water in pre-experiment trials. A Jiffy-24 cell seed starter planting tray (28 cm long x 18 cm wide) (Jiffy Products of America Inc., Batavia, IL) was clearly labeled and lined with paper towels (page 140, Fig. 24). Each cell (4 cm x 4 cm) received five seeds of the appropriate genotype and the tray was placed inside a plastic tub (32 cm long x 26 cm wide x 7 cm high) filled approximately 2.5-cm deep with 500 ml of R.O. water. Tubs were covered with Reynolds 900 clear wrap (Alcoa Inc., New York, NY) to prevent evaporation and placed approximately 1.5 m away from the lights in a growth chamber for 24 h.

Netted foam discs

One-cm thick rings were sliced from black foam tubes 2.5-cm outer diameter x 2-cm inner diameter. Black nylon netting was sandwiched between two rings and stitched together using a Micro Stitch Gun (Avery Dennison, Pasadena, California).

Seed germination

Pre-experiment trials on filter papers inside petri-dishes caused agravitropic or deformed roots. Hence, seeds were germinated over sand for better root establishment and to ensure natural root architecture. Washed sea-sand (Fisher Scientific, Pittsburgh, Pennsylvania) was used to fill a clear plastic box (40 cm long x 27 cm wide x 23 cm) to a depth of approximately 2.5 cm; the sand was moistened with R.O. water to the saturation point; then excess water was blotted away using paper towels to prevent puddling. Discs were uniformly spread over the sand (page 141, Fig. 25). Inside of each disc, sand was added until its upper surface was level with the netting which allowed sand to wick water up to each seed, providing uniform moisture at the center of each disc. A single pre-soaked seed per disc was placed with forceps at the center of the netting, covered with sand and moistened using a hand-held squirt bottle. The box was covered with Reynolds 900 Clear Wrap (Alcoa Inc., New York, NY) to retain moisture and placed approximately 1.5 m away from the lights in a growth chamber for two weeks for germination.

Preparation of seedlings

To loosen the 2-week old seedlings for placement into hydroponics, the germination box was filled with R.O. water to an approximate depth of 5 cm and gently agitated to loosen the roots from the sand with minimal injury (page 142,

Fig. 26). Each seedling with disc intact was carefully uprooted, and rinsed thoroughly with running water (R. O).

Preparation of Styrofoam coasters

In this study, it was decided that the root traits would be analyzed using a WinRhizo Pro (Regent Instruments, Quebec, Canada) system that required easy detachment and spreading while scanning from seedlings harvested just prior to the scanning process. Hence, seedlings were grown on floating Styrofoam coasters (page 143, Fig. 27). One major advantage of floating coasters is that the roots were always immersed in nutrient solution and never exposed to air or light. This is in contrast to hydroponic systems that use stationary plant supports where nutrients were refilled only on a daily or weekly basis, leaving parts of the root systems suspended in the air, which could possibly trigger stress responses. Using a power drill, Styrofoam sheets of 2.5-cm thickness were cut into 7.5-cm diameter circles with a 2-cm hole at the center to contain the seedling. A strip of sponge (2.5-cm long x 1-cm wide x 0.5-cm thick) that was covered in a black plastic sheet was wrapped around each seedling at the transition between the root and shoot and then inserted at the center of the coaster. Sponge was chosen as the seedling-plug because when it becomes soaked in the medium it provides a flexible cushion. This cushion is pliable enough to eliminate girdling of the seedling yet at the same time is sufficiently firm enough to mechanically support the seedling holding it in place at an upright

position in the coaster. The sponge was wrapped in the black plastic sheet to block light and to prevent the growth of algae which could alter root traits.

Hydroponic setup

Each seedling mounted on the coaster was transferred to a clear cylindrical glass vase 25-cm high x 8-cm wide wrapped with aluminum foil (page 144, Fig. 28). The coaster kept the seedlings afloat while the roots were immersed in hydroponic solution. Each vase contained 1 L of hydroponic medium comprised of 1 g L^{-1} Peters Professional 20-20-20 supplemented with 0.1 g L^{-1} $FeSO_4$ as per Adorada and Gregorio (2009), 0.74 g L^{-1} $CaNO_{3,}$ 0.005 g L^{-1} $CaCl_2$ and 0.03 g L^{-1} $CaCO_3$ (Salisbury and Ross, 1978) brought to volume with R.O. water. Each vase was aerated using a 0.5-mm inner diameter airline tubing (Fisher Scientific, Pittsburgh, PA) connected to Whisper® air pumps (Tetra Inc., Blacksburg, Virginia) through manifolds and syringe needles (Fisher Scientific, Pittsburgh, PA) of 25-gauge size and 0.5-mm outer diameter. The airline tubing was weighted near the outlet using a white paper clip without obstructing the air flow. Aeration provided oxygen to the roots and ensured movement in the solution that avoided stagnation. The medium was replaced once weekly with fresh hydroponic medium until plant harvest. The Styrofoam discs were topped with black foam felt cut into 8-cm diameter circles slit half way to contain the seedling. Together, the black foam felt on the surface of the medium and the aluminum foil wrapped around the vases blocked light and

prevented algae from growing in the medium, which could have subsequently altered root traits and nutrient availability.

Parameters measured at the time of transplanting

Shoot traits such as plant height, number of leaves, and leaf color were measured at the time of transplanting to ensure that all genotypes had uniform germination and had not encountered any variances during the two-week germination period on the sand culture. Plant height was determined from the base of the shoot to the highest reaching leaf tip. The number of leaves was recorded as the number of fully opened leaves followed by a decimal representing the percentage of the newest leaf emerged (e.g. 1.2 indicated one fully opened leaf plus approximately 20% of the second leaf emerged). Leaf color was determined using a leaf color chart (IRRI-Phil Rice, Manila, Philippines) by visually rating leaves on a scale from 1 (bright yellow) to 3 (dark green)

Parameters measured during the growing season

Solution pH and Oxidative Reductive Potential (ORP)

The pH and ORP of the nutrient solutions were measured at weekly intervals using a hand-held dual pH /ORP meter (HI 98121, Hanna Instruments, Woonsocket, RI) just prior to replacing the solution (page 145, Fig. 29).

Leaf photosynthesis

Photosynthesis was measured 2WAT (4WAG, ~V4 growth stage) using a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE). The ambient carbon dioxide concentration (400 µmoles $mole^{-1}$), temperature (30 °C), photosynthetic photon flux density (PPFD) (1500 µmol m^{-2} s^{-1}), and leaf area (6 cm^2) were set prior to taking measurements between 10:00 h and 12:00 h on the most recent fully opened leaves of the main tiller.

Parameters measured at the time of harvest

Shoot traits

Plants were harvested 15 DAT (29 DAG) and rinsed thoroughly under running R.O. water. Plant height, number of leaves, leaf color, and number of tillers were determined. Relative leaf area was determined from the most recently fully opened leaf by multiplying leaf blade length by leaf blade width at its maximum width.

Shoot biomass

Shoots were separated from roots and fresh and dry weights (dried at 70°C for 48 h) were determined.

Root traits

Roots were thoroughly rinsed with R.O. water. Excess water was removed by gently pressing roots between paper towels. Roots were carefully spread without overlap and scanned using a WinRhizo scanner (Regent Instruments, Quebec, Canada) (page 146, Fig. 30); root traits; namely root area, surface area, length, volume and diameter were analyzed from scanned digital root images using WinRhizo Pro software (Regent Instruments, Quebec, Canada).

Root respiration

After scanning roots, a portion of each root mass was used for measuring root respiration (Ota, 1970) (page 147, Fig. 31). Fifty mg of roots were weighed, cut into small pieces and transferred to a 2-ml centrifuge tube containing 1.25 ml of 20 µg g^{-1} α-naphthylamine solution (Fisher Scientific, Pittsburgh, PA). Roots were incubated at room temperature under continuous shaking for 2 h. Fifty µl α-naphthylamine solution was pipetted into a 2-ml centrifuge tube (Fisher Scientific, Pittsburgh, PA); containing 250 µl distilled water, 25 µl of 1% (w/v) sulphanilic acid in 30% acetic acid (v/v) and 25 µl of 100 µg g^{-1} sodium nitrite solution, before and after incubation with roots. After 60 minutes, samples were transferred to a 96-well plate and absorbance was recorded at 500 nm using a micro-plate spectrophotometer (PowerWaveX; Biotek Instruments Inc, Winooski, VT). Calibration curves were obtained using different concentrations of 20 ml α-

naphthylamine solutions, and the quantity of α-naphthylamine oxidized was calculated in μg g^{-1} g^{-1} h^{-1}, which is proportional to the rate of respiration.

Root biomass

Following root respiration measurements, root fresh weights were determined. Roots were collected in paper bags then kept at 70 °C for 48 h and dry weights were determined.

Element analysis

Root and shoot element concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS) courtesy of David Salt, University of Aberdeen, UK (Salt *et al.*, 2008). The samples were digested overnight with nitric acid at room temperature followed by heat-digestion using a heat block before being passed through ICP-MS (Danku *et al.*, 2012).

Data analysis

All data were subjected to analysis of variance (ANOVA) and means were separated using Tukey's least significance difference (LSD) at $P \leq 0.05$ using SPSS (IBM-SPSS Inc., Armonk, New York). Correlations were completed using SPSS (IBM-SPSS Inc., Armonk, New York) to identify associations between root traits and shoot elemental concentrations. Principal Component Analyses (PCA) with Varimax rotations were also conducted using SPSS (IBM-SPSS Inc.,

Armonk, New York) to identify a reduced set of independent variables. For the PCAs, independent variables were removed as needed to eliminate correlations among variables greater than 0.8, as well as to eliminate variables without any correlations to other variables greater than 0.2.

RESULTS AND DISCUSSION

None of the genotypes selected for high grain-Zn or-Fe concentration showed high shoot-Zn or -Fe concentration at 4WAG (~V4 growth stage). This is in contrast to the earlier vegetative leaf study (Chittoori et al. prepared, chapter II), where high grain-Zn genotype GSOR 310823 showed high leaf-Zn concentrations. However, among the five GSORs selected for their high grain-K concentrations (310356, 310742, 311007, 311012, and 31106), three (GSORs 310742, 3101007, and 31106) showed high shoot-K concentrations at 4WAG (~V4 growth stage) (page 148, Fig. 32). This is again in contrast to the earlier vegetative leaf study (Chittoori et al. prepared, chapter II), where these three genotypes showed relatively low leaf-K concentrations. It is interesting to note, however, that several genotypes which did not exhibit high grain-K concentrations in the prior study also showed high shoot-K concentrations (page 148, Fig. 32), suggesting that genotype variation in shoot/leaf-K accumulation is highly media dependent. Among GSORs 310354, 310355, 310356, 310823, 311643, and 311735 selected for high grain-Mo concentrations, GSORs 310355, 310356 and 311643 showed high shoot-Mo concentrations 4WAG (~V4 growth

stage) (page 149, Fig. 33). These are the same genotypes that showed high leaf-Mo concentrations in the earlier vegetative leaf study (Chittoori et al. prepared, chapter II) as well, indicating that genotype variation shoot/leaf-Mo accumulation is relatively independent of medium. Regardless of the differences in shoot-K and -Mo concentrations, genotypes showed no significant differences in their root fresh and dry weights, root area, root surface area, root volume, total root length and root respiration rates thus suggesting that none of the observed root morphological traits nor the physiological trait (root respiration) was associated with their differences in shoot elemental concentrations. Pearson correlation analyses did not detect significant associations between root traits and shoot-elemental concentrations. PCA was conducted to identify a recombined and reduced set of independent variables called principal components that explain the maximum variances. The PCA took the multivariate data cloud, consisting of the combined set of root and shoot morphological and physiological traits along with root and shoot element concentrations, and orthogonally rotated the axes to the directions that explained the maximum variances using a reduced set of independent variables (principal components). However, no root traits were among those found to play roles in the 1^{st} or 2^{nd} components found by PCA.

Shoot dry weight, a measure of seedling vigor and plant health among the study genotypes, showed significant and positive correlations with beneficial elements such as K, Zn, Mn, and Cu and was negatively correlated with Na, Cr,

Ni, As, and Se (page 176, Table 9). Deficiencies of K, Zn, Mn, and Cu are known to reduce shoot dry weights in rice (Arif *et al.*, 2008; Karim and Vlamis, 1962). Toxic elements such as Na, Cr, Ni, As, and Se have been shown to decrease shoot dry weight with an increase in their concentrations, even when not at toxic levels (Maheshwari and Dubey, 2009; Mahmood *et al.*, 2009; Mikkelsen and Wan, 1990; YiZhong *et al.*, 2010). Toxic elements disrupt redox homeostasis at the cellular level, thus adversely affecting photosynthesis and production of photosynthates resulting in shoots with reduced dry weight. All genotypes except GSORs 310167, 310356, 311007, 311621 and 311735 showed more shoot dry weight (page 150, Fig. 34) and lesser concentrations of toxic elements (data not shown), suggesting they likely have adequate production of ATP and NADPH, which are needed for active element uptake. For genotypes with high grain concentrations of K, Zn, Fe or Zn, the active uptake mechanisms might also be regulated differently, but this aspect was not examined in the present set of studies. All genotypes except for GSORs 310167, 310356, 311007, 311621 and 311735 serve as good candidates for plant breeders targeting the development of nutritionally enhanced genotypes also possessing low levels of toxic elements.

CONCLUSIONS

The observed root morphological and physiological traits were not found to be associated with shoot-K, -Fe, -Zn or -Mo concentrations, whereas

measures of seedling vigor and health were positively associated. Nor did any of the observed root traits explain the previously observed differences in grain element concentrations. Since the underlying causes of the observed shoot and grain differences were not explained even to a small degree by individual nor combinations of the observed root traits, other unobserved factors must be involved. For example, differences in the shoot elemental concentrations in these genotypes may instead be due to active uptake mechanisms.

CHAPTER V

ROOT MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSE TO AUXIN SEED TREATMENT IN RELATION TO SHOOT ELEMENTAL CONCENTRATIONS WITHIN A SET OF DIVERSE RICE (*ORYZA SATIVA*) GENOTYPES

INTRODUCTION

This study further addresses the hypothesis that rice (*Oryza sativa* L.) genotypes selected for extreme grain-K, -Fe, -Zn or -Mo concentration in a previous field study differ in root morphological and physiological traits that influence element absorption and uptake at a young vegetative stage in a manner consistent with an ultimate influence on grain element concentrations. The approach was to perturb root growth and development using a form of auxin known to alter roots. This perturbation would help to separate effects of gross root morphology and physiology from any heritable variation in gross root morphology and physiology among the genotypes that were relatively unrelated to element absorption and uptake.

Rice roots undergo morphological and physiological adaptation (e.g., aerenchyma) depending on flooded and unflooded conditions (Atkinson, 1990; Chen *et al.*, 2009; Clark, 1990; Counce *et al.*, 2002; Kirk, 1994; Zheng *et al.*, 2000). Root morphological traits like root length, area, volume, etc. influence

element absorption/uptake (Chen *et al.*, 2009; Wang *et al.*, 2008; Wang *et al.*, 2006). Root physiological processes like root respiration alter physical, chemical and biological properties of soil thus impacting element solubility, availability and mobility in soil and uptake into roots (Fageria and Stone, 2006). Root respiration and photosynthesis are interconnected (Osaki *et al.*, 1997). An increase in photosynthesis increases the photosynthates reaching the roots and this in turn can potentially increase element absorption by providing substrates for root exudates and energy-rich compounds to promote active uptake processes. Higher root respiration supplies higher rates of elements to shoots which can in turn ensure productivity (Osaki *et al.*, 1997). Chemical factors such as pH and redox potential of the growth media influence element availability, solubility and absorption by roots, thus influencing element concentrations (Norman *et al.*, 2002).

Prior flooded and unflooded field trials in 2007 and 2008 identified genotypes with extreme grain-K, -Fe, -Zn or -Mo concentrations hypothesized to be due to variation in root morphological and physiological traits. I hypothesized that these genotypes showed distinct root traits associated with their shoot-K, -Fe, -Zn and -Mo concentrations. To better analyze for these potentially related distinct root traits, a perturbation technique (Eng *et al.*, 2009) was used in this study. This is a common approach in plant biology research whereby the function of the biological system is altered to compare the effects in control (untreated) and treated plants. Perturbation was accomplished using a seed

treatment of 1-naphthalene acetic acid (NAA), an auxin plant growth regulator (PGR), that alters root growth and development (Aloni *et al.*, 2006). The goal of the previous study (Chapter IV) was to identify distinct root traits responsible for the observed differences in grain and shoot element concentrations, with the idea that the rood differences could be used as bases for identifying genotypes and genes associated with enhanced uptake, transport and accumulation of these elements. This study wherein root differences were induced with NAA treatment was conducted to clarify how root differences can impact grain element uptake. The specific objectives of this study were 1) to analyze and compare root traits 3WAG (~ V3 growth stage), in control versus PGR-treated plants of 24 genotypes which included Lemont (a standard US genotype) and 23 genotypes from around the world known to have genetic differences in their grain-K, -Fe, -Zn and -Mo concentrations, and 2) to identify distinct root traits and their associations with shoot-K, -Fe, -Zn, and -Mo concentrations.

MATERIALS AND METHODS

Plant environmental conditions and general setup

This sand-culture experiment was conducted in 2012 at the Texas A&M AgriLife Research Center at Beaumont, Texas, USA, in a growth chamber at 30/25 °C (day/night) under a 10-h photoperiod (daylength from 8:00 h to 18:00 h) using white fluorescent lamps (55 µmol m^{-2} s^{-1}) and incandescent bulbs (40 W) producing 200 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The

experiment had a randomized complete block design which included 24 genotypes consisting of 'Lemont,' representing US germplasm, plus 23 other genotypes from around the world known to have extreme grain-K, -Fe, -Zn or -Mo concentrations. The experiment was repeated four times (repeated over time in the same growth chamber) and each repetition had two plants per genotype per treatment and pots were randomized each time.

Preparation of plants and sand culture

Seed treatments

A Jiffy-24 cell seed starter planting tray (28 cm long x 18 cm wide) (Jiffy Products of America Inc., Batavia, IL) was lined with paper towels (page 151, Fig. 35). Each cell (4 cm x 4 cm) was clearly labeled and 10 seeds of the appropriate genotype were placed in each cell, one cell per genotype. The tray was placed in a plastic tub (32 cm x 26 cm x 7 cm high) filled approximately 2-cm deep with 500 ml of 2 µg g^{-1} NAA (Sigma-Aldrich, St. Louis, MO) solution. The solution was made by dissolving 1 mg of NAA in 500 ml of reverse osmosis (R.O.) water. I chose R.O. water throughout this experiment as seeds gave better germination percentages in R.O. water when compared to distilled water or nano-pure water in pre-experiment trials. The control group was housed in a similar way in a different tub filled with R.O. water. The tubs were covered using Reynolds 900 Clear Wrap (Alcoa Inc., New York, NY) to prevent evaporation

and were placed approximately 1.5 m away from the lights in the growth chamber for 24 h.

Sand culture setup

Clear cylinder glass vases (15-cm high x 7-cm wide) wrapped with aluminum foil were filled with washed sea-sand (Fisher Scientific, Pittsburgh, PA) approximately 13-cm deep leaving 2 cm space on the top for nutrient fertilization (page 152, Fig. 36). Sand was moistened with R.O. water and one platinum electrode (personal communication, Richard Loeppert, locally constructed by inserting 10-gauge 2-cm long platinum wire 1-cm deep inside 30-cm long Cu wire and junction glued using epoxy glue) per vase was placed 10 cm deep. Five seeds per genotype were planted in each vase with approximately 1-cm spacing and at a 2-cm depth. A week after sowing, seedlings were thinned to just one plant per vase. Seedlings were fertilized with nutrient solution containing 1 g L^{-1} Peters Professional 20-20-20 (Everris International B. V., Geldermalsen, The Netherlands) supplemented with 0.1 g L^{-1} $FeSO_4$ (2009), 0.74 g L^{-1} $CaNO_3$, 0.005 g L^{-1} $CaCl_2$, and 0.03 g L^{-1} $CaCO_3$ (Salisbury and Ross, 1978). Following the first nutrient fertilization, the surface of the sand was covered with a black plastic sheet with a slit at the center to contain the seedling. The black plastic sheet on the surface along with aluminum foil wrapped around the vases blocked light and prevented algae from growing in the medium, which could alter nutrient availability and root traits. Fertilization was completed on a weekly basis until harvest and was poured into the vase

until the saturation point (~ 250 ml). Nutrient solutions were made fresh at the time of fertilization.

Parameters measured during the growing season

Redox potential

Redox potential was manually measured every week until harvest, just before fertilization, using a hand-held digital voltmeter connected to an Orion 900100 Ag/AgCl reference electrode, which was placed temporarily on the soil surface at the time of measurements (Thermo Scientific, Pittsburgh, PA) (page 153, Fig. 37) and prior to fertilization.

Leaf photosynthesis

Photosynthesis was measured 3 WAG (~ V3 growth stage) using a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE). The ambient carbon dioxide concentration (400 µmoles $mole^{-1}$), temperature (30 °C), photosynthetic photon flux density or PPFD (1500 µmol m^{-2} s^{-1}), and leaf area (6 cm^2) were set prior to taking measurements between 1000 h and 1200 h on the recent fully opened leaves of the main tiller.

Parameters measured at the time of harvest

pH of soil solution

Five g of sand was sampled from each vase at approximately a 2-cm depth and transferred into a plastic cup filled with 25 ml of distilled water to get a

1:5 ratio of soil to water (page 154, Fig 38) (Sparks, 1996). The mixture was stirred well using a glass rod and after 60 minutes, pH was measured using a hand-held pH meter (HI 98121, Hanna Instruments, Woonsocket, RI).

Shoot traits

Plants were carefully uprooted 22 DAG and rinsed thoroughly with running R.O. water. Plant height (from the base to the highest reaching leaf tip), and number of tillers were determined. The number of leaves was recorded as the number of fully opened leaves along the main culm followed by a decimal representing the percentage of the newest leaf emerged (e.g., 5.2 indicated five fully opened leaves plus approximately 20% of the sixth leaf emerged). Relative leaf area was determined from the most recently fully emerged leaf by multiplying leaf blade length by leaf blade width at its maximum width. Leaf color was determined using a leaf color chart (IRRI-Phil Rice, Manila, Philippines) by visually rating them on a scale from 1 (bright yellow) to 3 (dark green).

Shoot biomass

Shoot fresh and dry weights (dried at 70°C for 48 h) were determined.

Root traits

Roots were thoroughly rinsed with running R.O. water then were gently pressed between paper towels to remove excess water. Roots were evenly

spread without overlap and scanned using a WinRhizo scanner (Regent Instruments, Quebec, Canada) scanner; root traits, namely root area, surface area, length, volume and diameter were analyzed from scanned digital root images using WinRhizo Pro (Regent Instruments, Quebec, Canada) software.

Root respiration

After scanning roots, 50 mg of roots were weighed and cut into small pieces and transferred to a 2-ml centrifuge tube containing 1.25 ml of 20 µg g^{-1} (w/v) α–naphthylamine solution (Fisher Scientific, Pittsburgh, PA) (Ota, 1970). Roots were incubated under continuous shaking for 2 h at room temperature. After incubating with roots, 50 µl of α–naphthylamine solution was pipetted into a 2-ml centrifuge tube (Fisher Scientific, Pittsburgh, PA) containing 250 µl of distilled water, 25 µl of 1% (w/v) in 30% [v/v] acetic acid) sulphanilic acid and 25 µl of 100 µg g^{-1} sodium nitrite solution. After an hour, samples were transferred to a 96-well plate and absorbance was recorded at 500 nm using a spectrophotometer (PowerWaveX; Biotek Instruments Inc, Winooski, VT). Calibration curves were obtained using different concentrations of 20-ml α–naphthylamine solutions and the quantity of α–naphthylamine oxidized was calculated in µg g^{-1} g^{-1} $h^{-1,}$ which is proportional to the rate of respiration.

Root biomass

Following root respiration measurements, root fresh and dry weight (kept at 70°C for 48 h) were determined.

Element analysis

Root and shoot element concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS) courtesy of David. Salt, University of Aberdeen, UK (Salt *et al.*, 2008). The samples were digested overnight with nitric acid at room temperature before being passed through an ICP-MS (Danku *et al.*, 2012).

Data analysis

Data were subjected to analysis of variance (ANOVA) and means were separated using Tukey's least significance difference (LSD) at $P \leq 0.05$ using SPSS (IBM-SPSS Inc., Armonk, New York). Correlations were completed using SPSS (IBM-SPSS Inc., Armonk, New York) to identify associations between root traits and shoot elemental concentrations. Principal Component Analyses using mean-centered and Z-scaled data (i.e., correlation table as input) with Varimax rotation were also conducted using SPSS (IBM-SPSS Inc., Armonk, New York) to identify a reduced set of independent variables from among the set of root and shoot morphological and physiological traits and element concentrations. No effort was made to account for NAA treatments in the PCA. For the PCAs, independent variables were removed as needed to eliminate correlations among

variables greater than 0.8, as well as to eliminate variables without any correlations to other variables greater than 0.2. Genotype means of control and NAA treatments were used to examine the likelihood that the number of genotypes showing an increase or decrease in the level of a gross morphological trait, physiological trait, or root or shoot element concentration was not random. For this, standard deviations were calculated according to Gnedenko and Khinchin (1962) and used to determine Z-scores for the event counts according to Snedecor and Cochran (1980). A conservative test value ($P < 0.001$) was used for these treatment comparisons.

RESULTS AND DISCUSSION

Control plants of high grain-K, -Fe, -Zn and -Mo genotypes were hypothesized to show high shoot-K, -Fe, -Zn and -Mo concentrations with different associable root morphological and physiological traits 3WAG (~ V3 growth stage) for the particular elements. However, in agreement with the hydroponics experiment (Chapter IV), only control plants of high grain-Mo genotypes GSORs 310354, 310355, 310356, 311643, and 311735 showed high shoot-Mo concentrations at 3WAG (~ V3 growth stage) (page 155, Fig. 39), again indicating that genotype variation in shoot/leaf-Mo accumulation is relatively independent of media effects. However, within-genotype variation in root morphological and physiological traits was relatively high (data not shown), indicating that these high shoot-Mo genotypes (GSORs 310354, 310355,

310356, 311643, and 311735) did not possess distinct root morphological or physiological traits that contributed to or that were affected by high shoot-Mo concentrations.

A weakness of a study that compares different phenotypic traits, such as morphology and element concentrations, is the potential to detect associations that include unrelated genotypic variation in traits, for example in root morphology. Therefore seed treatments with NAA were provided to perturb development and clarify associations between distinct root traits with shoot-K, -Fe, -Zn or -Mo concentration. Preliminary trials on these genotypes with different concentrations of NAA showed visual differences in root biomass & root diameter at 2 WAG when treated with 2 µg g^{-1} NAA (results not shown). Hence 2 µg g^{-1} NAA was chosen for the current study. I also considered that auxin seed-treated plants cultured under field conditions retained auxin effects on root biomass until at least 6WAG (personal communication, Lee Tarpley, Texas A&M AgriLife Research at Beaumont, Texas). Hence, treated plants in the current study were assumed to retain NAA effects until 3WAG (~ V3 growth stage), the chosen length of the study. However, only one of the 24 genotypes, GSOR 310715, displayed NAA effects at 3WAG (~ V3 growth stage) by showing within-genotype differences in root traits when compared to untreated controls. The rest of the genotypes presumably had diminished NAA efficacy by 3WAG resulting in similar root traits as that of the control (but see results from comparisons across genotype means below). The NAA-treated plants of GSOR

310715 showed more total root length (page 156, Fig. 40) and smaller root diameters (page 135, Fig. 41) than control plants. GSOR 310715 was selected for high grain-Fe and -Zn concentrations and although treatment differences in total root length and diameter were present, PGR-treated plants showed similar shoot-Fe and -Zn concentrations as that of control plants (data not shown). This suggests that neither total root length nor diameter was a key factor determining the shoot-Fe or -Zn concentration of GSOR 310715. The PGR-treated plants of GSOR 310715 also showed more shoot dry weight when compared to the control (page 136, Fig. 42). It may also be possible that the increased shoot dry weight caused dilution of shoot-Fe and -Zn concentrations resulting in similar concentrations as that of control plants.

Although an examination of individual genotypes failed to show treatment differences in root morphology or physiology that could be related to genotypic differences in shoot concentrations of K, Fe, Zn or Mo, the possibility remained that differences that involved relatively small changes in numerous morphological/physiological root traits occurred resulting in a shift in overall root architecture and/or physiology that could be important in affecting or being affected by variation in tissue element concentrations. Principal Component Analysis (PCA) was used for examining for possible multivariate effects. The PCA took the multivariate data cloud, consisting of the combined set of root and shoot morphological and physiological traits along with root and shoot element concentrations, and orthogonally rotated the axes to the directions that

explained the maximum variances using a reduced set of independent variables (principal components). Although this analysis identified a number of principal components with an eigenvalue greater than 1 (a common cut-off point for possible significance in PCA), the first two components strikingly stood out from the rest with respect to the amount of variance explained (high eigenvalue) (page 159, Fig. 43). The first component (PC1) was positively governed by total root length (0.824) and root fresh weight (0.719) (page 177, Table 10). The first component was also positively governed by shoot dry weight (0.824) leaf area (0.812), plant height (0.778), shoot-K concentrations (0.761), leaf number (0.563), and photosynthesis (0.523) and negatively governed by shoot-Se (-0.834), shoot-Cd (-0.646), shoot-Ca (-0.559), shoot-Fe (-0.549), shoot-As (-0.516), shoot-Na (-0.504), and shoot-S (-0.500). The root and shoot gross morphological traits, along with photosynthesis, that positively loaded on PC1, tentatively identify PC1 as primarily a seedling vigor/plant health component. K is an important macro element and its deficiency has been shown to affect shoot growth, shoot dry weight, number of leaves, leaf area, plant height and photosynthesis in various crops (Arif *et al.*, 2008; Pervez *et al.*, 2006; Pettigrew, 2008). Heavy elements such as Se, Cd, As and Na, though not at toxic levels, affect cell redox and metabolic activities, thus affecting plant growth (Mahmood *et al.*, 2009; Mikkelsen and Wan, 1990; Waisel *et al.*, 1996; YiZhong *et al.*, 2010). The negative loadings of Ca, Fe and S hint at an indirect effect of soil pH

or redox potential, but the lack of loading of soil pH and redox potential on PC1 do not support this relationship.

Although a comparison of treatment effects within genotypes might not show significant differences, such a comparison does not eliminate the possibility that NAA caused small, consistent effects that an analysis looking across genotypes could detect. Based on comparisons of genotype means for treated and control plants, NAA treated plants of the genotypes showed increases ($P < 0.001$) in root-As and -Fe concentrations and decreases in soil pH, soil redox potential 1WAG, root and shoot-Zn concentrations and root-Ni concentrations, root fresh weight, and shoot fresh and dry weights, when compared to the control (pages 160-169, Figs. 44- 53). The NAA was used in this study based on the expectation that the treatment would affect root morphology. The preliminary results from 2WAG indicated that the morphology was affected. However, based on this analysis, the NAA effects were stronger on soil pH and redox potential and/or directly on the elements than on the root morphological traits. The NAA transporter, *AtPIN1*, has been shown to transport arsenic acids (Muller *et al.*, 1998), and it may be due to up regulation of this transporter that root-As concentration increased with NAA treatments (page 160, Fig. 44). The NAA has been shown to up-regulate ferric reductase oxidase enzyme activity accompanied by proton extrusions which enhance Fe uptake (Chen *et al.*, 2010; Wu *et al.*, 2011). These may have caused an increase in root-Fe concentrations (page 161, Fig. 45) and a decrease in pH (page 162, Fig.

46). In addition, auxin is well-known for promoting proton extrusion, and thus decreasing pH of the local soil environment (Hager *et al.*, 1991). The pH also impacts element availability (Fageria and Stone, 2006). The NAA treatments resulted in pH 7.5. Because Zn and Ni are not readily available to plants at neutral pH (Sanders *et al.*, 1986), this change in pH could possibly have caused the observed decreased root-Ni (page 164, Fig. 48) concentrations and root and shoot-Zn concentrations (pages 165--166, Figs. 49- 50). Zinc is needed for the normal growth of the shoots (Sommer and Lipman, 1926). Because Zn is not available at pH 7.5, shoot growth may have been affected resulting in decreased shoot fresh and dry weights (pages 167-168, Figs. 51-52). Auxin can inhibit root elongation in some circumstances (Eliasson *et al.*, 1989) and may have resulted in decreased root fresh weights with NAA treatment (page 169, Fig. 53). Neither pattern of change in root concentrations of all elements examined or of root-to-shoot ratios of element concentrations indicate that the observed changes in some of the root element concentrations due to NAA treatment was an indirect effect on root adsorption of the elements associated with the NAA effect on soil pH (results not shown).

CONCLUSIONS

Shoot K concentrations were positively associated with measures of seedling vigor/plant health, but not with distinct root morphological traits. Shoot Fe concentrations were increased and root and shoot Zn concentrations were

decreased with NAA treatment when examined across the 24 genotypes. However, no distinct root morphological or physiological characters were affected by the NAA treatment. Instead NAA affected several shoot and root biomass characters along with soil pH and redox, indicating that the observed NAA effects on Fe and Zn concentrations were directly through effects on element uptake or root-to-shoot transfer or indirectly through pH and redox potential effects. In agreement with the findings of Chapter 2, genotype variation in shoot Mo concentrations appears to be largely independent of variation in root and shoot morphology and of soil pH. Although root morphological and physiological characteristics are not associated with shoot K, Fe, Zn or Mo concentrations, the reasons for the lack of association appear to differ by element, thus necessitating different approaches for these different elements in determining influences, and ultimately mechanisms, alleles and genes, affecting their shoot concentrations.

CHAPTER VI

SUMMARY

The goal of this research was to develop knowledge on the mechanisms underlying grain element accumulation. This improved understanding of the underlying physical and chemical mechanisms could lead to defined studies aimed at identifying the genes responsible for the uptake and accumulation of K, Fe, Zn and Mo in rice grain. Specifically the objective of my research was to determine which root gross morphological and physiological traits govern the uptake and accumulation of these individual elements in select genotypes. Different growth mediums including clay, hydroponics and sand culture were used to study root traits with respect to element uptake/accumulation including varying redox potential conditions. These root traits could then be used as bases for identifying genes for the same.

Different genotypes have different mechanisms of accumulating elements in grains which may or may not be noticeable in leaves. The first study investigated if genotypic variations in K, Fe, Zn and Mo accumulation patterns in grains were present also in leaves at a particular range of vegetative growth stages. The approach involved planting high grain-K, -Fe, -Zn and -Mo genotypes at set intervals to generate a wide range of vegetative growth stages on a single sampling date. Grain concentrations of elements would be expected to be sometimes controlled at the plant uptake level, leading to similar

accumulation patterns in leaves and grain, thus this study tested the hypothesis that the genotypes would show similar accumulation patterns to that of grain for many of these elements in leaves at some vegetative growth stages. High and low grain-K, -Fe, -Zn and -Mo genotypes were evaluated for their leaf-K, -Fe, -Zn or -Mo concentrations across different growth stages. Growth stages less than V2 were avoided for not having enough material for element analyses and the stages V2 to V4 were eliminated as showing nutritional dependence on seeds. The V4 to V6 range was concluded to be the optimal growth-stage range to potentially screen element accumulation patterns as several genotypes developed flag leaves by V7, which was not representative of vegetative leaf material. The V4 to V6 growth stage range was found viable when Mo and Co element concentrations, known for organ to organ associations of concentrations, showed similar leaf accumulation patterns as in their grains. Of the high grain-K, -Fe, -Zn and -Mo genotypes, the high grain-Mo genotypes showed similar accumulation patterns in leaves as in grains suggesting the roots' influence across a population of rice that was diverse genetically and phenotypically. Individual or a few genotypes with high grain-K, -Fe, or -Zn also had leaf accumulation patterns suggesting that roots could be influencing the grain accumulation patterns for these genotypes, thus providing the possibility of screening genetically segregating progeny when these genotypes were cross-parents.

The second study evaluated distinct root traits of high grain-Mo genotypes. Most of the high grain-Mo genotypes originated from Malaysia where soils are acidic with pH as low as 4.7. Mo is strongly adsorbed to soil and not available to plants at acidic pH. My hypothesis was that these high grain-Mo genotypes showed an acid-tolerance mechanism, which was root-localized, thus enabling them to efficiently mine Mo. The approach involved hydroponically growing a Malaysian genotype, GSOR 310356, in three pH regimes – 4.7, 5.4 and 6.1, and comparing root trait differences. A high grain-Mo genotype from Iraq GSOR 310823, and a low grain-Mo genotype from the US, Lemont, were also included in the study to represent different Mo uptake mechanisms. The Malaysian genotype showed distinct root traits at pH 4.7 with corresponding high shoot-Mo concentrations when compared to the other two genotypes; however, the differences among genotypes and among pH's indicated that the high Mo levels of the Malaysian genotype were not reflecting an acid-tolerance mechanism. It is not clear as to why a number of the Malaysian genotypes mine Mo in excess of their apparent needs, given that there was no corresponding significant increase in GSOR 310356 of shoot dry weight, leaf color or plant height.

The third study investigated root traits of high grain-K, -Fe, -Zn and -Mo genotypes in hydroponics media. My hypothesis was that these genotypes showed differences in their root gross morphological or physiological traits enabling them to accumulate high levels of K, Fe, Zn or Mo, respectively. The

approach involved growing genotypes in hydroponic media and analyzing root traits at 4 weeks after germination (WAG) or ~ V4 growth stages. However, for none of the elements did we find any root trait differences associated with shoot element concentration differences. Also, correlation analyses between individual root gross morphological and physiological traits with shoot element concentrations did not show any linear relationships that could help explain genotype variation in element accumulation patterns. Similarly, Principal Component Analysis (PCA) showed no associations between components of root gross morphology and physiology traits with shoot-K, -Fe, -Zn or -Mo levels. Of note, both correlation analysis and PCA showed positive associations of seedling vigor/plant health characteristics, e.g. shoot dry weight, with shoot concentrations of beneficial elements like K, Zn, Mn and Cu and showed negative associations with toxic elements like Na, Cr, Ni, As and Se. In the case of the toxic elements, this might indicate disruption of redox homeostasis at the cellular level even if the elements are present at sub-toxic levels. As in the first study, many of the high grain-Mo genotypes also displayed high shoot-Mo levels, thus supporting the notion that control is at the plant uptake level, but not one manifested as variation in one of the root gross morphological or physiological mechanisms documented in this study.

The fourth study investigated root gross morphological and physiological traits of high grain-K, -Fe, -Zn and -Mo genotypes in sand-culture media, which exposed plants to a soil redox potential (~ 310 mV) different (more reduced)

than that of the aerated hydroponics (~ 160 mV) experiment. NAA (auxin-type plant growth regulator) seed treatments were introduced as a perturbation of root development to further evaluate relationships between root traits and tissue element concentrations. These genotypes were hypothesized to show root trait differences between control and treated plants with corresponding differences in shoot elemental concentrations. The approach involved treating seeds with NAA and growing the control and NAA-treated plants until 3WAG (~ V3 growth stage). Root traits and corresponding shoot element concentrations were analyzed among and between controls and treated plants. Only one genotype, GSOR 310715, showed root trait differences (increased total root length and decreased root diameter) with NAA treatment. GSOR 310715 was selected for high grain-K and -Fe concentrations. Regardless of the root trait differences, the NAA treated and untreated GSOR 310715 plants showed similar shoot-K and -Fe concentrations. It is not clear why NAA treatment did not induce measurable root trait differences in other genotypes as observed at 3WAG (~ V3 growth stage) and showed no root gross morphological or physiological trait differences between control and treated plants, especially since the shoot traits indicated a lingering effect on shoot development and health. As was the case for the third study, PCA showed a positive association between shoot dry weight, and other measures of seedling vigor/plant health, with shoot-K concentration and a negative association with the toxic elements, Se, Cd, As and Na. In analysis of the differences between genotype means of the untreated controls and the NAA-

treated plants, NAA decreased soil pH and soil redox potential, along with affecting several shoot and root biomass characters, indicating that NAA effects on tissue Fe (increased) and Zn (decreased) concentrations were either directly through effects on element uptake or root-to-shoot transfer or indirectly through pH and redox potential effects. The more significant effects of NAA on soil pH and soil redox potential than on root gross morphological and physiological traits were unexpected. In agreement with the findings of the other studies, variation in shoot Mo concentration appears to be largely determined by genetics but independent of variation in root and shoot morphology and physiology and of soil pH.

Based on the results of my experiments, I conclude that there is no association between the observed root gross morphological and physiological traits and shoot-K, Fe, Zn or Mo concentrations among these genotypes and hence, these root traits may not serve as bases for identifying genes affecting grain element concentrations. In the first experiment, moderately high grain-K genotype, GSOR 310823 showed moderately high leaf-K concentrations. In the second experiment, GSOR 310823 showed moderately high shoot-K concentrations that increased with increase in pH as K is more available at higher pH. In the third experiment, GSOR 310823 showed moderately high shoot-K concentrations in hydroponics and Correlation Analysis showed shoot-K concentrations to be positively associated with root fresh weight. Most of the high grain-Mo genotypes, GSORs 310355, 310356, 311643 and 311735 showed

high shoot or leaf-Mo concentrations in all the experiments regardless of the growth medium- clay, hydroponics or sand. These genotypes showed diverse shoot phenotypes ranging from short to tall plants, and early flowering to late flowering, suggesting that their high grain-Mo was not driven by these attributes. Among the 24 genotypes characterized for root traits under hydroponic (Chapter IV) and sand culture (Chapter V), the high-Mo genotypes did not prove to be exceptional in terms of root number nor for any other of the measured root traits. The fact that Mo genotypes, GSORs 310355, 310356, 311643 and 311735 showed consistently high leaf or shoot-Mo concentrations in all of these experiments makes an exciting conclusion that neither the root nor the shoot phenotype or the candidate MOT1 drove Mo uptake or accumulation in these genotypes. Leaf iron concentrations varied little among genotypes compared to the variation for other elements (Chapter II), and there were no genotype differences in shoot-Fe concentrations in either the hydroponics study (Chapter IV) or the sand culture study (Chapter V). This relative lack of variation in shoot/leaf Fe concentration may reflect Fe homeostasis where plants maintain steady Fe levels to maintain redox equilibrium at the cellular level. Hence, in spite of high Fe in the rhizosphere, plants do not take up Fe in excess of their needs or if they did, excess Fe will be sequestered in the vacuoles (Stacey *et al.*, 2008).

Genetic variation in seedling vigor/plant health was found positively associated with several beneficial elements like K, Cu, Mn and Zn and

negatively associated with several toxic elements like Na, Cr, Ni, Se, As, and Cd. Hence, the identification of this variation might assist plant breeders seeking to develop genotypes with high beneficial elements and less toxic elements.

Future study on high grain-K and -Zn genotypes may focus on root hair traits such as length, width and number of root hairs which have been shown to be positively associated with K and Zn uptake. Because of Fe speciation at different pH and redox potentials, future study on high grain-Fe genotypes may focus on pH and redox-potential interactions with root traits and corresponding changes in Fe concentrations. Future study on high grain-Fe and -Zn genotypes may include root exudates as Fe uptake in rice involves Strategy II whereby roots secrete phytosiderophores (PS) forming PS-Fe and PS-Zn complex. In addition, other exudates have indirect effects on Fe and Zn availability. Since high grain-Mo genotypes showed consistently high grain and leaf-Mo concentrations, future study to identify genes and mechanisms underlying the enhanced Mo concentrations can be conducted using any and all of the growth conditions and plant stages documented in these studies.

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APPENDIX A

FIGURES

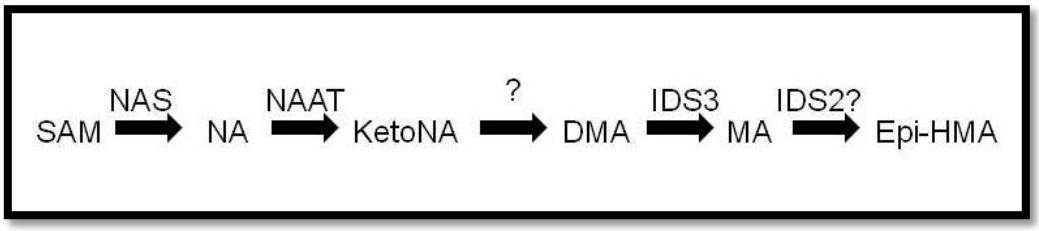


Figure 1. Phytosiderophore synthetic pathway in barley (*Hordeum vulgare*). SAM, S-adenosyl-L-methionine;NAS, nicotianamine synthase;NA, nicotianamine;NAAT, nicotianamineaminotransferase; KetoNA, Keto-nicotianamine; DMA, 2-deoxymugineic acid; IDS3, mugineic acid synthase; MA, mugineic acid; IDS2, dioxygenase; and Epi- HMA (Fushiya *et al.*, 1982), 3-hydroxymugineic acid (Negishi *et al.*, 2002).

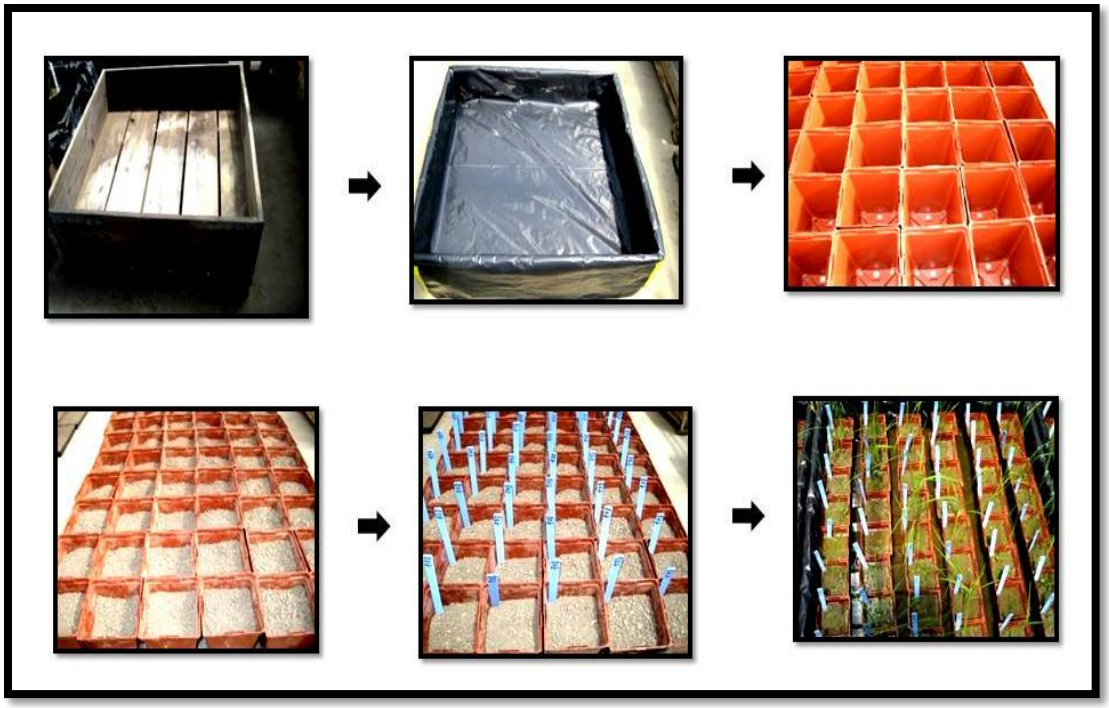


Figure 2. Different stages in the making of a set.
A wooden box (1m x 1m) was used to house plants. It was covered with black plastic sheet to contain water. Plants were grown in square pots (10 cm x 10 cm x 12 cm) filled 10-cm deep with Beaumont clay soil. Each pot was appropriately labeled for each genotype and five seeds per genotype were planted at 2.5-cm spacing between seeds and covered with 2.5-cm soil.

Figure 3. Plants at different growth stages.
The set of 40 genotypes was planted repeatedly at 7 to 10 day intervals to provide plants with a wide range of vegetative growth stages from which to obtain leaf samples on a single sampling date (the eldest set in extreme left and the youngest in extreme right).

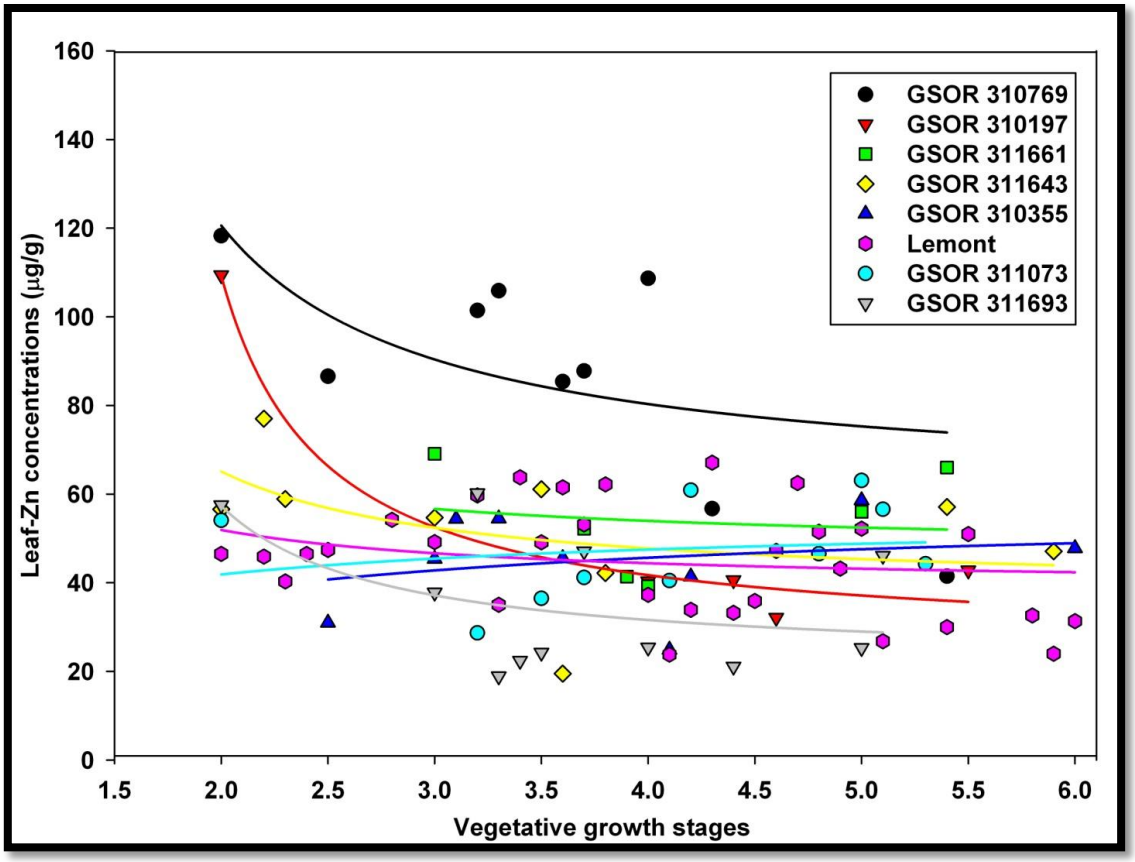
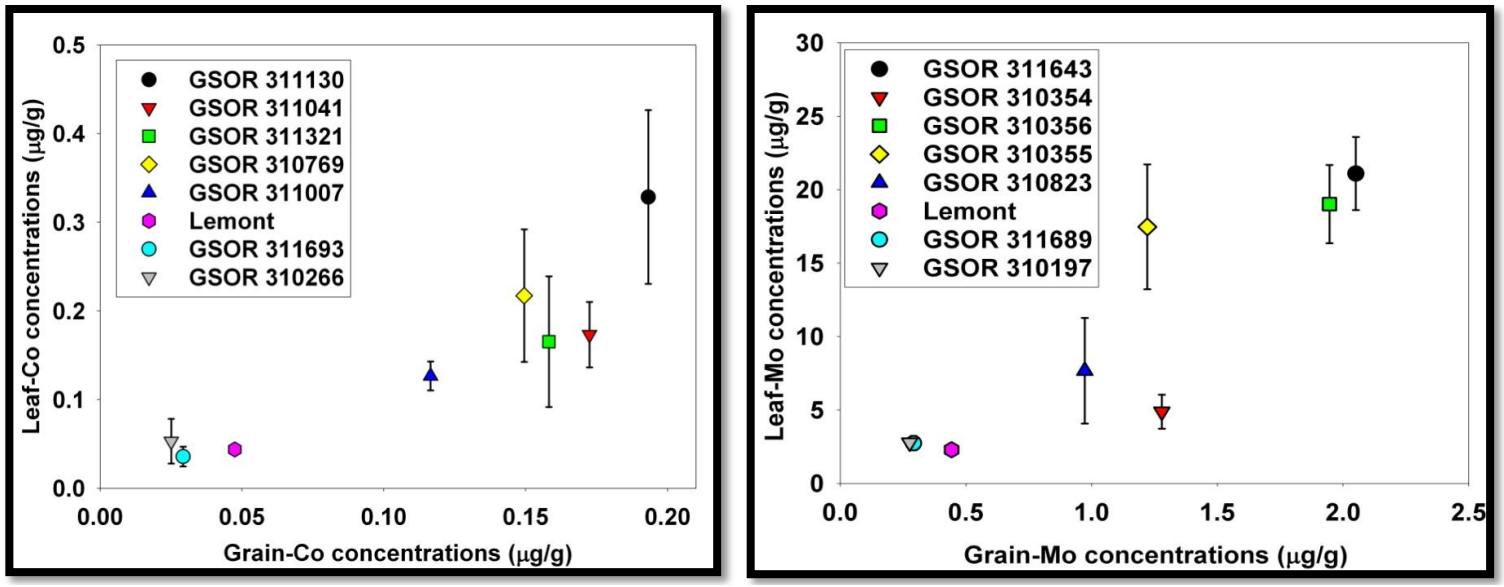


Figure 4. Nutritional dependence of genotypes between V2 to V4 stages. In unflooded conditions, GSORs 310769, 310197, 311661, 311643 and 310355 were selected for high grain-Zn concentrations and GSORs 311073 and 311693 were selected for low grain-Zn concentrations. GSORs 310769, 310197, and 311693 exhibited a decline in leaf-Zn concentrations from the V2 to the V4 growth stages hinting nutritional dependence on seed.



(a) (b)

Figure 5. V4 to V6 growth stages -the youngest and viable growth stages for leaf screening.
a) In flooded conditions, GSORs 311130, 311041, 311321, 310769, and 311007 were selected for high grain-Co concentrations and GSORs 311693 and 310266 were selected for low grain-Co concentrations. GSORs 311130, 311041, and 310769 exhibited high leaf-Co concentrations and GSORs 311693 and 310266 exhibited low leaf-Co concentrations. b) In flooded conditions, GSORs 31643, 310354, 310355, 310356, and 310823 were selected for high grain-Mo concentrations and GSORs 311689 and 310197 were selected for low grain-Mo concentrations. GSORs 311643, 310356, and 310355 exhibited high leaf-Mo concentrations and GSORs 311689 and 310197 showed low leaf-Mo concentrations. Each symbol represents the mean with standard error of the leaf element concentrations of all plants of the genotype between V4 and V6 growth stages. Some error bars are obscured by the symbol.

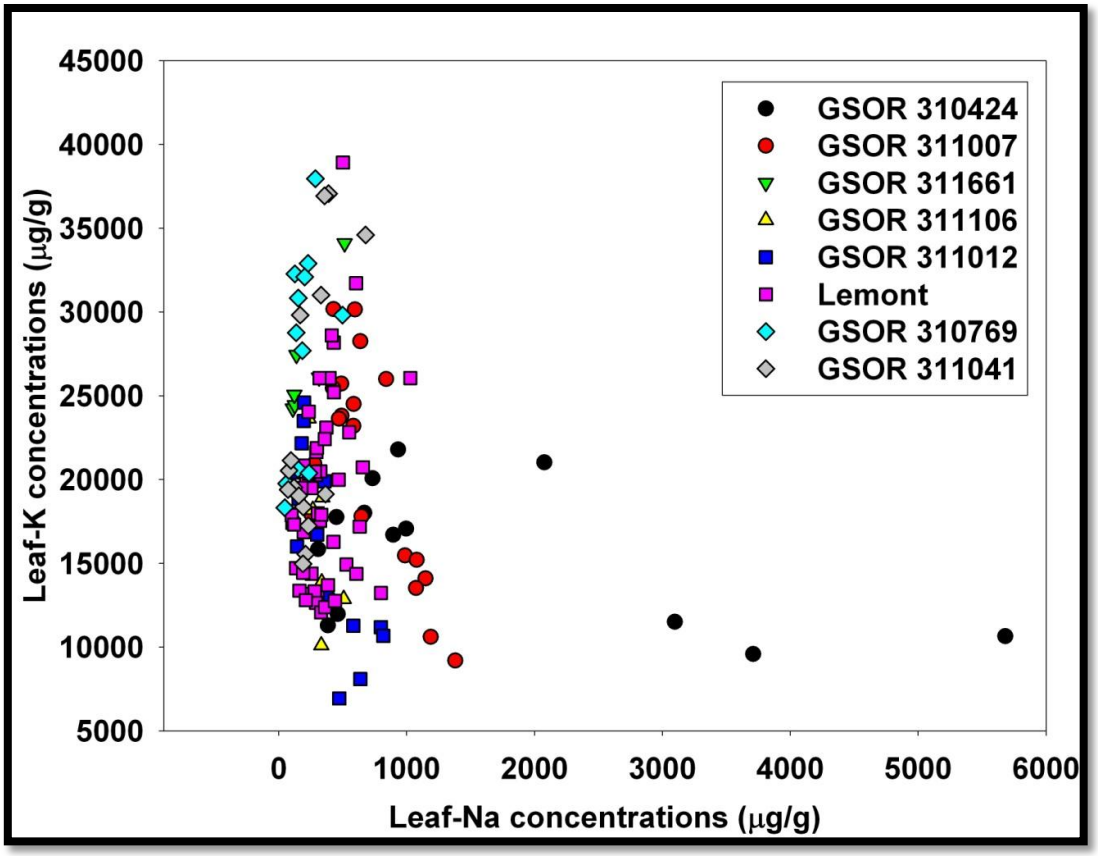


Figure 6. High grain-K genotypes and leaf-Na concentrations
In unflooded conditions, GSORs 310424, 311007, 31661, 311106 and 311012 exhibited high grain-K concentrations while GSORs 310769 and 311041 exhibited low grain-K concentrations. GSOR 310424, and to a lesser extent GSOR 311007, exhibited high leaf-Na concentrations but not high leaf-K concentrations.

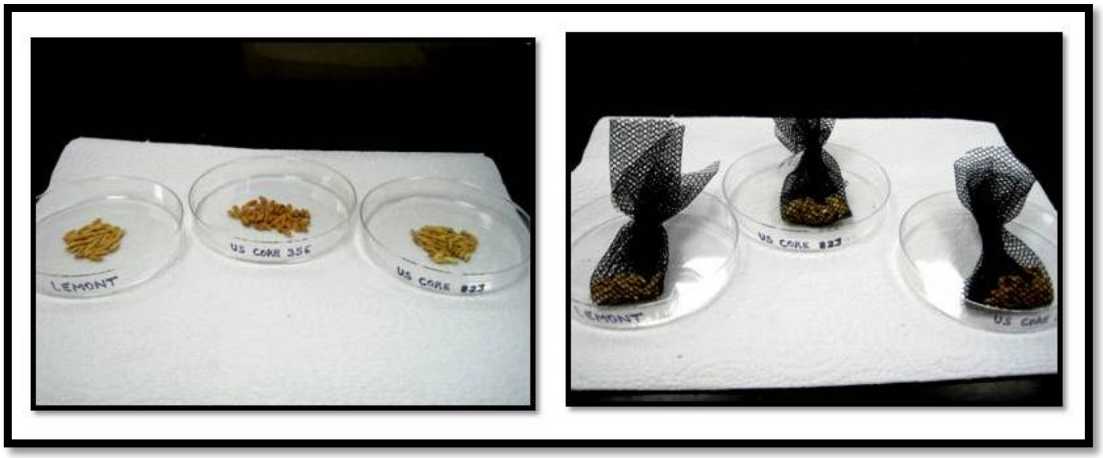


Figure 7. Seed sterilization.
Seeds of GSOR 310356, GSOR 310823 and Lemont were sterilized using hydrogen peroxide (10%) for 10 minutes followed by ethyl alcohol (70%) for a minute (Iyer-Pascuzzi *et al.*, 2010).

Figure 8. Different stages in the preparation of a netted foam disc.
Black foam insulation tubes of 2.5-cm outer diameter and 2-cm inner diameter were sliced to 1-cm thick rings. Two of these rings were stitched together with black nylon netting placed between using a Micro Stitch Gun (Avery Dennison, Pasadena, California).

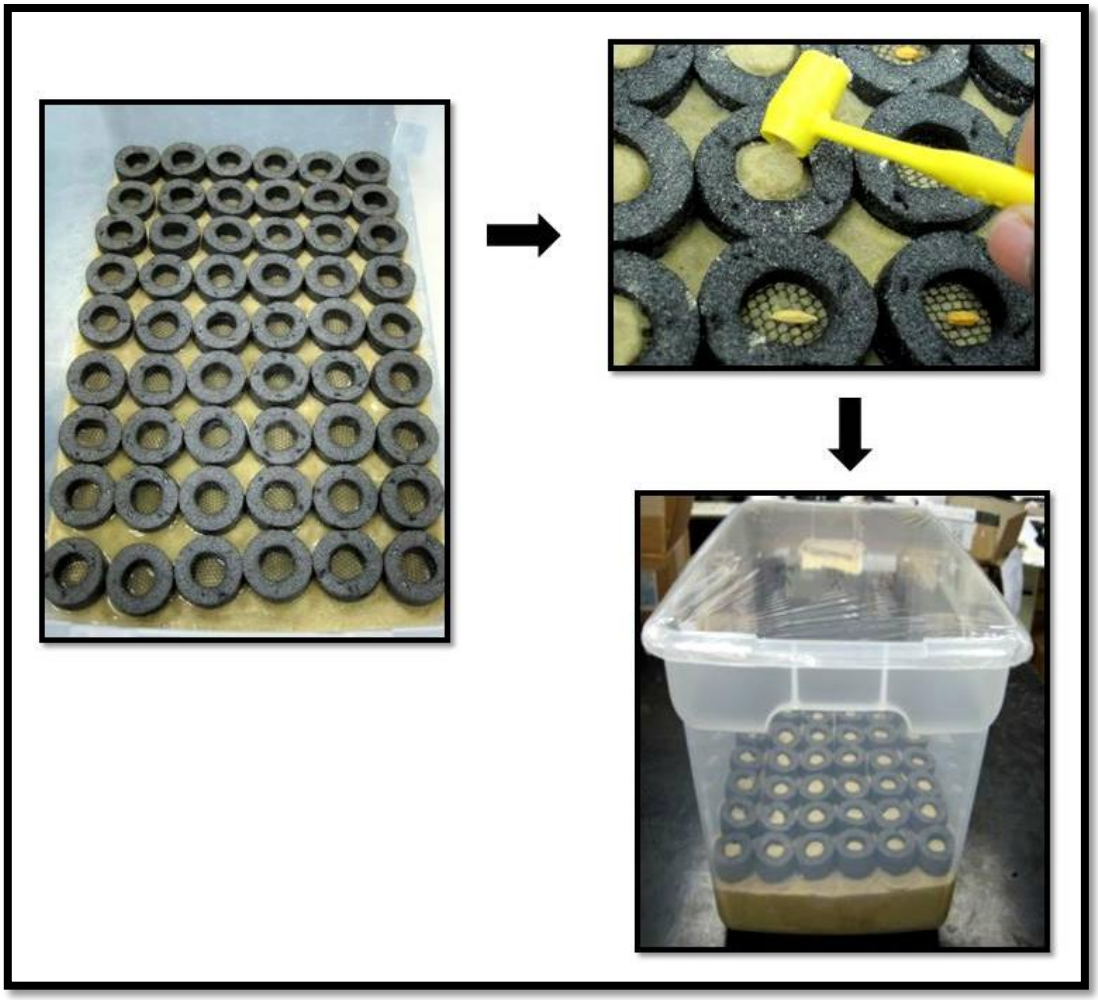


Figure 9. Pre-germination stages.
Sand was filled (approximately 2.5-cm deep) in a clear plastic box and moistened with just enough R.O. water. Netted foam discs were uniformly spread over the sand. Inside each disc, sand was added until its upper surface was level with the netting allowing sand to wick water up to each seed, providing uniform moisture at the center of each disc. One sterilized seed per disc was placed with forceps at the center of the netting, covered with sand to a 0.5-cm depth and then moistened with approximately 5 ml R.O. water per disc with a hand-held squirt bottle. The box was covered with Reynolds 900 Clear Wrap to retain moisture and placed in a growth chamber for two weeks for germination.

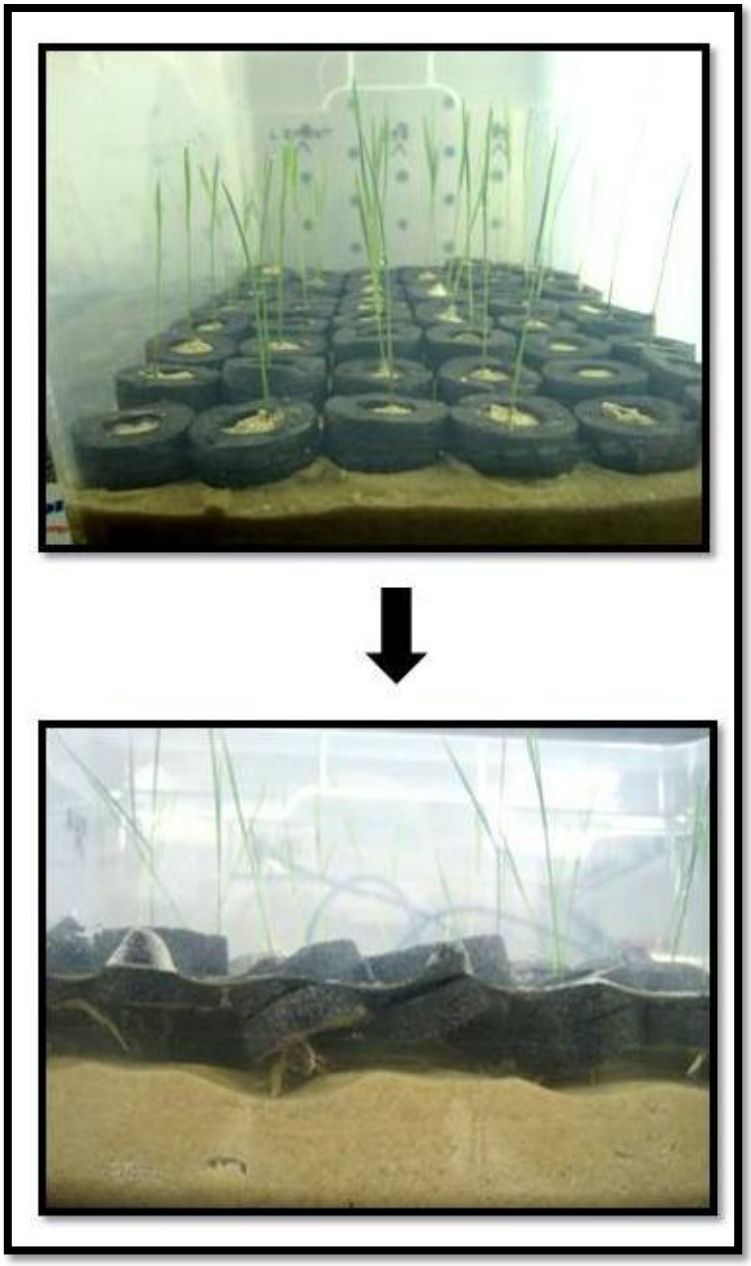


Figure 10. Seedling preparation.
To loosen the 2-week old seedlings for placement into hydroponics, the germination box was filled approximately 5-cm deep with R.O. water and gently agitated to loosen the roots from the sand while sustaining minimal injury. Each seedling with disc intact was carefully uprooted, rinsed thoroughly with running water (R.O), and then placed in its appropriate hydroponic vase.

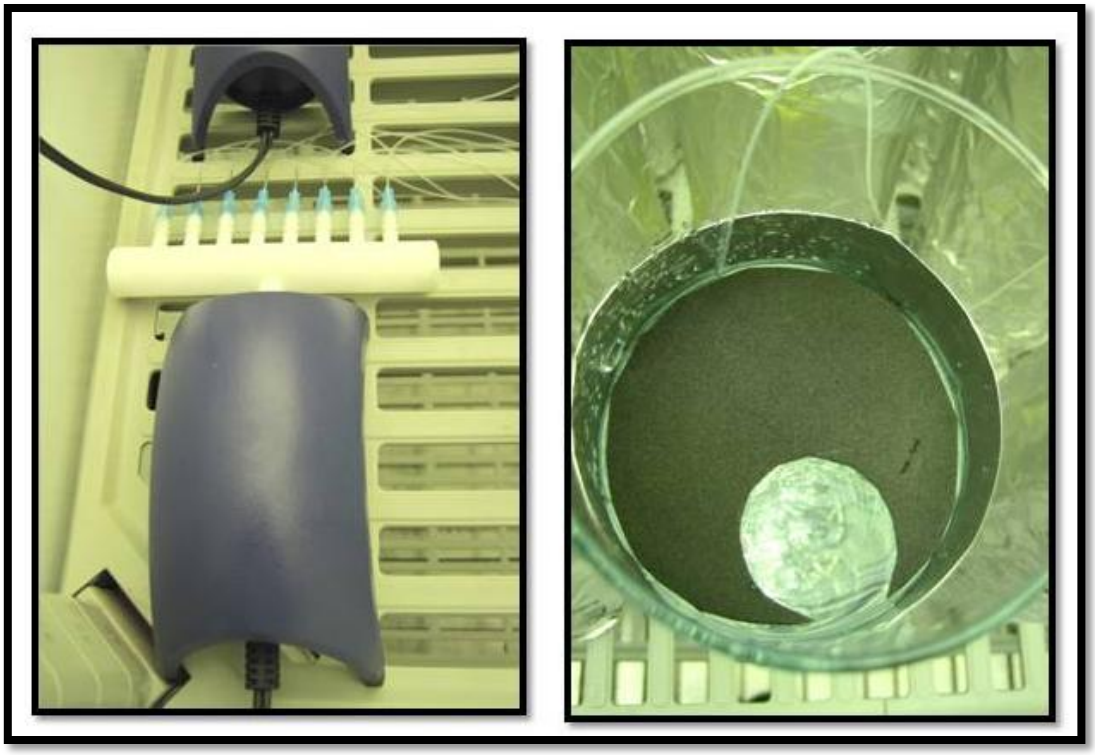


Fig. 4a Fig. 4b

Figure 11. Basic components of hydroponic -setup.
5a. Air pump, manifold, syringe and airline tubings; 5b. Clear glass cylinder wrapped in aluminum foil with hydroponic solution covered with horse-shoe shaped black foam felt to contain the seedling.

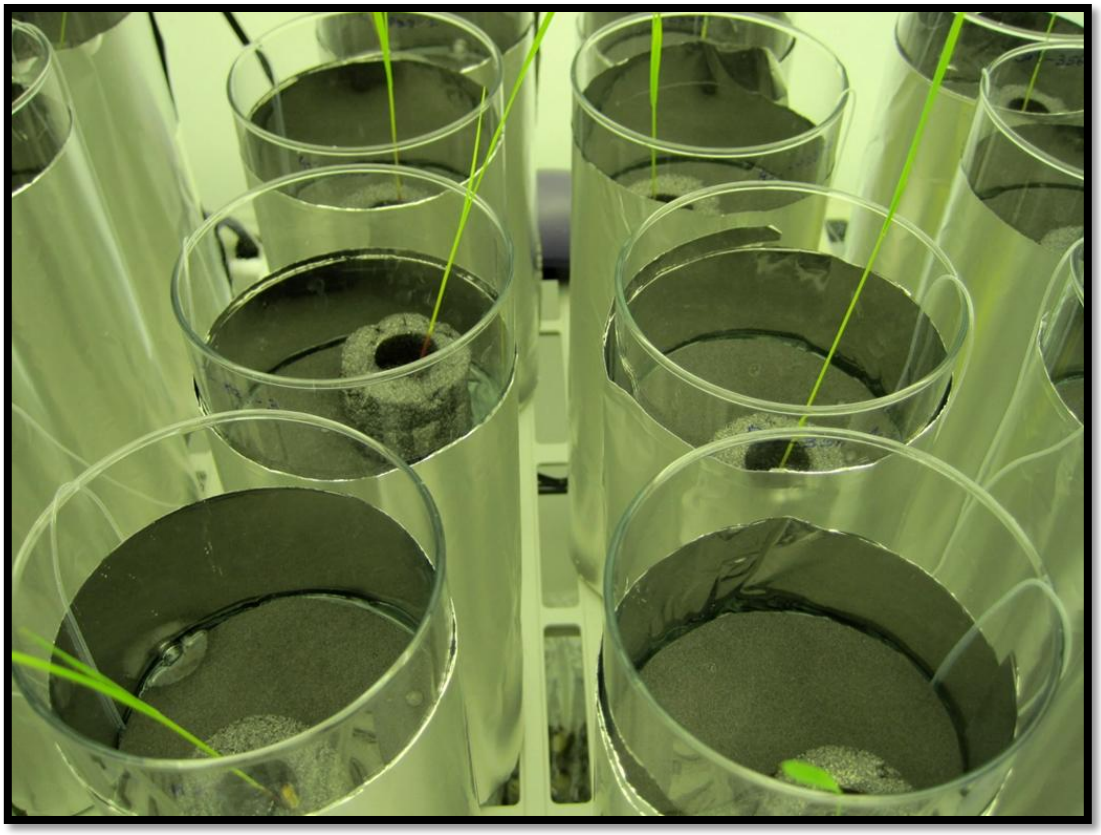


Figure 12. Hydroponic setup at transplant.
Each vase contained 1 L of hydroponic medium and received a seedling that floated on the netting of its foam disc. Black foam felt on the surface of the medium, along with aluminum foil wrapped around the vases, blocked light and prevented algae from growing in the medium.

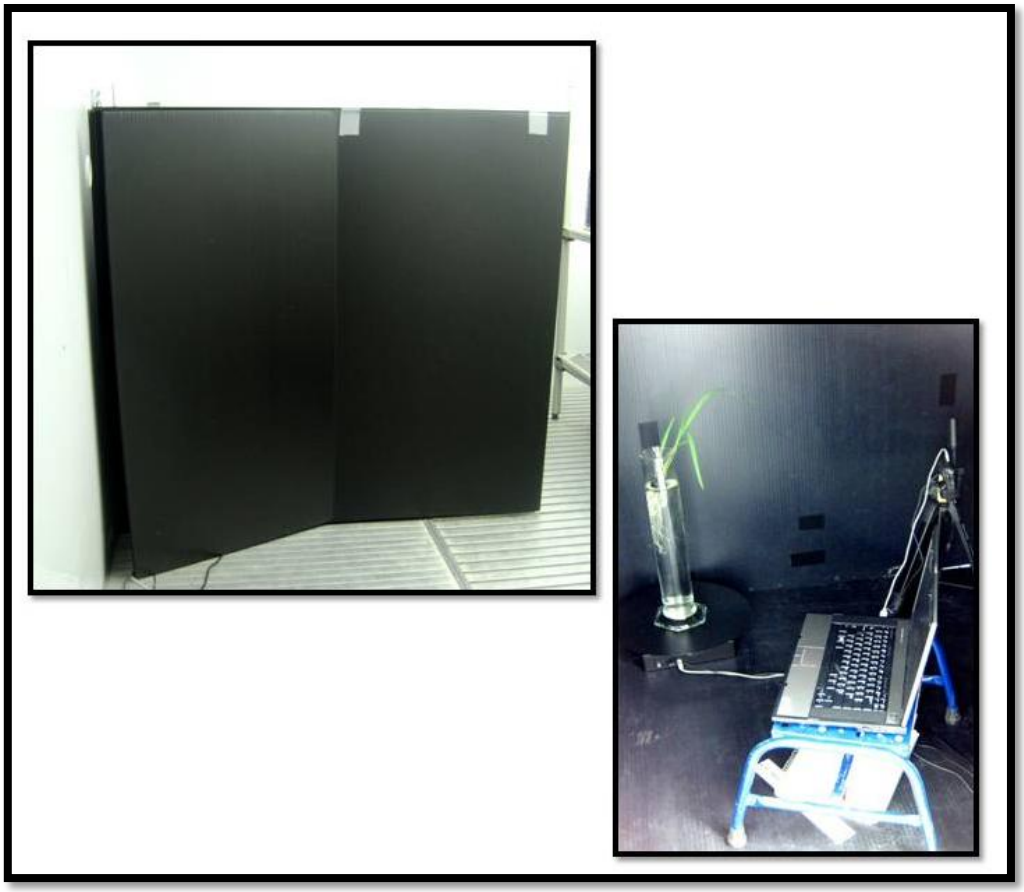


Figure 13. Photo Studio and its components.
Images were captured inside a photo studio made by taping six pieces of black coroplast boards (48-cm long x 36-cm wide) to form a closed chamber. Root imaging consisted of three components, 1) a laptop which was connected to 2) a turntable, and 3) a camera mounted on a tripod (Iyer-Pascuzzi *et al.*, 2010). Plants were mounted in a 2L ungraduated cylinder and mounted on the turntable. PhotoCapture 360 (Ortery Technologies Inc., Irvine, California) imaged roots every 18° rotation giving a total of 20 images per plant.

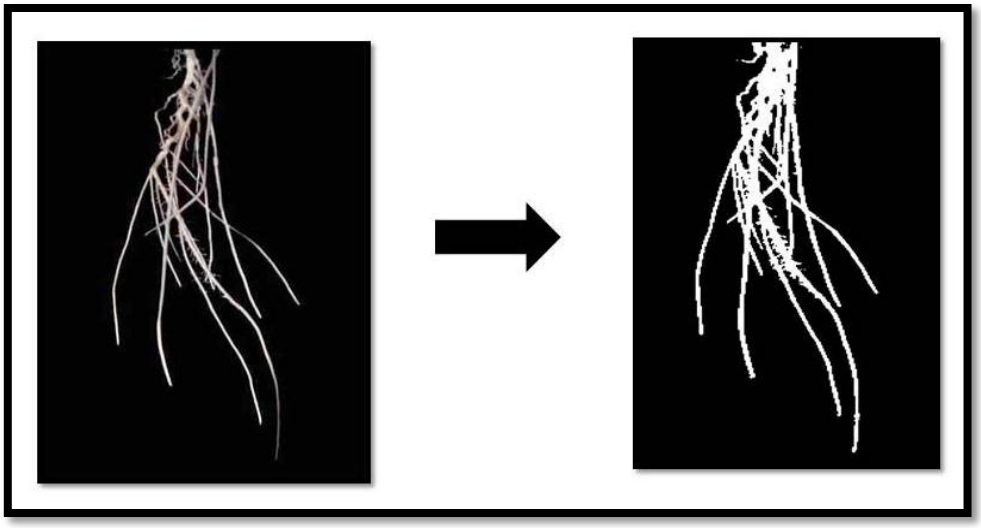


Figure 14. Adaptive thresholding in MATLAB software.
Original root images in color were converted to binary images using adaptive threshold coded in MATLAB software. These binary images were then used to quantify 16 different root traits.

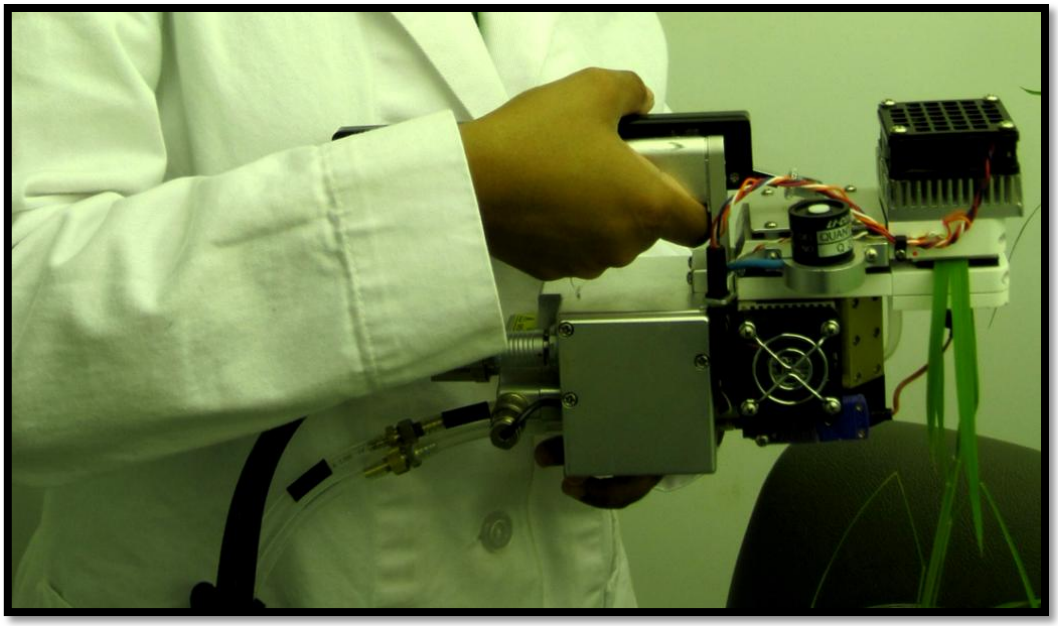


Figure 15. Photosynthesis measurements.
Photosynthesis measured between 1000 h and 1200 h on the most recent fully opened leaf of the main tiller on each plant using a portable photosynthesis system LICOR 6400 (LI-6400, LI-COR Inc., Lincoln, NE).

Figure 16. Chlorophyll fluorescence measurements.
Photosynthetic efficiency (Fv/Fm) determined using a fluorometer (Opti-Sciences, Tyngsboro, MA) on the same leaves used for photosynthesis measurements after dark-acclimating leaves using dark-adaption cuvettes (Opti-Sciences, Tyngsboro, MA) 20 minutes prior to taking measurements.

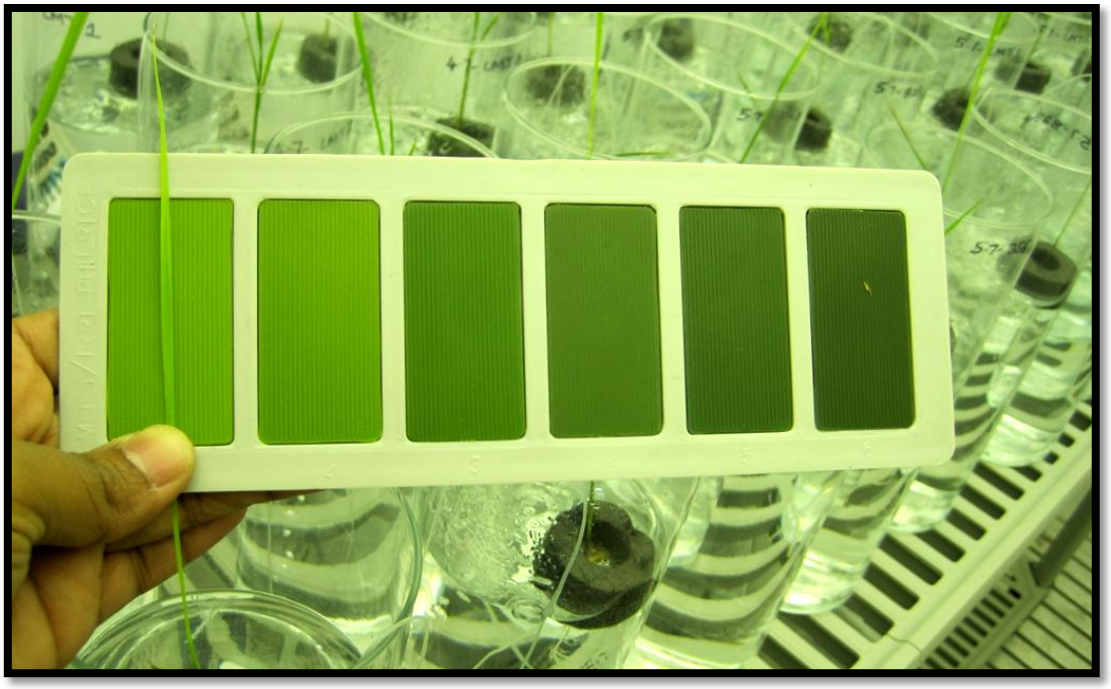


Figure 17. Leaf color quantification.
Leaf color was determined by visually rating against a leaf color chart (IRRI-Phil Rice, Manila, Philippines) on a scale from 1 (light yellow) to 6 (dark green).

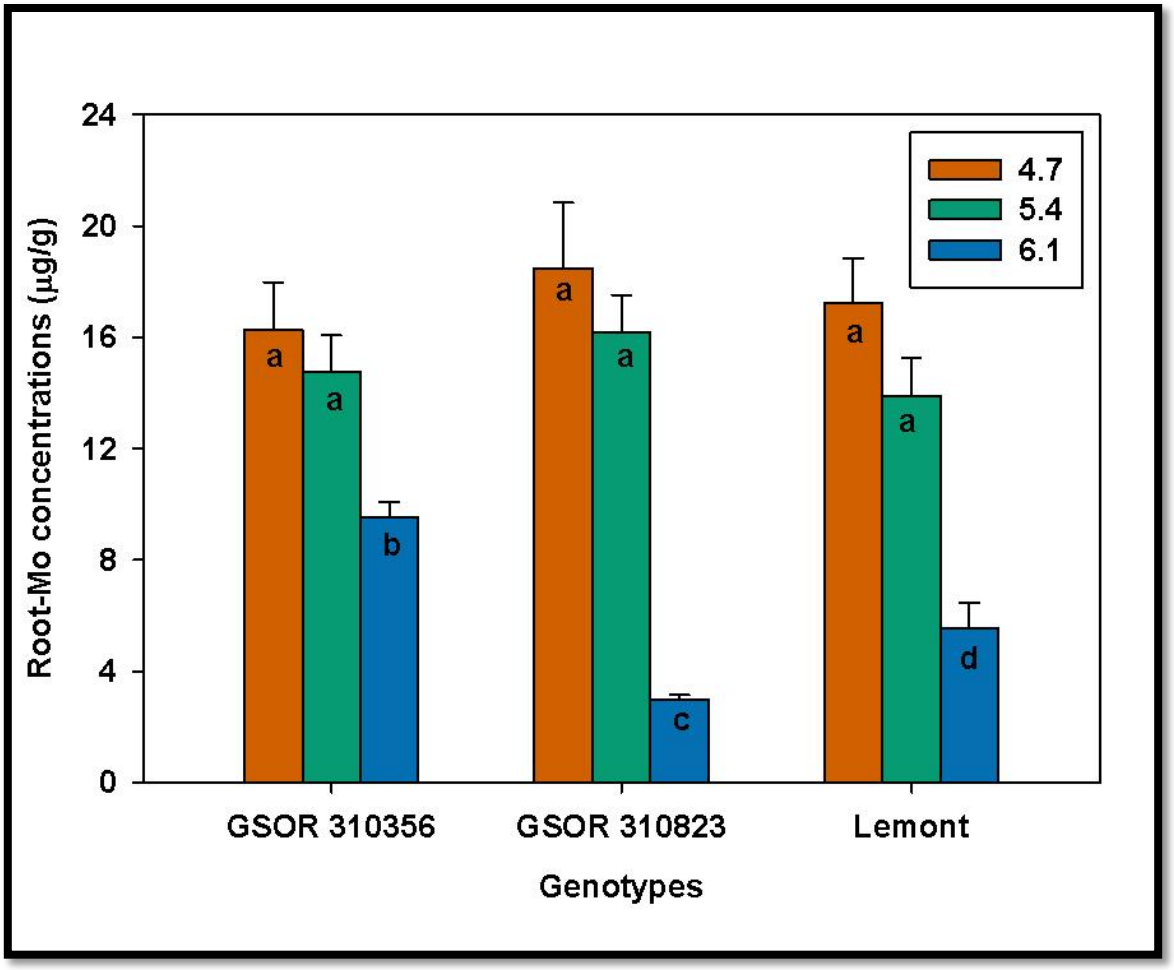


Figure 18. Root-Mo concentrations of different genotypes in different pH. Similar root-Mo concentrations in all genotypes in acidic pH regimes were likely due to increased adsorption to roots at pH 4.7 and 5.4 (Different letters indicate statistically significant differences at α = 0.05).

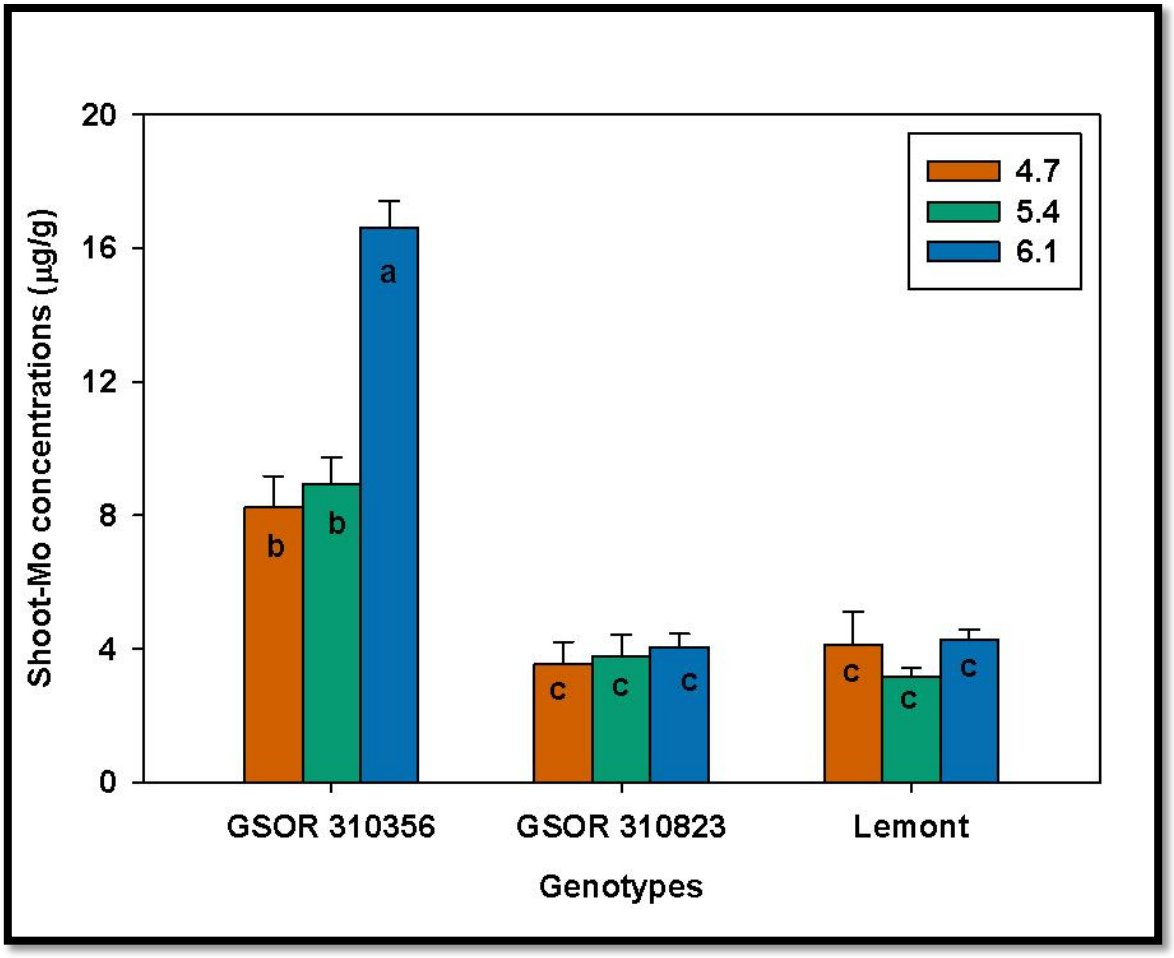


Figure 19. Shoot-Mo concentrations of different genotypes in different pH. GSOR 310356 (Malaysia) showed high shoot-Mo concentrations across all pH regimes and genotypes. Shoot-Mo concentrations of GSOR 310356 doubled at pH 6.1 when compared to 4.7 and 5.4 (Different letters indicate statistically significant differences at $\alpha = 0.05$).

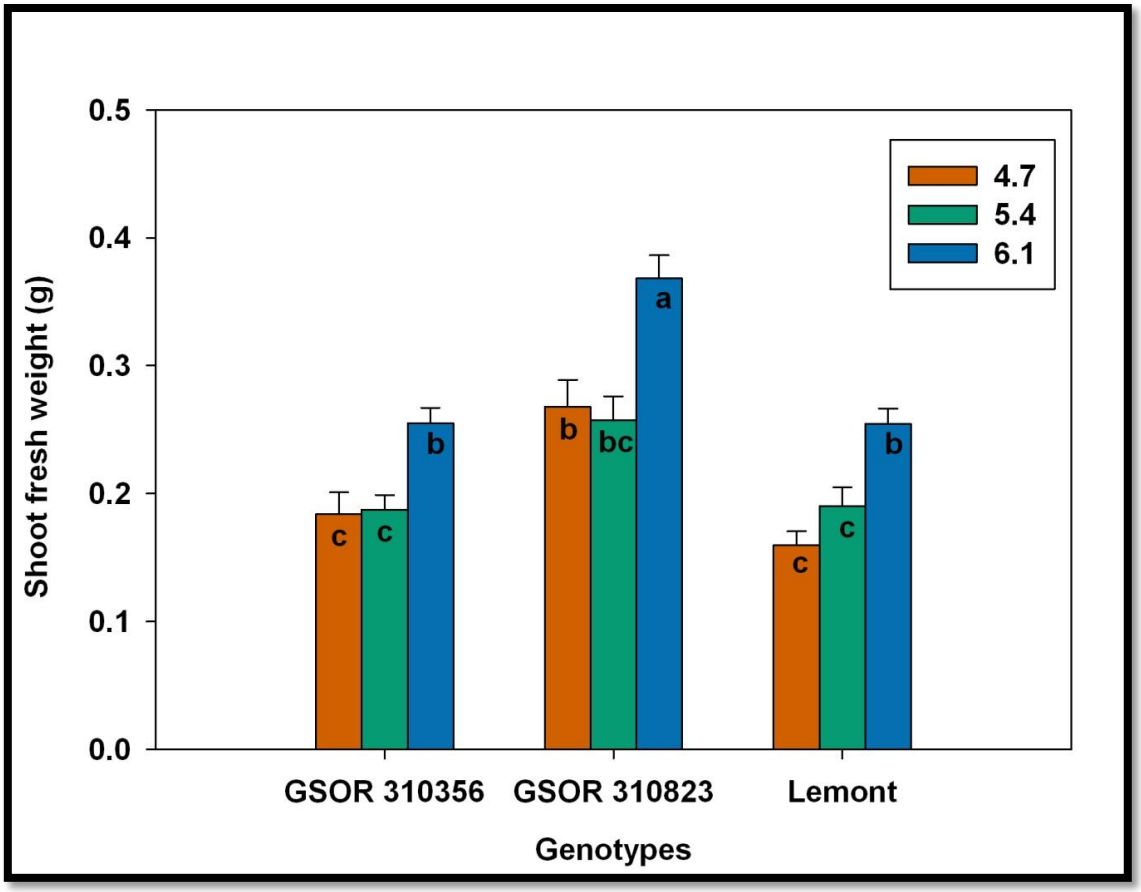


Figure 20. Shoot fresh weights of different genotypes in different pH. GSOR 310356 showed lesser shoot fresh weight at pH 4.7 when compared to 6.1 and less/similar shoot fresh weight when compared to the other two genotypes at pH 4.7 (Different letters indicate statistically significant differences at $\alpha = 0.05$).

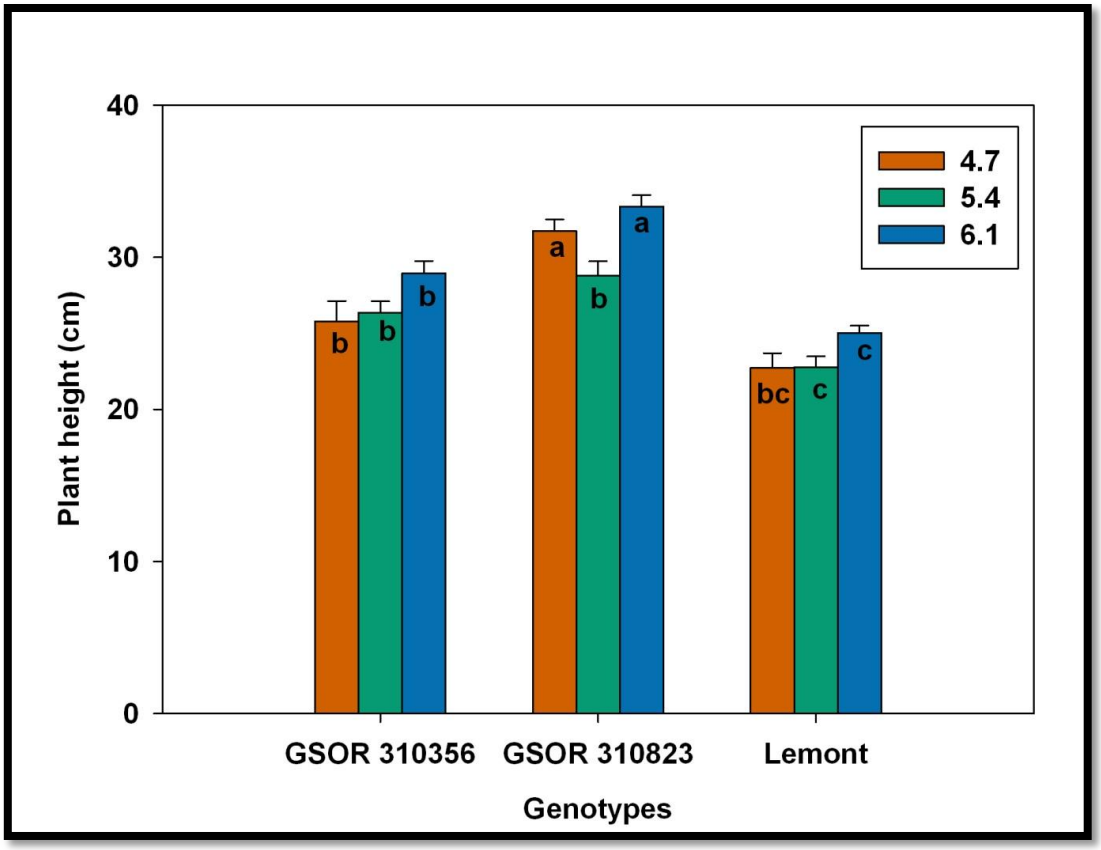


Figure 21. Plant heights of different genotypes in different pH.
GSOR 310356 showed similar plant height in all pH regimes and similar/lesser plant height when compared to other genotypes at pH 4.7 (Different letters indicate statistically significant differences at $\alpha = 0.05$).

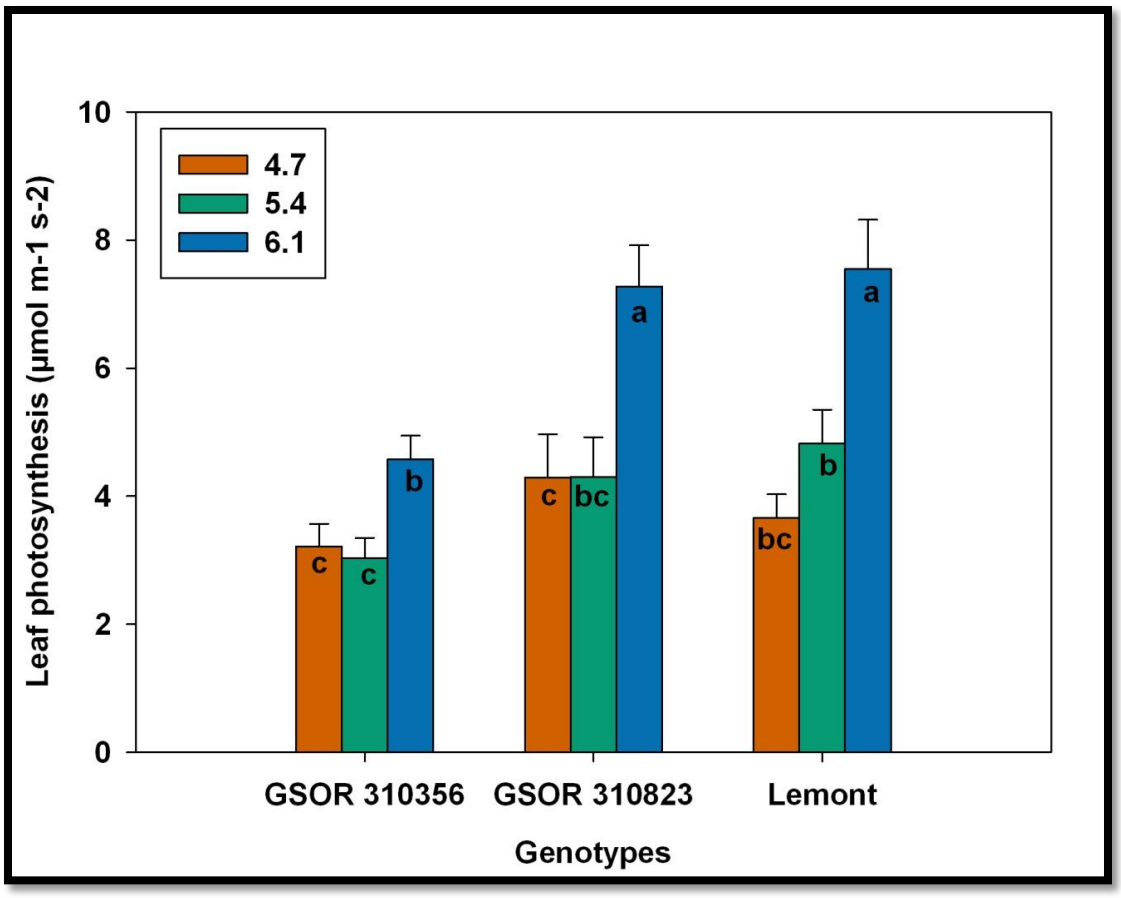


Figure 22. Leaf photosynthetic rates of different genotypes in different pH. GSOR 310356 showed a lesser rate of photosynthesis at pH 4.7 when compared to pH 6.1 and similar photosynthetic rates as that of other genotypes at pH 4.7 (Different letters indicate statistically significant differences at α = 0.05).

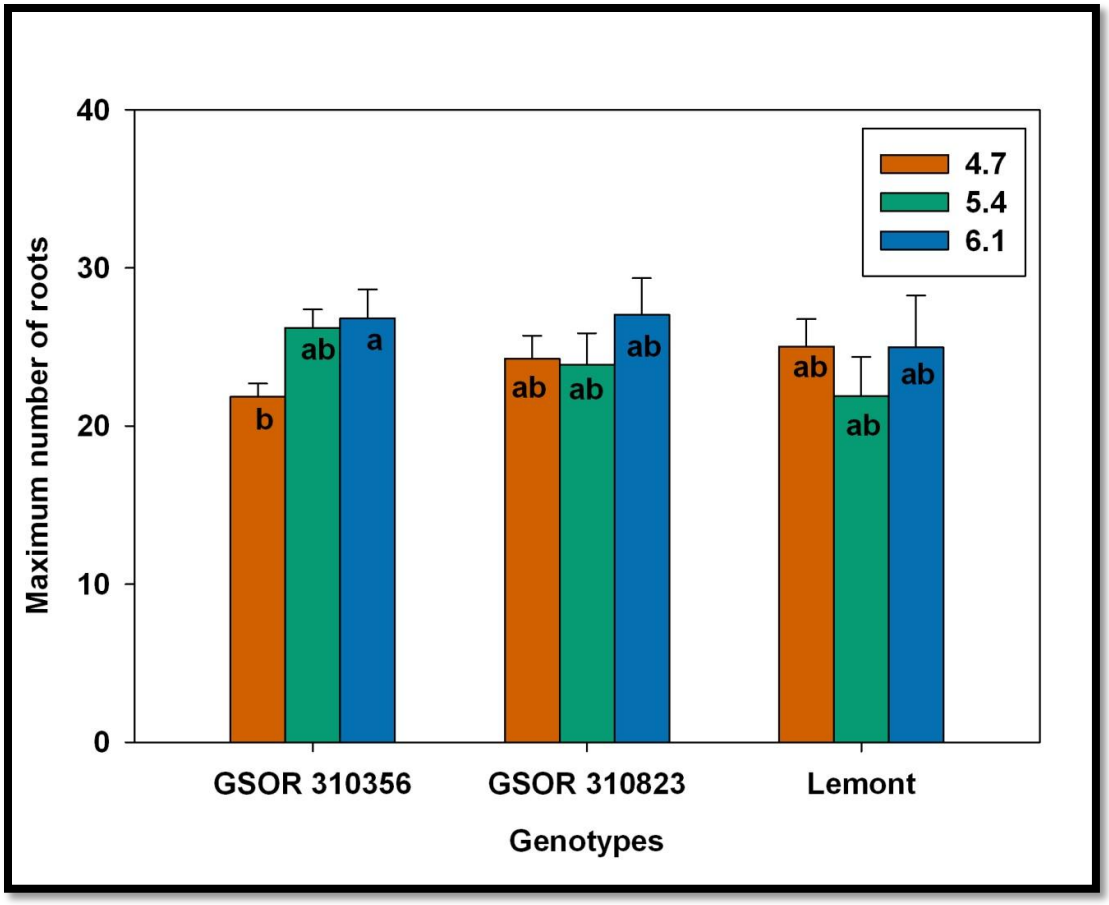


Figure 23. Maximum number of roots of different genotypes in different pH. GSOR 310356 showed lesser number of roots in pH 4.7 when compared to pH 6.1 but showed similar number of roots as that of other genotypes at pH 4.7 (Different letters indicate statistically significant differences at α = 0.05).

Figure 24. 24-h pre-soak of seeds.
Seeds were soaked in reverse osmosis water for better, faster and more uniform germination.

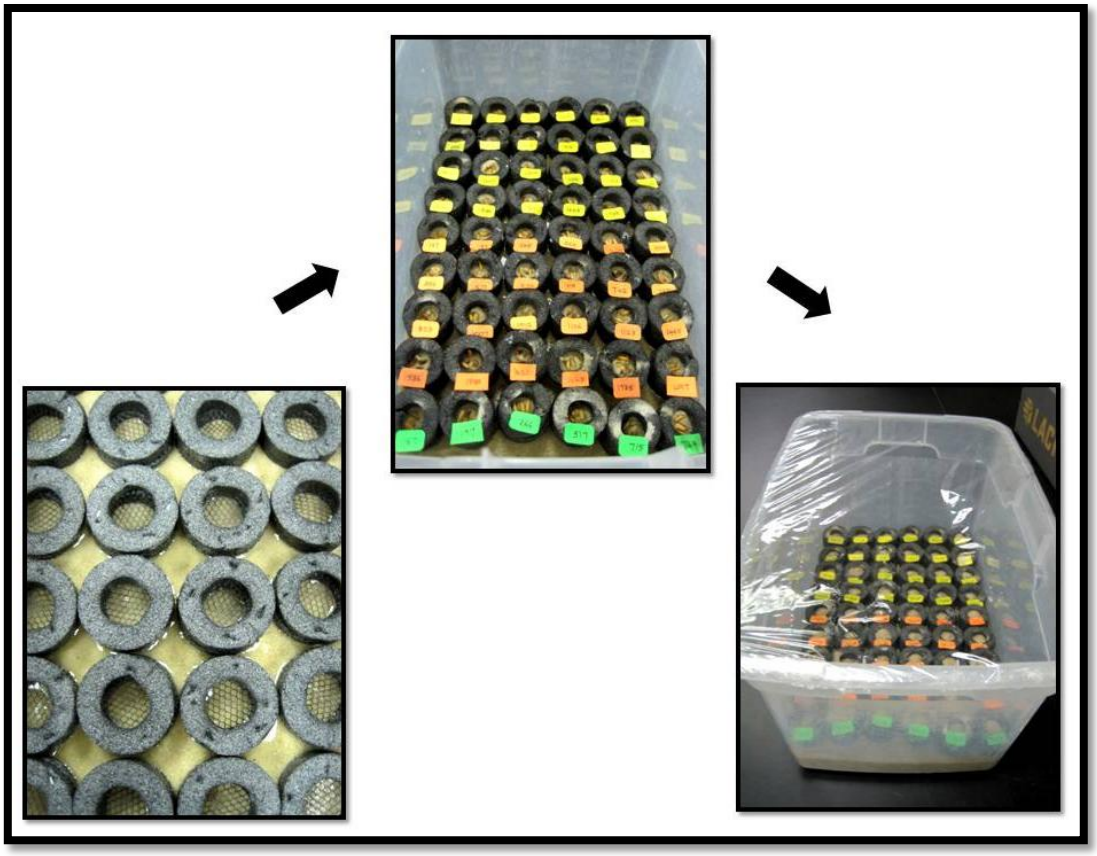


Figure 25. Pre-germination stages.
Sand was filled (approximately 2.5-cm deep) in a clear plastic box and moistened with just enough R.O. water. Netted foam discs were uniformly spread over the sand. Inside each disc, sand was added until its upper surface was level with the netting allowing sand to wick water up to each seed, providing uniform moisture at the center of each disc. One sterilized seed per disc was placed with forceps at the center of the netting, covered with sand to a 0.5-cm depth and then moistened with approximately 5 ml R.O. water per disc with a hand-held squirt bottle. The box was covered with Reynolds 900 Clear Wrap to retain moisture and placed in growth chamber for two weeks for germination.

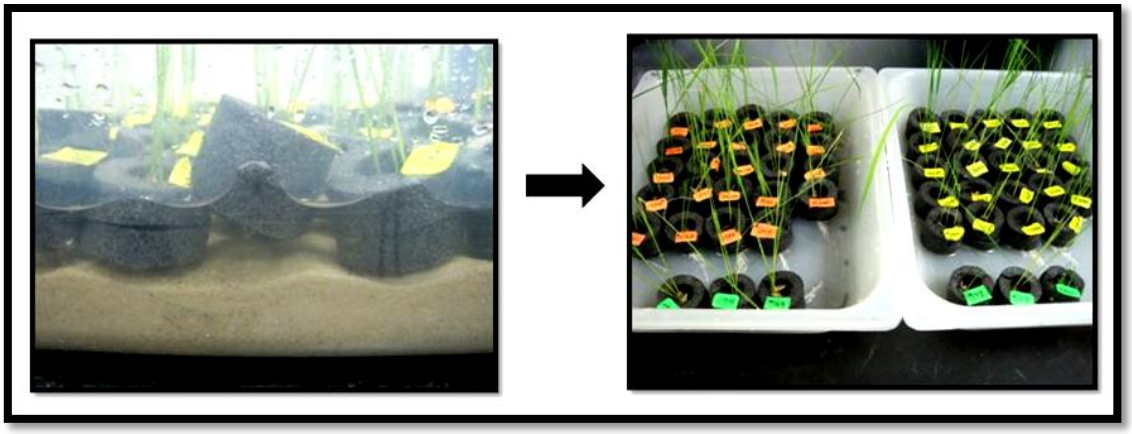


Figure 26. Preparation of seedlings.
To loosen the 2-week old seedlings for placement into hydroponics, the germination box was filled with R.O. water to an approximate depth of 5 cm and gently agitated to loosen the roots from the sand with minimal injury. Each seedling with disc intact was carefully uprooted, and rinsed thoroughly with running water (R.O).

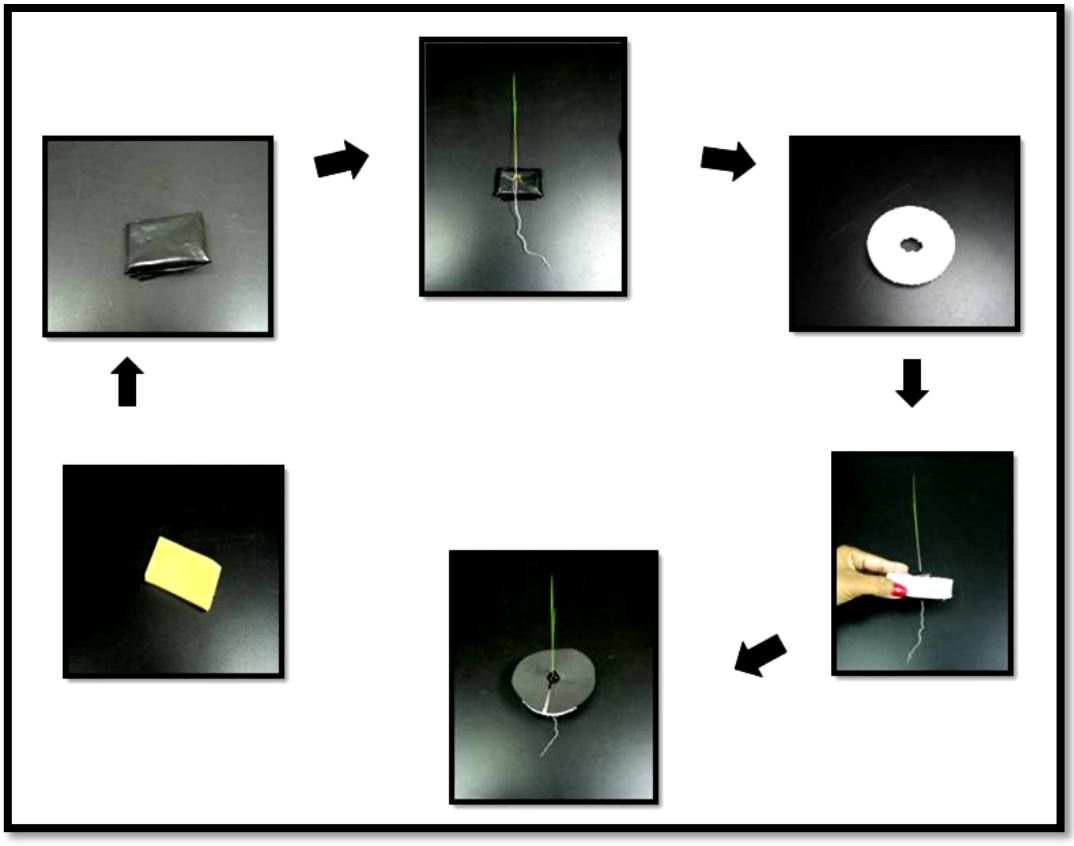


Figure 27. Preparation of a seedling float.
Styrofoam sheets of 2.5-cm thickness were cut into 7.5-cm diameter circles with a 2-cm hole at the center to contain the seedling. A strip of sponge (2.5-cm long x 1-cm wide x 0.5-cm thick) that was covered in a black plastic sheet was wrapped around each seedling at the transition from root to shoot and then inserted at the center of the coaster. Styrofoam coasters were then topped off with a black foam felt slit half-way with 0.5 cm hole at the center to contain the seedling.

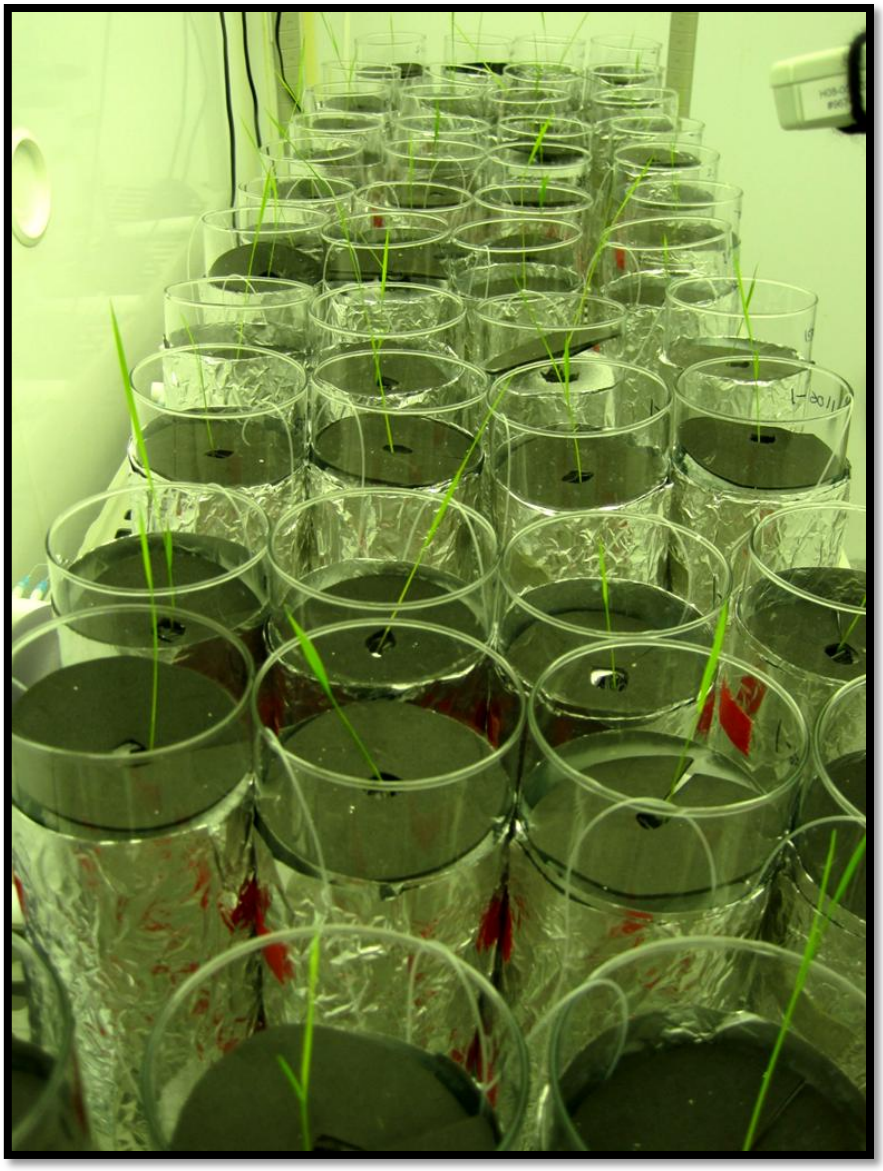


Figure 28. Hydroponic setup at transplant.
Each vase contained 1 L of hydroponic medium and received a seedling that floated on the netting of its foam disc. Black foam felt on the surface of the medium, along with aluminum foil wrapped around the vases, blocked light and prevented algae from growing in the medium.

Figure 29. Nutrient solution pH and ORP measurements.
pH and ORP of the nutrient solutions was measured at weekly intervals using a hand-held dual pH /ORP meter (HI 98121, Hanna Instruments, Woonsocket, RI) just prior to replacing the solution.

Figure 30. A scanned root image.
Roots scanned using WinRhizo scanner (Regent Instruments, Quebec, Canada) and then analyzed for root area, surface area, volume, length and diameter using WinRhizo Pro software (Regent Instruments, Quebec, Canada).

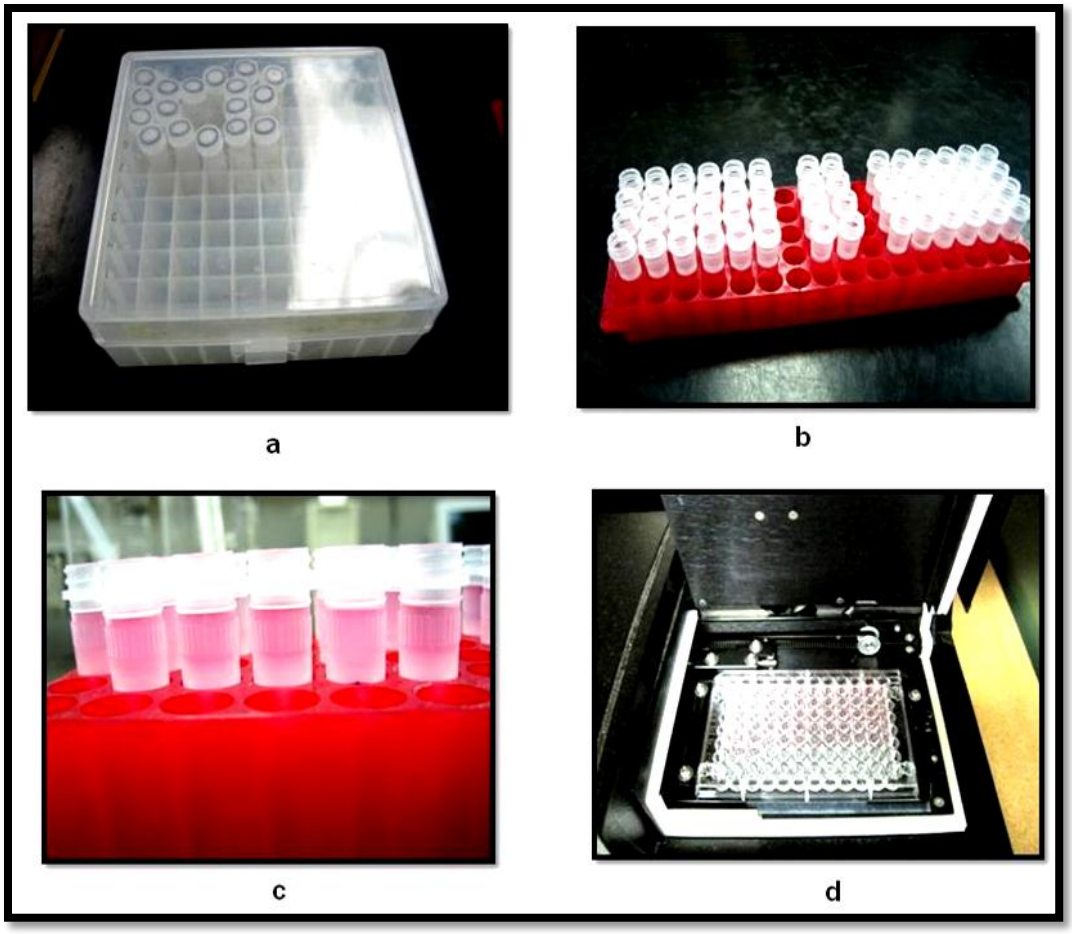


Figure 31. Different stages of measuring root respiration.
a. Roots incubated in 20 µg g^{-1} naphthylamine solution for 2 h. b. A sub sample of naphthylamine solution after root incubation transferred to 2 ml centrifuge vial containing sulphanilic acid, sodium nitrite and distilled water. c. Naphthylamine oxidation after 1 h noticeable as different shades of pink d. Samples transferred onto 96-well plate for quantifying naphthylamine oxidation in Spectrophotometer at 500 nm.

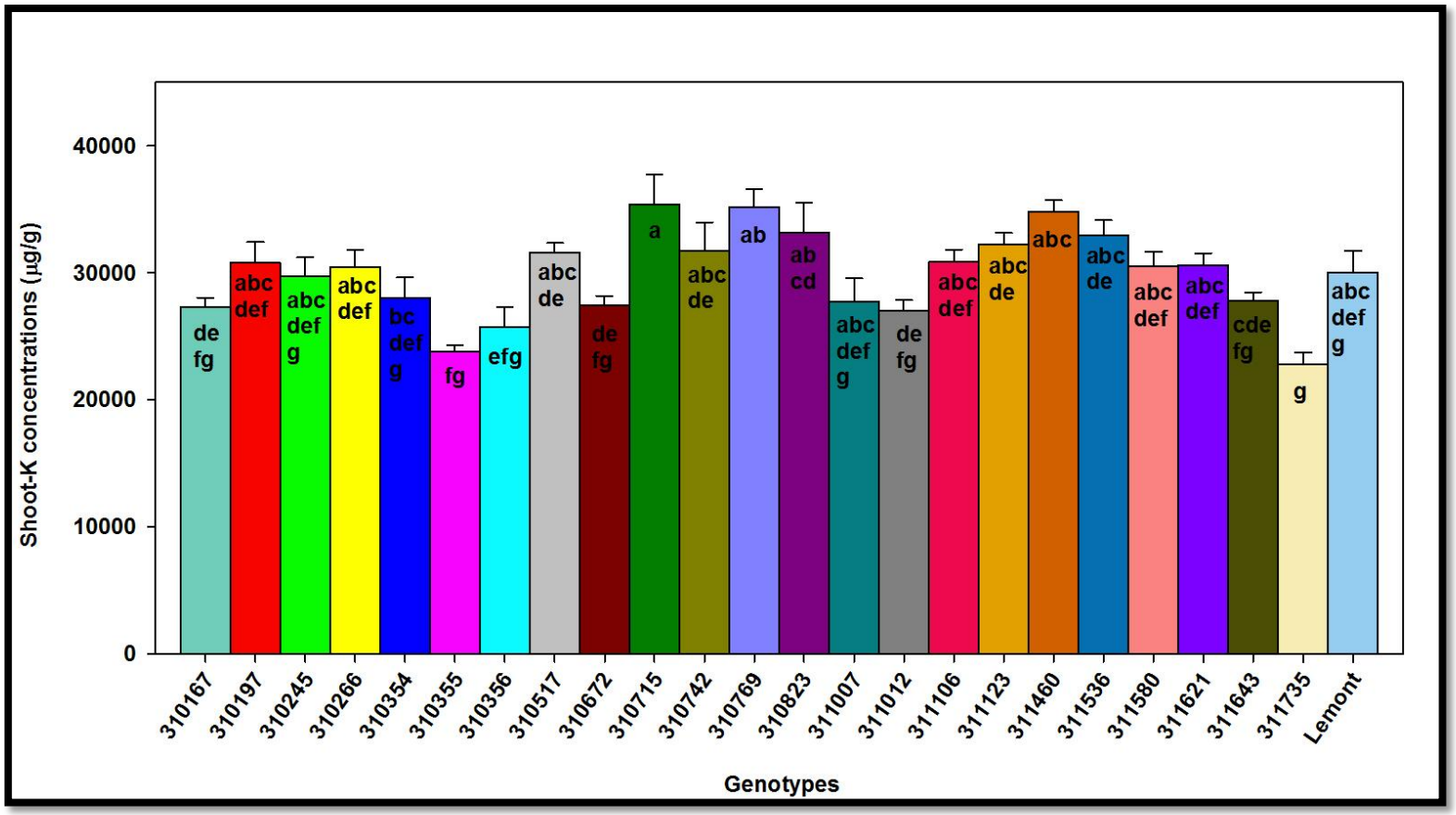


Figure 32. Shoot-K concentrations of different genotypes.
GSORs 310742, 3101007, and 311106 selected for high grain-K genotypes and several genotypes with less grain-K concentrations showed high shoot-K concentrations 4WAG (Different letters indicate statistically significant differences at α = 0.05).

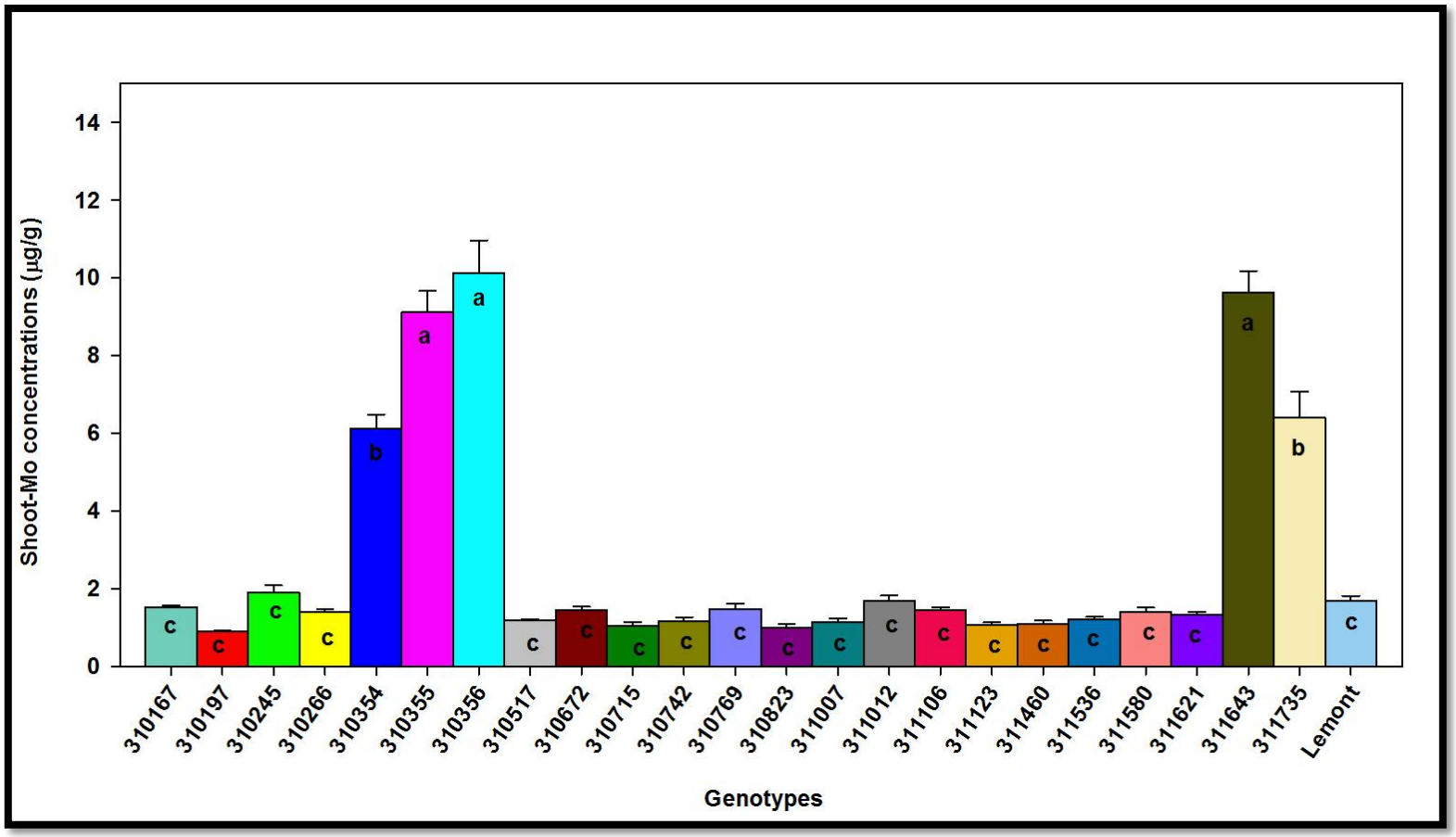


Figure 33. Shoot-Mo concentrations of different genotypes.
GSORs 310355, 310356, and 311643 selected for high grain-Mo concentrations showed high shoot-Mo concentrations 4WAG (Different letters indicate statistically significant differences at α = 0.05).

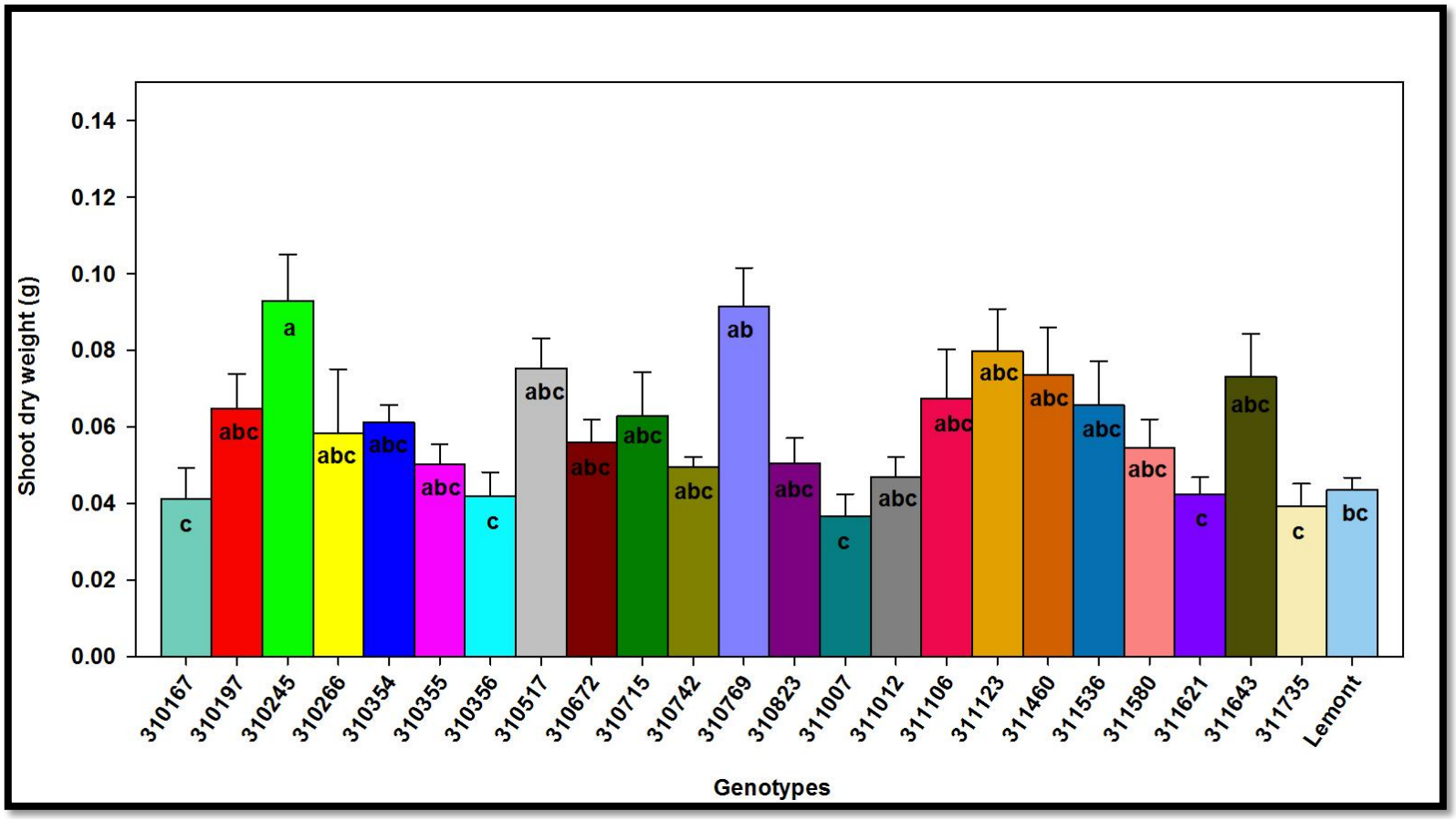


Figure 34. Shoot dry weights of different genotypes.
All genotypes except GSORs 310167, 310356, 311007, 311621 and 311735 showed high shoot dry weight (Different letters indicate statistically significant differences at α = 0.05).

Figure 35. Seed treatments with 2 µg g^{-1} NAA.
Jiffy-24 cell seed starter planting tray was lined with paper towels. Each cell was clearly labeled and 10 seeds of the appropriate genotype were placed in each cell. The tray was placed in a plastic tub filled approximately 2-cm deep with 500 ml of 2 µg g^{-1} NAA solution [Control (left) and NAA (right)].

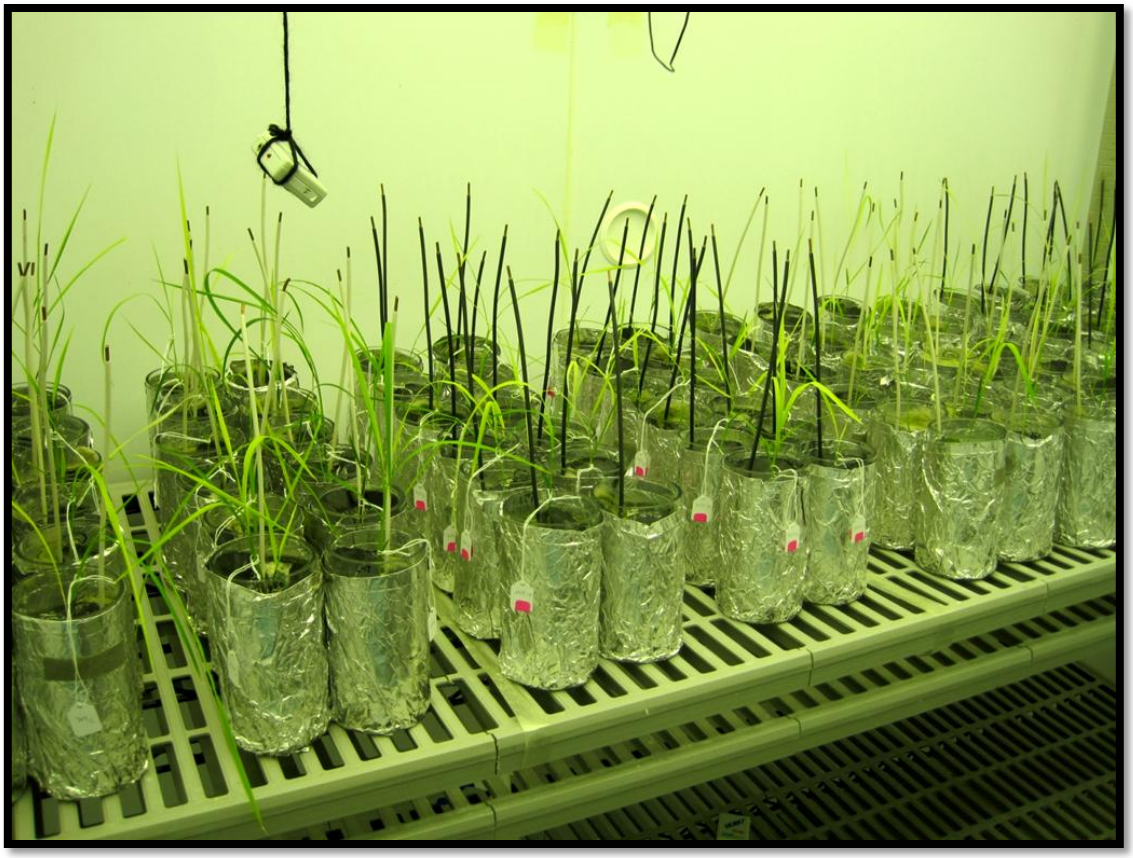


Figure 36. Sand culture setup.
Clear cylinder glass vases (15-cm high x 7-cm wide) wrapped with aluminum foil were filled with washed sea-sand (Fisher Scientific, Pittsburgh, PA) approximately 13 cm deep leaving 2 cm space on the top for nutrient fertilization. Sand was moistened with R.O. water and one platinum electrode per vase was placed.The vase was topped off with a circular black plastic sheet slit half way to contain the seedling. Together, the aluminum foil and black plastic sheet blocked light and prevented algal growth in the sand.

Figure 37. Soil redox potential measurements.
Redox potential measured using a hand-held digital voltmeter connected to Orion 900100 Ag/AgCl reference electrode, which was placed temporarily on the soil surface at the time of measurements.

Figure 38. Soil pH measurements.
Five g of sand was sampled from each vase at approximately a 2-cm depth and transferred into a plastic cup filled with 25 ml of distilled water to get a 1:5 ratio of soil to water (Sparks et al., 1996). The mixture was stirred well using a glass rod and after 60 minutes, pH was measured using a hand-held pH meter (HI 98121, Hanna Instruments, Woonsocket, RI).

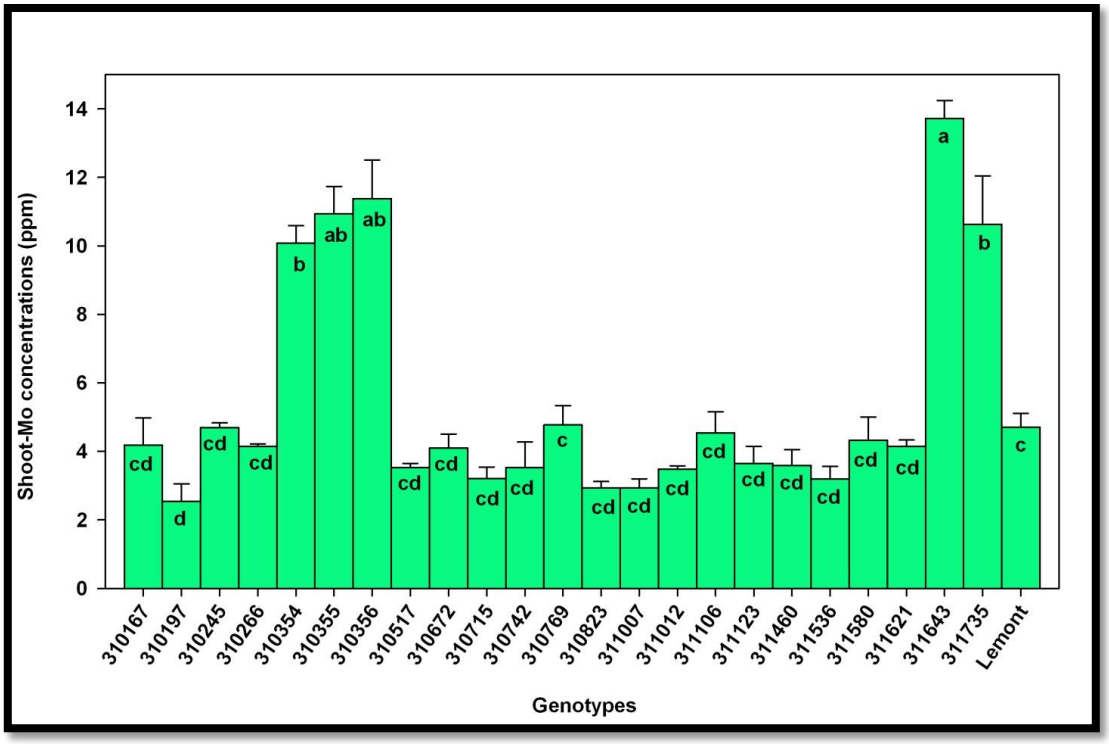


Figure 39. Shoot-Mo concentrations of different genotypes.
Control plants of high grain-Mo genotypes, GSORs 310354, 310355, 310356, 311643, 311735 showed high shoot-Mo concentrations 3WAG.

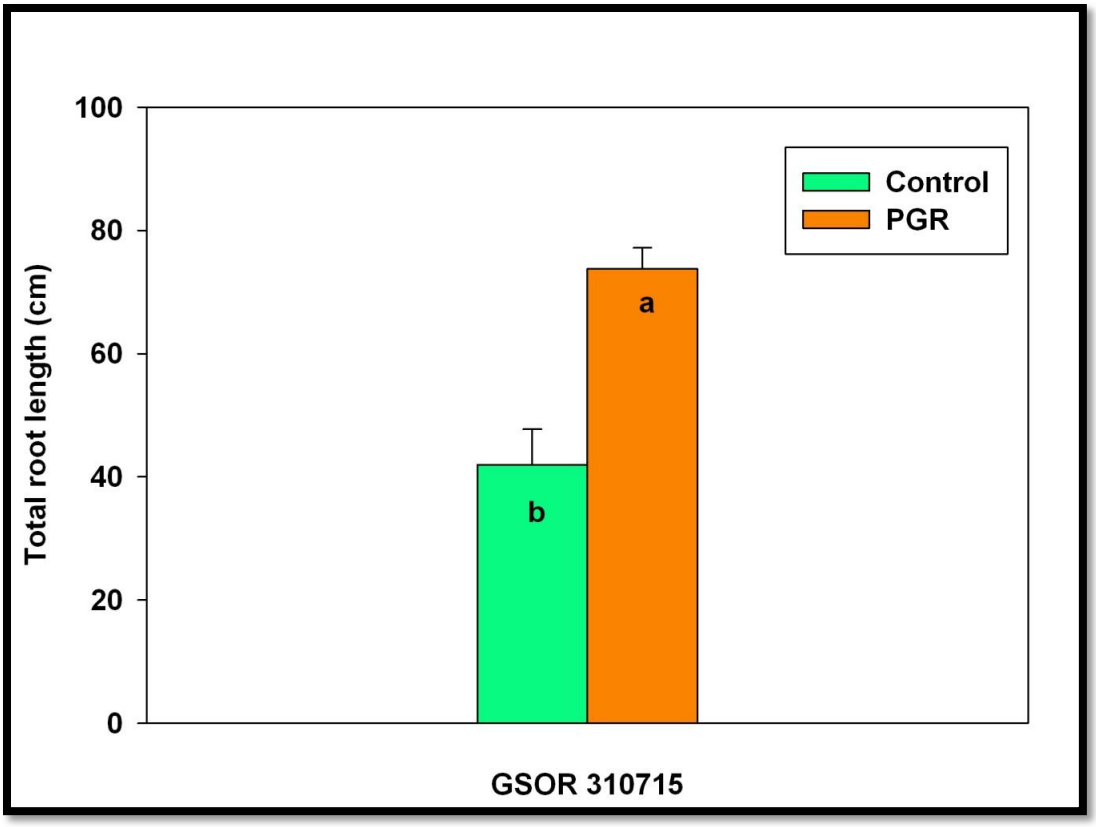


Figure 40. Total root length of GSOR 310715.
GSOR 310715 showed more total root length with PGR treatment when compared to the control.

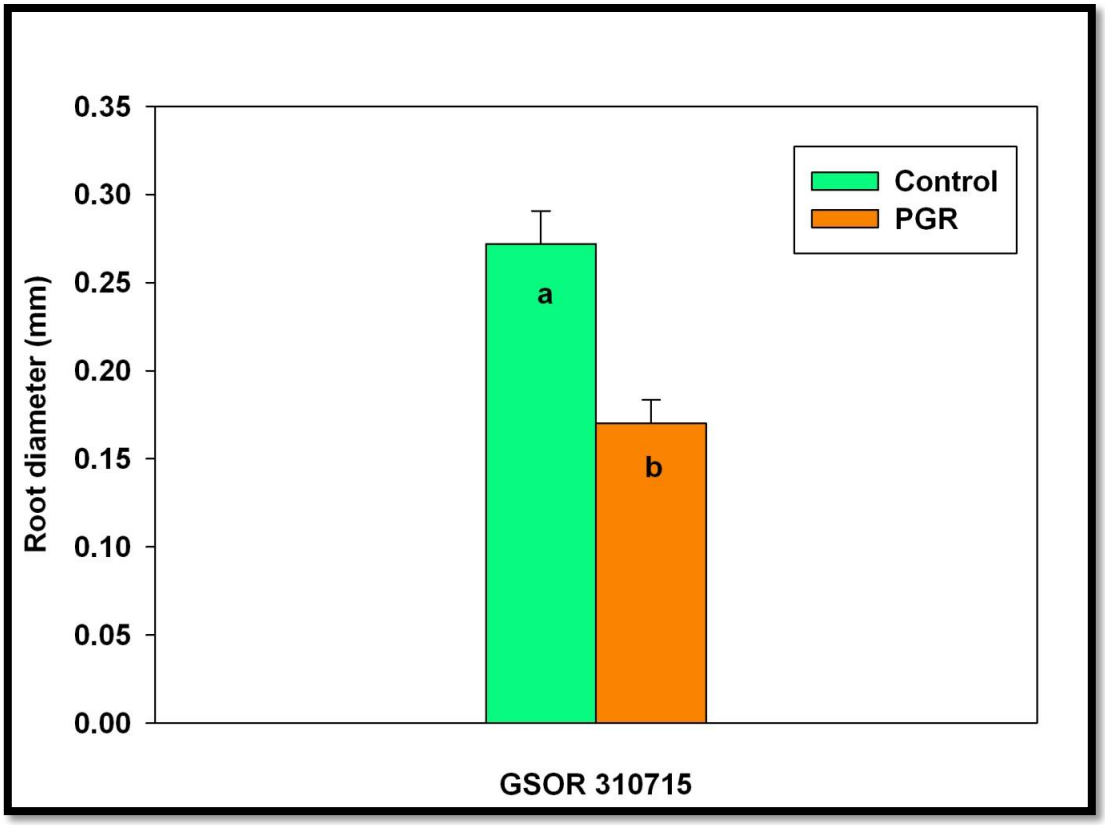


Figure 41. Root diameter of GSOR 310715.
GSOR 310715 showed smaller root diameter with PGR treatment when compared to the control.

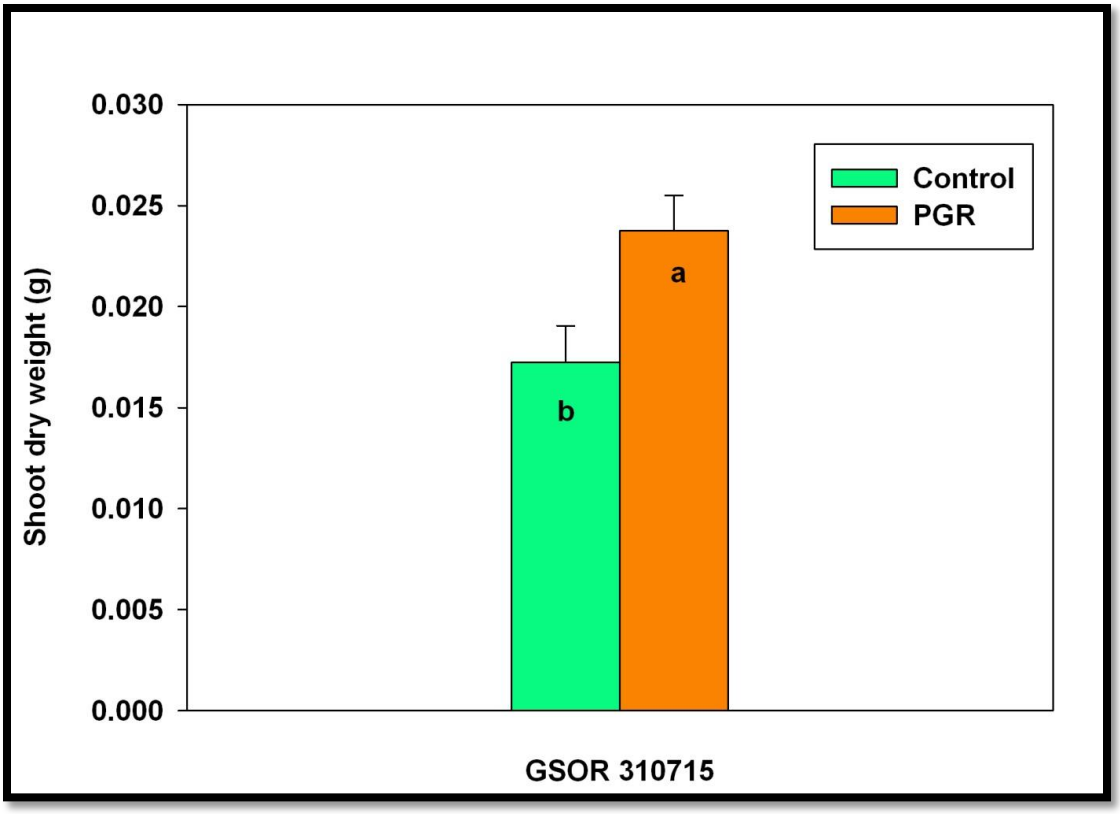


Figure 42. Shoot dry weight of GSOR 310715.
GSOR 310715 showed more shoot dry weight with PGR treatment when compared to the control.

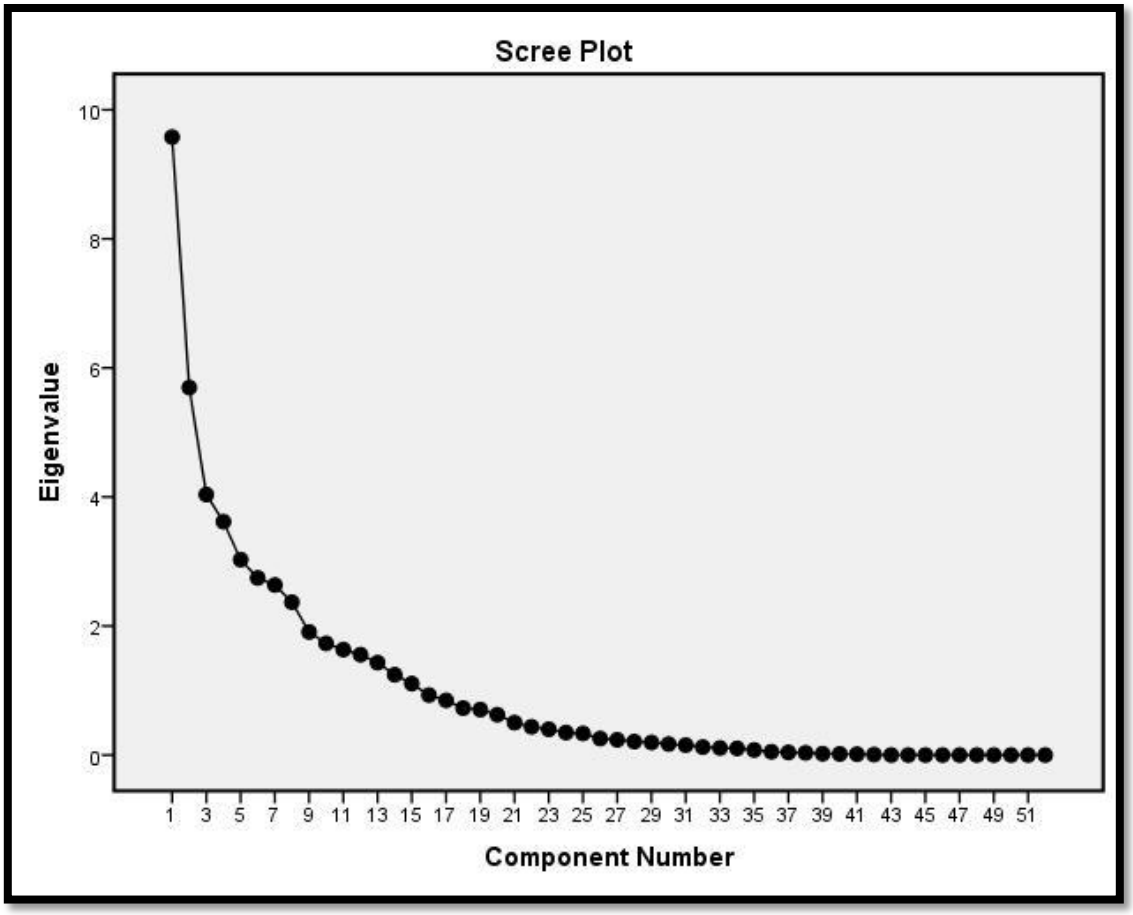


Figure 43. Scree plot - Principal Component Analysis.
Scree plot from Principal Component Analysis showed a number of components with an eigenvalue greater than 1, from which the first two components stood out from the rest.

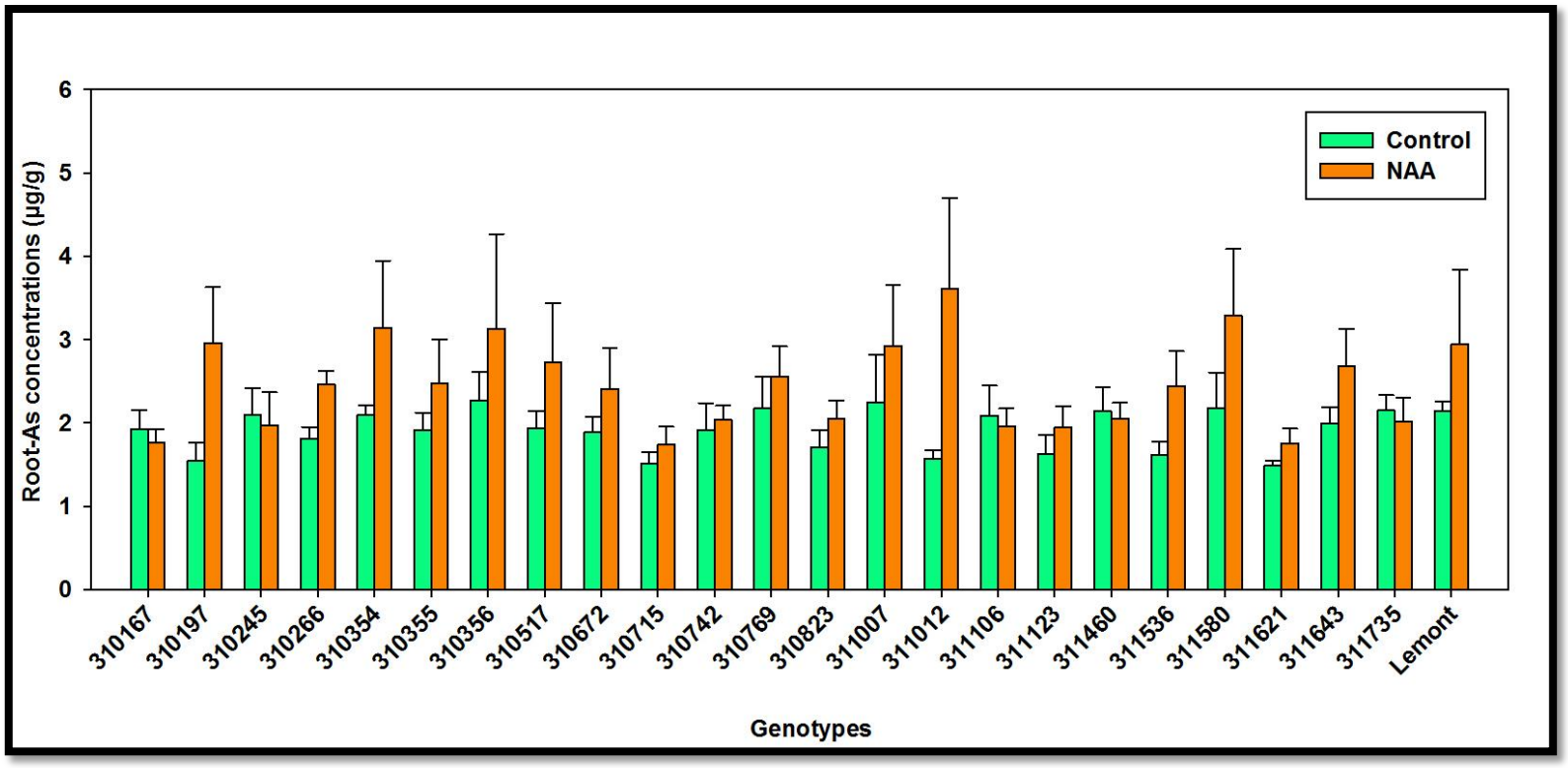


Figure 44. Root-As concentrations of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed increases in root-As concentration when compared to control plants ($P < 0.001$).

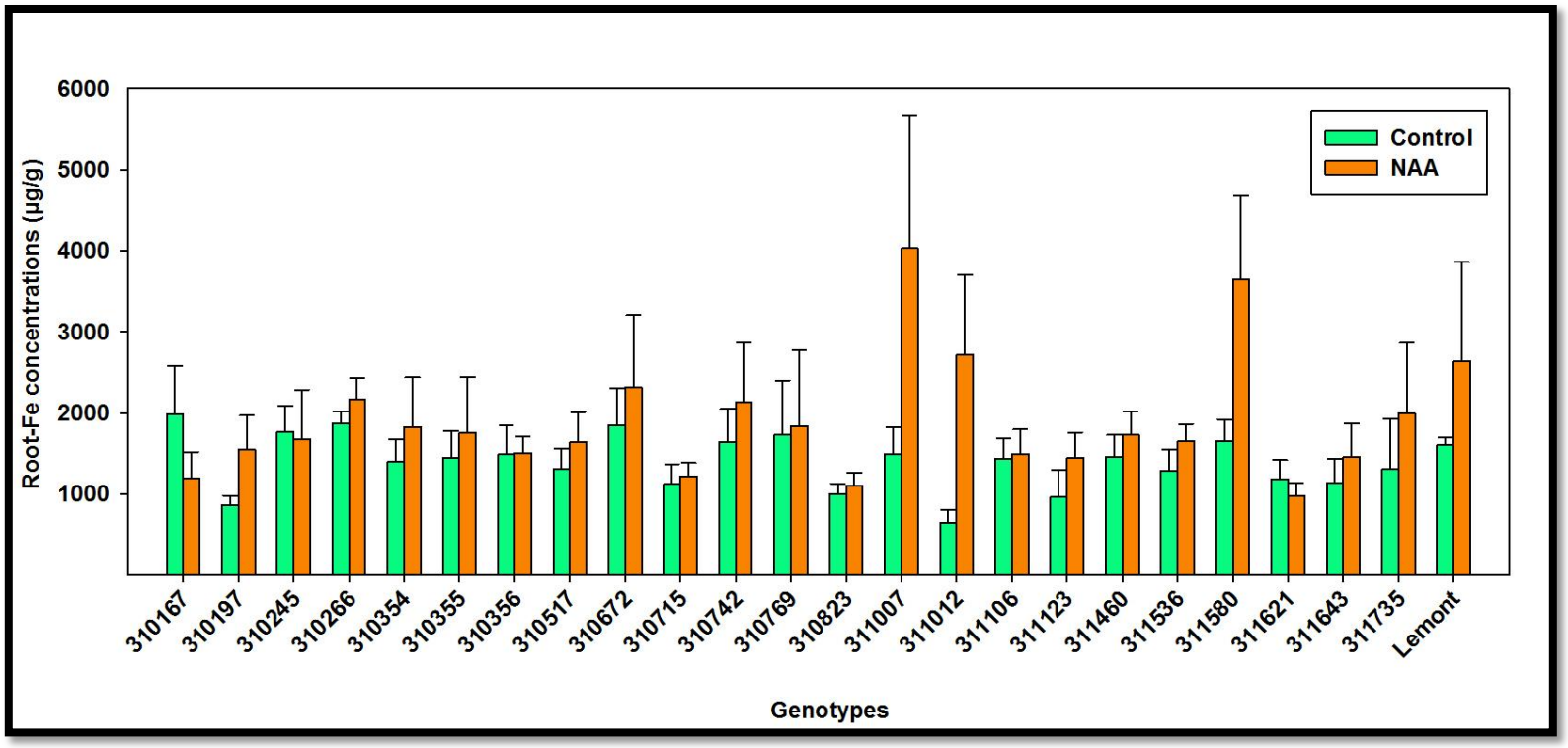


Figure 45. Root-Fe concentrations of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed increases in root-Fe concentrations when compared to control plants ($P < 0.001$).

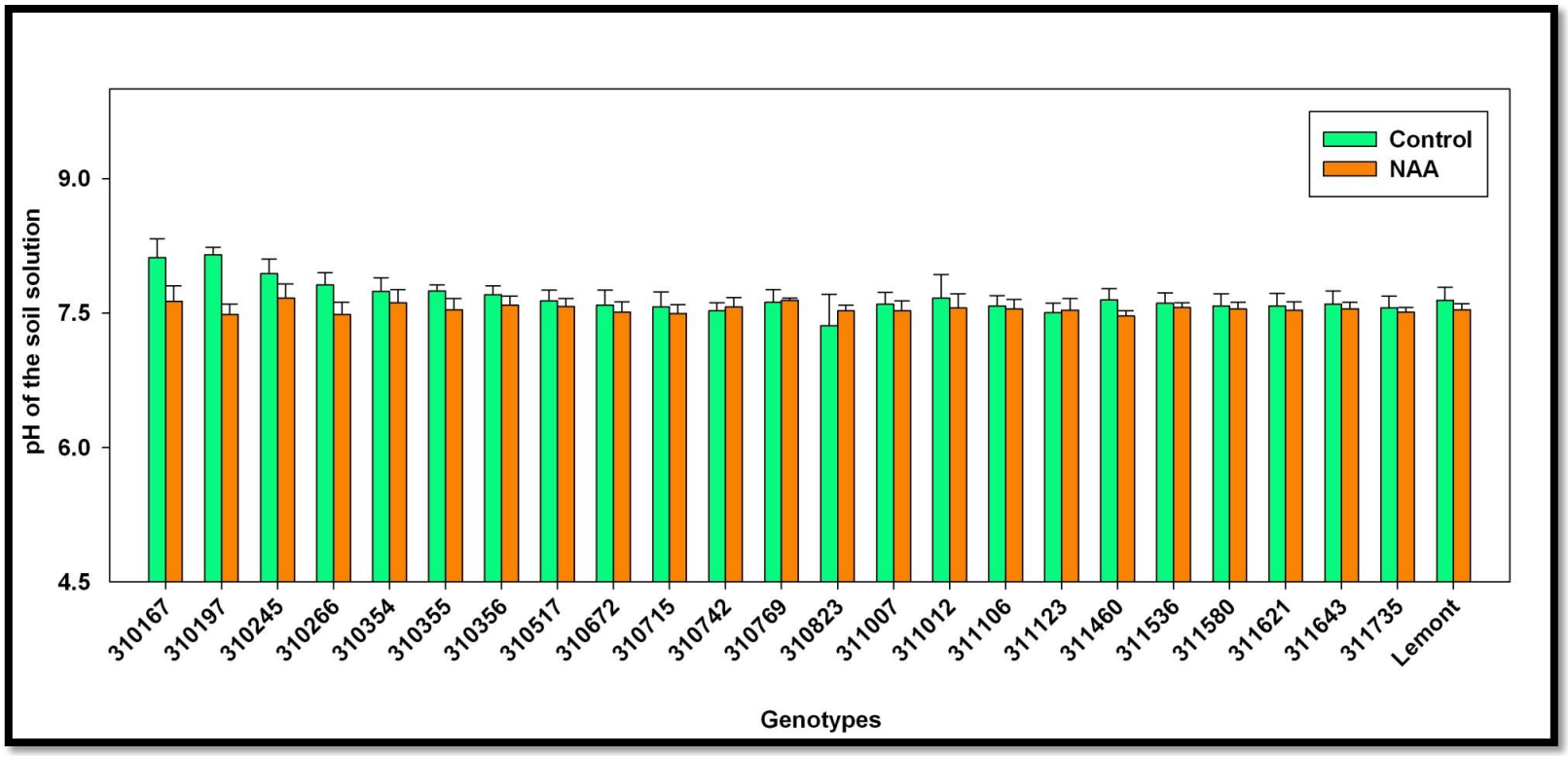


Figure 46. Soil pH of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed decreases in soil pH when compared to control plants ($P < 0.001$).

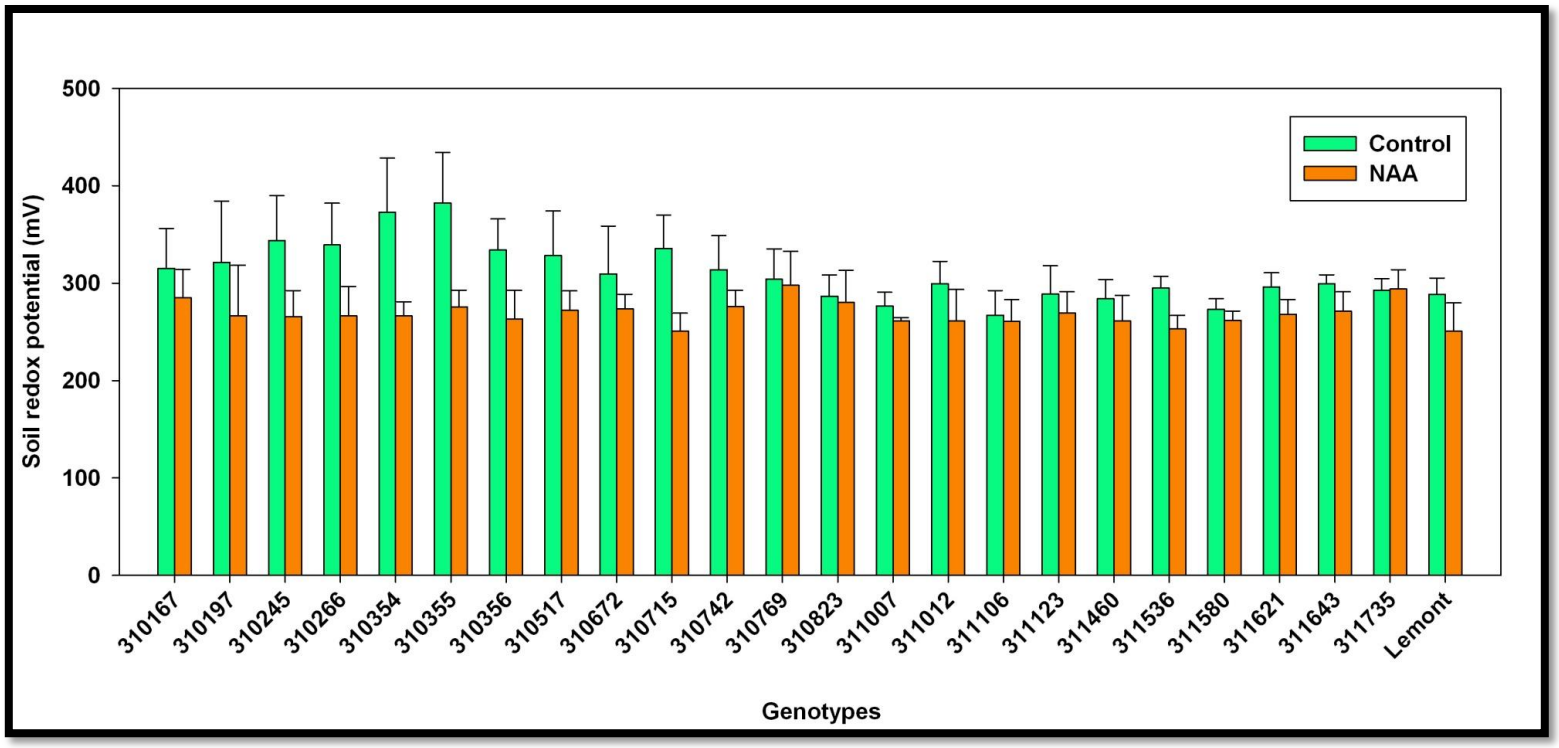


Figure 47. Soil redox potential of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed decreases in soil redox potential when compared to control plants ($P < 0.001$).

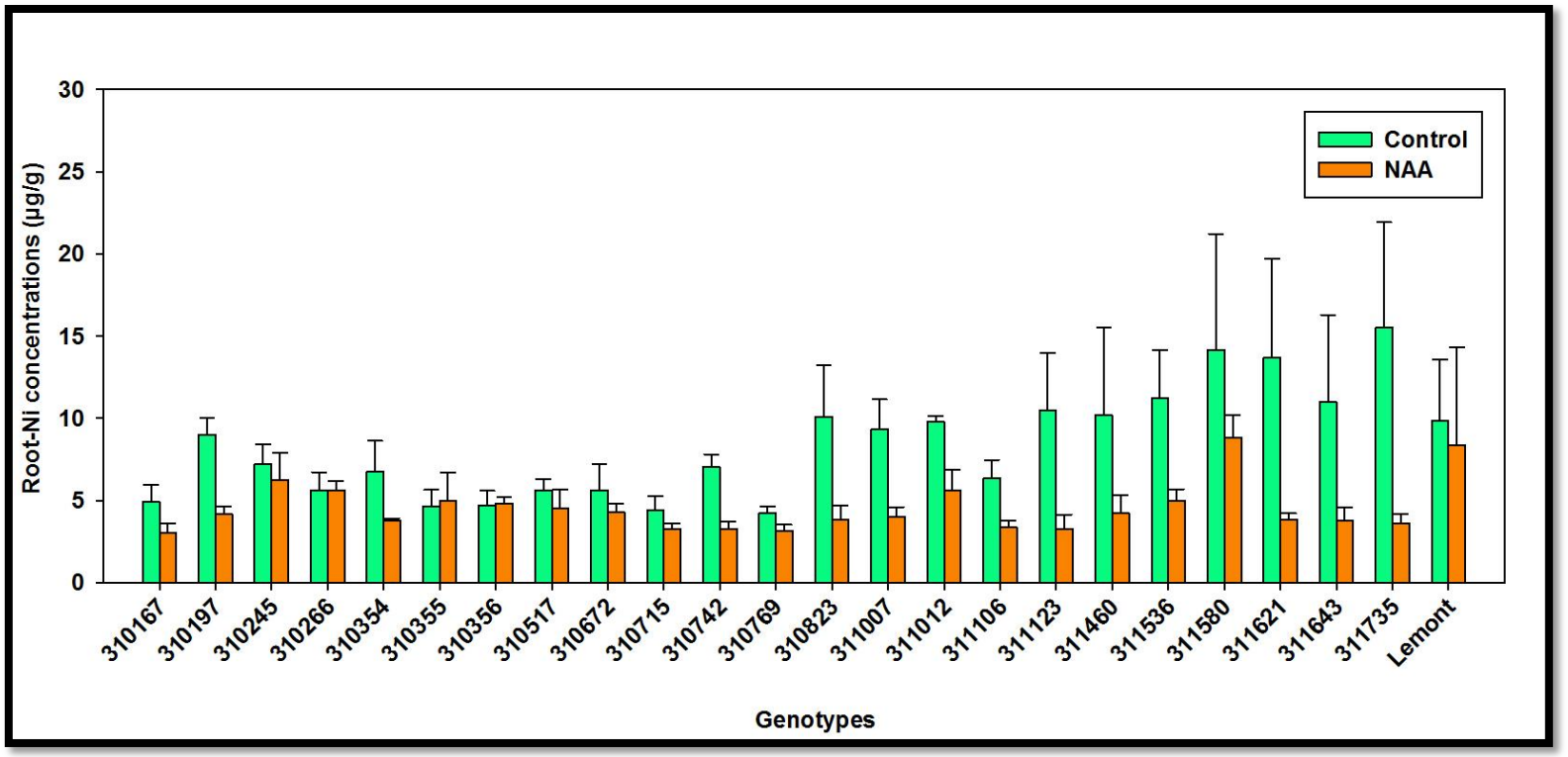


Figure 48. Root-Ni concentrations of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed decreases in root-Ni concentrations when compared to control plants ($P < 0.001$).

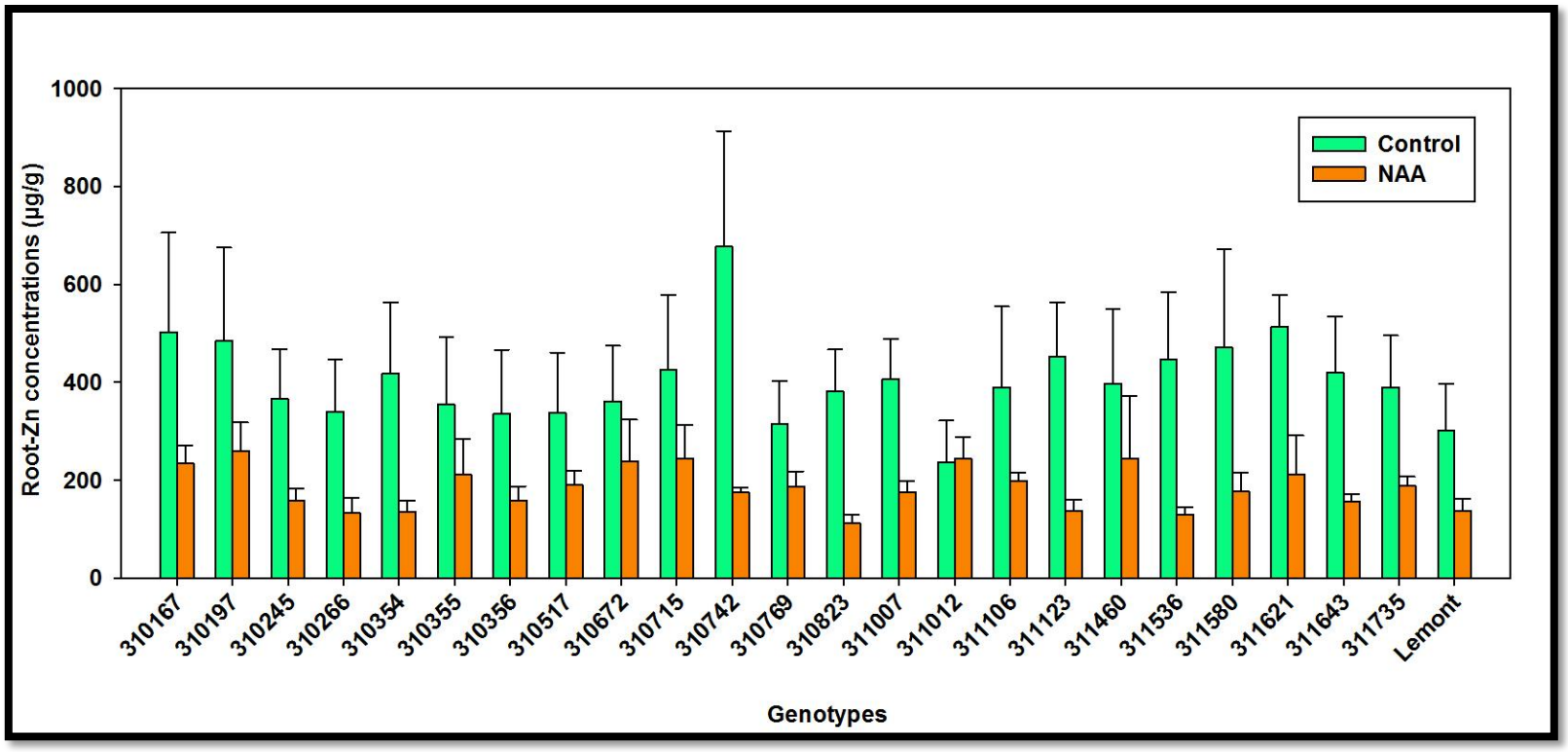


Figure 49. Root-Zn concentrations of control and NAA-treated plants of different genotypes.
NAA-treated plants of all of the genotypes showed decreases in root-Zn concentrations when compared to control plants ($P < 0.001$).

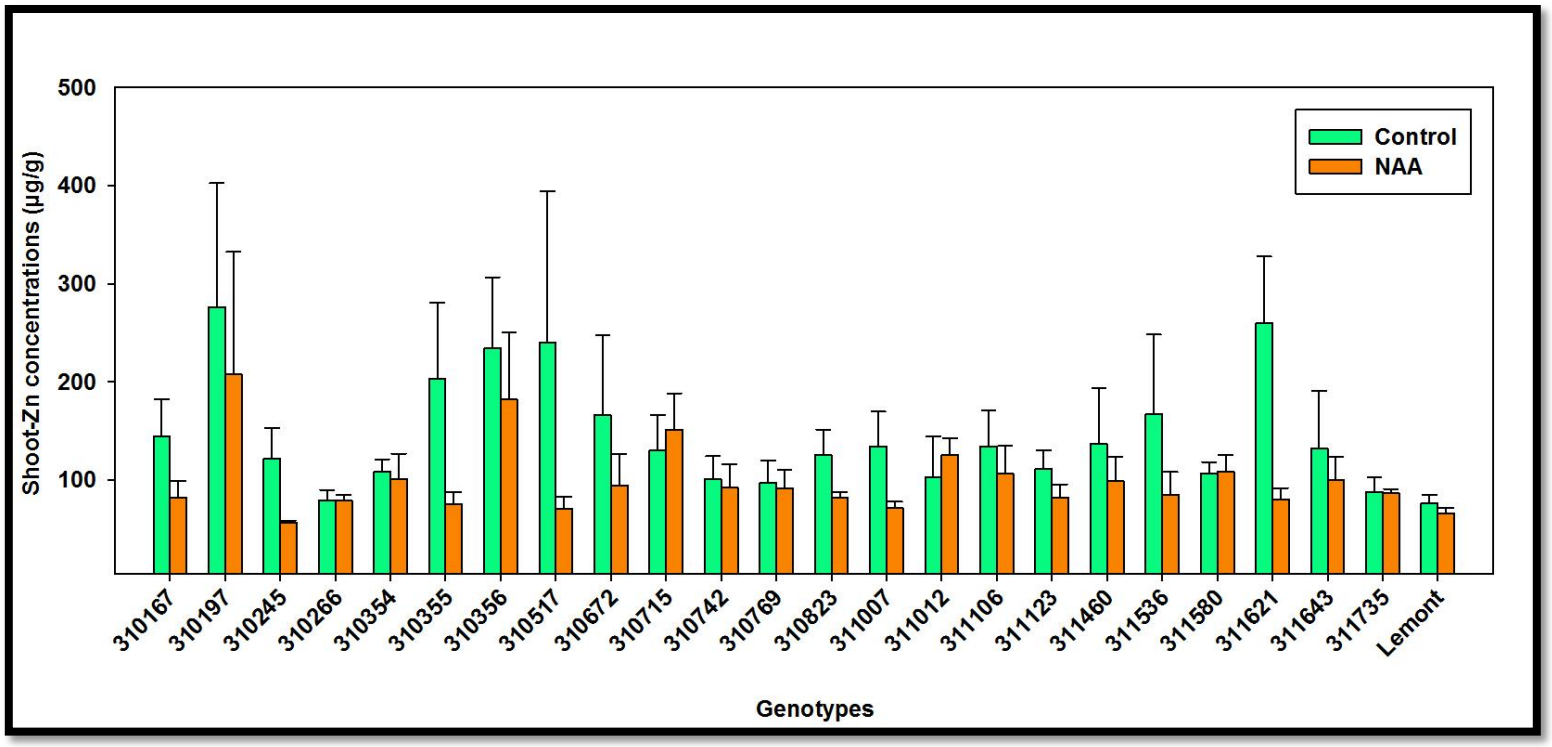


Figure 50. Shoot-Zn concentrations of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed decreases in shoot-Zn concentrations when compared to control plants ($P < 0.001$).

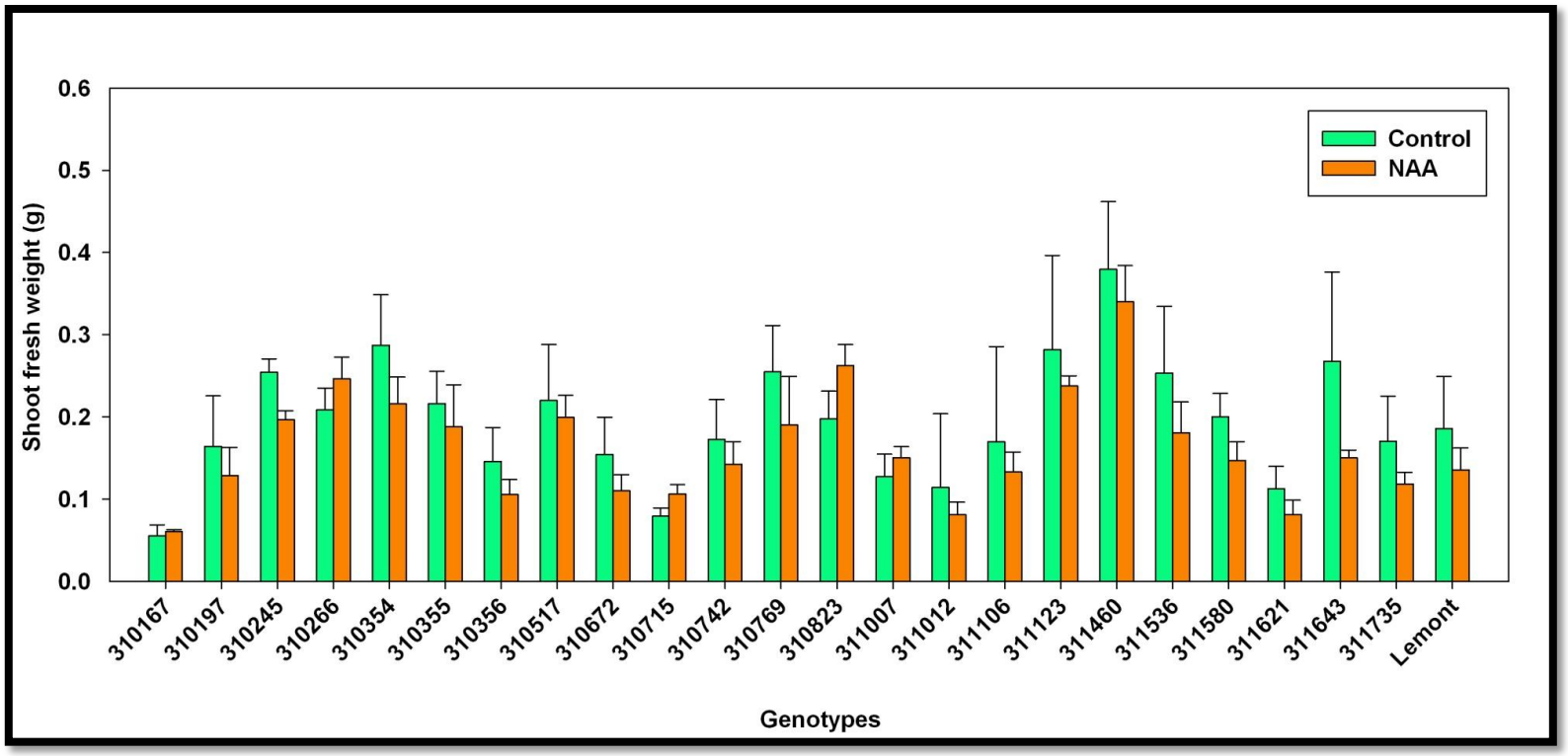


Figure 51. Shoot fresh weights of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed decreases in shoot fresh weight when compared to control plants ($P < 0.001$).

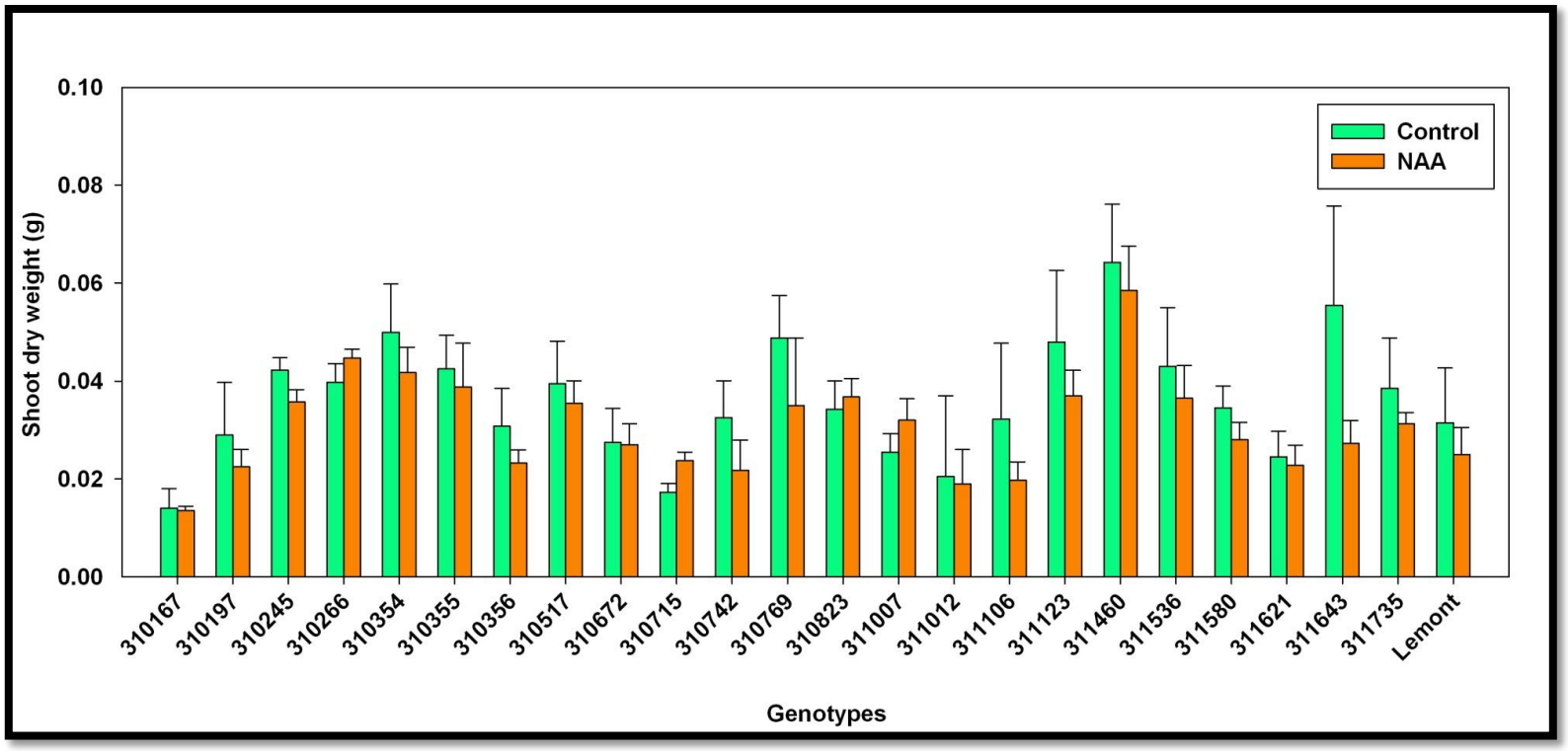


Figure 52. Shoot fresh weights of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed decreases in shoot dry weight when compared to control plants ($P < 0.001$).

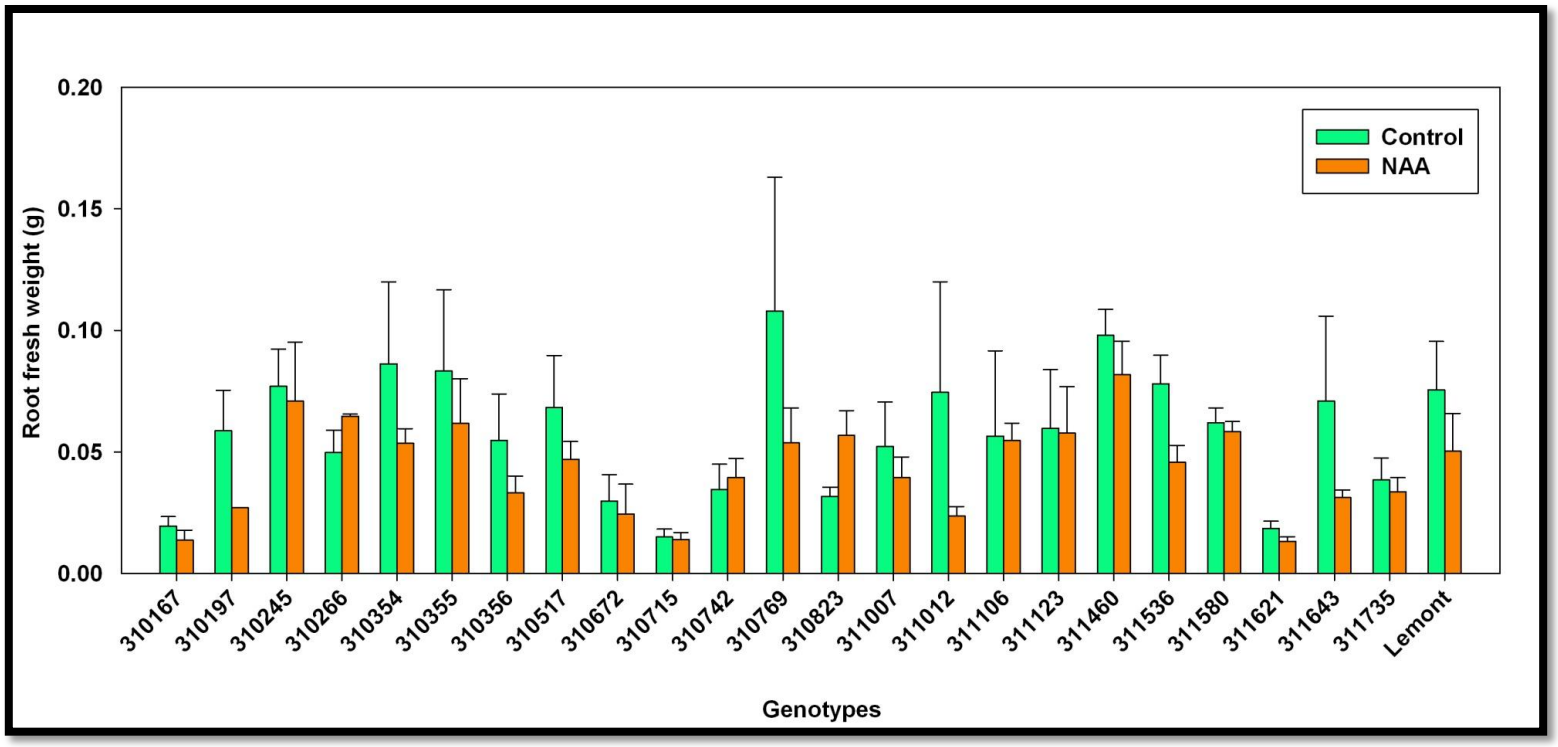


Figure 53. Root fresh weights of control and NAA-treated plants of different genotypes.
NAA-treated plants of most of the genotypes showed decreases in root fresh weight when compared to control plants ($P < 0.001$).

APPENDIX B

TABLES

Table 1. List of genotypes selected for each element, the basis of their selection, p-value resulting from t-test of mean vs. unselecteds for that element, and the Origin and Sub-species.

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
Arsenic (As)	Combined selected (flooded)	HF	0.135	2.33	0.302	NA	NA
	Combined selected (unflooded)	HU	0.896	1.84	0.200	NA	NA
	Unselected	Not HF or HU	NA	1.87	0.085	NA	NA
	311693	HF	0.067	0.67	0.221	Africa (Cameroon)	Unknown
	310491	HF	0.494	2.23	0.369	Africa (Ghana)	IND
	311735[x]	HF	0.004[*]	3.14	0.365	South Pacific (Brunei)	TRJ
	311123	HU	0.348	1.45	0.308	Western Europe (Italy)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310442	HU	0.701	2.02	0.461	Subcontinent (Sri Lanka)	IND
	310811	HU	0.359	2.40	0.551	North Pacific (Japan)	IND
	310769	HU, LF	0.311	1.36	0.361	Eastern Europe (Hungary)	TEJ
	310979	HU	0.547	1.63	0.341	North Pacific (Japan)	IND
	Lemont	US check	0.007*L	1.50	0.104	North America (U.S.A.)	TRJ
Cadmium (Cd)	Combined selected (flooded)	HF	0.000*	0.22	0.026	NA	NA
	Combined selected (unflooded)	HU	0.000*	0.15	0.017	NA	NA
	Unselected	Not HF or HU	NA	0.07	0.003	NA	NA
	310993	HF, HU	0.004*	0.26	0.047	Central Asia (Uzbekistan)	TEJ
	310245	HF, HU	0.065	0.23	0.060	Eastern Europe (Hungary)	TEJ

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	311621	HF, HU	0.053	0.22	0.067	Subcontinent (Sri Lanka)	AUS
	310672	HF, HU	0.117	0.21	0.074	North Pacific (Japan)	AUS
	310364	HF, HU	0.005*	0.18	0.027	Subcontinent (India)	AUS
	311007	HU	0.664	0.07	0.011	South Pacific (Philippines)	IND
	311661	HU	0.813	0.08	0.014	Subcontinent (Sri Lanka)	IND
	311073	HU	0.446	0.08	0.016	Oceania (Indonesia)	IND
	310517	HU	0.086	0.05	0.017	China (Hong Kong)	IND
	Lemont	US check	0.000*L	0.06	0.004	North America (U.S.A.)	TRJ
Calcium (Ca)	Combined selected (flooded)	HF	0.059	7301.18	287.34	NA	NA
	Combined selected (unflooded)	HU	0.244	6930.58	255.88	NA	NA

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	Unselected	Not HF or HU	NA	6548.77	168.56	NA	NA
	310364	HF	0.027*	8525.75	698.39	Subcontinent (India)	AUS
	310155	HF	0.407	6261.38	289.52	Subcontinent (Afghanistan)	AR
	310167	HF, HU	0.978	6576.50	783.75	North America (Mexico)	AUS
	311661	HF, HU	0.488	7417.75	918.94	Subcontinent (Sri Lanka)	IND
	310491	HF, HU	0.002*	9768.83	578.06	Africa (Ghana)	IND
	311106	HF, HU	0.709	6131.40	518.28	Southeast Asia (Vietnam)	IND
	310672	HF, HU	0.846	6365.00	404.65	North Pacific (Japan)	AUS
	311424	HU	0.104	7883.40	1058.63	China (China)	IND
	310517	HU	0.464	6250.00	356.94	China (Hong Kong)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	311007	HU	0.648	6238.71	508.07	South Pacific (Philippines)	IND
	311621	HU	0.824	6712.58	675.89	Subcontinent (Sri Lanka)	AUS
	Lemont (US check)	NF, NU	0.050[*L]	5871.26	241.65	North America (U.S.A.)	TRJ
Cobalt (Co)	Combined selected (flooded)	HF	0.000[*]	0.20	0.020	NA	NA
	Combined selected (unflooded)	HU	0.447	0.12	0.017	NA	NA
	Unselected	Not HF or HU	NA	0.11	0.005	NA	NA
	311130	HF	0.000[*]	0.39	0.049	Subcontinent (India)	TEJ
	311041	HF	0.004[*]	0.18	0.023	Central Asia (Kazakhstan)	TEJ
	311321	HF	0.246	0.17	0.074	Central Asia (Kazakhstan)	TEJ
	311007	HF	0.684	0.12	0.014	South Pacific (Philippines)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310769	HF, HU	0.001*	0.22	0.045	Eastern Europe (Hungary)	TEJ
	311261	HU	0.636	0.08	0.004	South Pacific (Malaysia)	TRJ
	311012	HU	0.109	0.06	0.016	North America (Mexico)	TRJ
	Lemont	US check	0.000*L	0.06	0.005	North America (U.S.A.)	TRJ
Copper (Cu)	Combined selected (flooded)	HF	0.279	6.89	0.284	NA	NA
	Combined selected (unflooded)	HU	0.341	6.80	0.298	NA	NA
	Unselected	Not HF or HU	NA	6.49	0.131	NA	NA
	310244	HF	0.072	7.59	0.613	Eastern Europe (Hungary)	TEJ
	310769	HF, HU	0.174	7.56	0.346	Eastern Europe (Hungary)	TEJ
	311514	HF, HU	0.380	6.02	0.273	China (China)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	311073	HU	0.748	6.71	0.638	Oceania (Indonesia)	IND
	311041	HU	0.225	7.25	0.808	Central Asia (Kazakhstan)	TEJ
	310245	HU	0.466	7.18	1.536	Eastern Europe (Hungary)	TEJ
	Lemont	US check	0.319	6.673	0.128	North America (U.S.A.)	TRJ
Iron (Fe)	Combined selected (flooded)	HF	0.126	53.18	1.992	NA	NA
	Combined selected (unflooded)	HU	0.209	54.09	1.622	NA	NA
	Unselected	Not HF or HU	NA	56.48	0.976	NA	NA
	311261	HF	0.354	49.00	1.986	Africa (Zaire)	TRJ
	310672	HF	0.818	55.26	3.599	North Pacific (Japan)	AUS
	310811	HF	0.040*	65.02	6.028	North Pacific (Japan)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310769	HF, HU	0.013	43.17	2.561	Eastern Europe (Hungary)	TEJ
	311514	HF, HU	0.436	53.63	2.338	China (China)	IND
	310424	HU, LF	0.110	63.98	7.334	China (China)	IND
	311536	HU	0.098	63.87	2.572	Subcontinent (India)	IND
	311106	HU	0.245	49.20	3.088	Southeast Asia (Vietnam)	IND
	311321	HU	0.248	47.13	4.776	Central Asia (Kazakhstan)	TEJ
	311041	HU	0.039[*L]	47.93	3.703	Central Asia (Kazakhstan)	TEJ
	310742	HU	0.525	52.48	5.808	Africa (Mali)	IND
	310245	HU	0.954	56.84	3.372	Eastern Europe (Hungary)	TEJ
	Lemont	US check	0.000[*L]	46.323	1.128	North America (U.S.A.)	TRJ

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
Magnesium (Mg)	Combined selected (flooded)	HF	0.951	2072.2	87.5	NA	NA
	Combined selected (unflooded)	HU	0.652	2035.1	70.6	NA	NA
	Unselected	Not HF or HU	NA	2079.0	37.8	NA	NA
	310491	HF	0.496	1910.3	135.7	Africa (Ghana)	IND
	311106	HF	0.731	2172.4	231.1	Southeast Asia (Vietnam)	IND
	311689	HF, HU	0.042*	2547.6	118.5	Africa (Mali)	AUS
	311007	HF, HU	0.200	1868.1	128.8	South Pacific (Philippines)	IND
	310197	HU	0.805	2022.4	120.4	South America (Suriname)	TRJ-IND-AUS
	310424	HU	0.686	2000.8	146.4	China (China)	IND
	311661	HU	0.411	1830.3	56.0	Subcontinent (Sri Lanka)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	Lemont	US check	0.001*	2287.4	47.5	North America (U.S.A.)	TRJ
Manganese (Mn)	Combined selected (flooded)	HF	0.336	366.93	44.17	NA	NA
	Combined selected (unflooded)	HU	0.812	312.67	20.79	NA	NA
	Unselected	Not HF or HU	NA	319.53	11.37	NA	NA
	310155	HF	0.324	256.38	35.37	Subcontinent (Afghanistan)	AR
	310356	HF	0.009*	514.33	43.53	South Pacific (Malaysia)	TRJ
	311007	HU	0.242	376.86	36.94	South Pacific (Philippines)	IND
	310167	HU	0.578	360.67	47.58	North America (Mexico)	AUS
	310424	HU	0.612	290.20	49.13	China (China)	IND
	311012	HU	0.039*L	260.00	22.11	North America (Mexico)	TRJ

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310355	HU	0.154	213.83	48.89	South Pacific (Malaysia)	TRJ
	Lemont	US check	0.002*	443.13	37.47	North America (U.S.A.)	TRJ
Molybdenum (Mo)	Combined selected (flooded)	HF	0.000*	12.24	1.189	NA	NA
	Combined selected (unflooded)	HU	0.000*	9.11	0.904	NA	NA
	Unselected	Not HF or HU	NA	5.05	0.209	NA	NA
	310823	HF	0.042*	8.08	2.013	Mideast (Iraq)	AUS
	311012	HF	0.764	5.42	1.295	North America (Mexico)	TRJ
	311735	HF, HU	0.000*	12.61	1.239	South Pacific (Brunei)	TRJ
	311643	HF, HU	0.000*	21.10	2.484	South Pacific (Malaysia)	TRJ
	310354	HF, HU	0.649	4.31	0.906	South Pacific (Malaysia)	TRJ

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310356	HF, HU	0.000[*]	19.41	2.024	South Pacific (Malaysia)	TRJ
	310355	HF, HU	0.027[*]	16.83	3.824	South Pacific (Malaysia)	TRJ
	310197	HU	0.000[*L]	2.70	0.185	South America (Suriname)	TRJ-IND-AUS
	310364	HU	0.825	4.79	1.078	Subcontinent (India)	AUS
	310167	HU	0.658	5.65	1.428	North America (Mexico)	AUS
	311693	HU	0.174	2.83	0.668	Africa (Cameroon)	Unknown
	Lemont	US check	0.000[*L]	3.191	0.150	North America (U.S.A.)	TRJ
Nickel (Ni)	Combined selected (flooded)	HF	0.416	0.50	0.083	NA	NA
	Combined selected (unflooded)	HU	0.924	0.43	0.048	NA	NA
	Unselected	Not HF or HU	NA	0.43	0.017	NA	NA

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310244	HF	0.356	0.64	0.214	Eastern Europe (Hungary)	TEJ
	311514	HF	0.778	0.41	0.041	China (China)	IND
	310742	HF	0.938	0.44	0.052	Africa (Mali)	IND
	311106	HU	0.332	0.31	0.063	Southeast Asia (Vietnam)	IND
	310197	HU	0.191	0.30	0.069	South America (Suriname)	TRJ-IND-AUS
	311012	HU, LF	0.824	0.46	0.167	North America (Mexico)	TRJ
	310442	HU	0.240	0.33	0.027	Subcontinent (Sri Lanka)	IND
	310424	HU	0.328	0.51	0.116	China (China)	IND
	310491	HU	0.412	0.68	0.284	Africa (Ghana)	IND
	310742	HU	0.913	0.44	0.052	Africa (Mali)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	Lemont	US check	0.01[*L]	0.33	0.034	North America (U.S.A.)	TRJ
Phosphorus (P)	Combined selected (flooded)	HF	0.550	2288.2	91.5	NA	NA
	Combined selected (unflooded)	HU	0.415	2446.4	87.0	NA	NA
	Unselected	Not HF or HU	NA	2364.2	49.5	NA	NA
	311106	HF	0.000[*L]	1955.2	54.9	Southeast Asia (Vietnam)	IND
	310491	HF	0.539	2165.7	209.4	Africa (Ghana)	IND
	310424	HF	0.002[*L]	1763.0	141.2	China (China)	IND
	310197	HF, HU	0.256	2705.3	204.7	South America (Suriname)	TRJ-IND-AUS
	311007	HF, HU	0.219	2626.1	134.0	South Pacific (Philippines)	IND
	311661	HU	0.342	2260.0	86.8	Subcontinent (Sri Lanka)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310356	HU	0.407	2096.5	162.9	South Pacific (Malaysia)	TRJ
	311261	HU	0.366	1952.3	87.8	South Pacific (Malaysia)	TRJ
	Lemont	US check	0.000[*L]	1865.5	34.2	North America (U.S.A.)	TRJ
Potassium (K)	Combined selected (flooded)	HF	0.160	22044	1035	NA	NA
	Combined selected (unflooded)	HU	0.118	21732	1139	NA	NA
	Unselected	Not HF or HU	NA	23532	450	NA	NA
	310197	HF	0.000[*]	33750	2815	South America (Suriname)	TRJ-IND-AUS
	310742	HF	0.104	28731	3711	Africa (Mali)	IND
	310356	HF	0.000[*L]	19279	567	South Pacific (Malaysia)	TRJ
	311693	HF	0.080	17322	1100	Africa (Cameroon)	Unknown

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	311661	HF, HU	0.347	26869	2767	Subcontinent (Sri Lanka)	IND
	311012	HF, HU	0.003[*L]	15573	1378	North America (Mexico)	TRJ
	310424	HU	0.000[*L]	15371	1516	China (China)	IND
	311007	HU	0.653	22962	1162	South Pacific (Philippines)	IND
	311106	HU	0.458	21183	1917	Southeast Asia (Vietnam)	IND
	Lemont	US check	0.000[*L]	20432	664	North America (U.S.A.)	TRJ
Rubidium (Rb)	Combined selected (flooded)	HF	0.132	4.75	0.301	NA	NA
	Combined selected (unflooded)	HU	0.005[*]	4.43	0.217	NA	NA
	Unselected	Not HF or HU	NA	5.17	0.103	NA	NA
	311661	HF	0.886	5.29	0.468	Subcontinent (Sri Lanka)	IND

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310742	HF	0.013*	7.00	0.784	Africa (Mali)	IND
	310491	HF	0.149	4.22	0.618	Africa (Ghana)	IND
	311012	HF	0.002*L	3.27	0.312	North America (Mexico)	TRJ
	310197	HF, HU	0.198	5.96	0.234	South America (Suriname)	TRJ-IND-AUS
	310424	HF, HU	0.016*L	3.91	0.674	China (China)	IND
	311073	HU	0.309	4.63	0.262	Oceania (Indonesia)	IND
	310442	HU	0.008*L	3.87	0.263	Subcontinent (Sri Lanka)	IND
	311106	HU	0.184	4.22	0.361	Southeast Asia (Vietnam)	IND
	Lemont	US check	0.003*L	4.61	0.150	North America (U.S.A.)	TRJ
Strontium (Sr)	Combined selected (flooded)	HF	0.011*	37.20	1.599	NA	NA

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	Combined selected (unflooded)	HU	na	na	na	NA	NA
	Unselected	Not HF or HU	NA	32.31	0.762	NA	NA
	310167	HF, NU	0.719	34.13	4.259	North America (Mexico)	AUS
	310364	HF, NU	0.001*	46.46	3.617	Subcontinent (India)	AUS
	310491	HF, NU	0.006*	46.38	2.578	Africa (Ghana)	IND
	310672	HF, NU	0.635	34.53	2.365	North Pacific (Japan)	AUS
	311621	HF, NU	0.381	35.50	3.650	Subcontinent (Sri Lanka)	AUS
	310155	HF, NU	0.015*L	28.24	1.235	Subcontinent (Afghanistan)	ARO
	Lemont	US check	0.002*L	27.09	1.223	North America (U.S.A.)	TRJ
Sulfur (S)	Combined selected (flooded)	HF	0.279	3795.5	265.2	NA	NA

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	Combined selected (unflooded)	HU	0.000*	5482.8	257.8	NA	NA
	Unselected	Not HF or HU	NA	4151.3	138.0	NA	NA
	310197	HF	0.129	2977.3	406.4	South America (Suriname)	TRJ-IND-AUS
	310266	HF	0.014*L	2083.3	415.1	Central America (El Salvador)	TRJ-IND
	311073	HF, HU	0.550	3744.0	358.3	Oceania (Indonesia)	IND
	310979	HF, HU	0.064	5268.8	605.8	North Pacific (Japan)	IND
	310424	HF, LU	0.461	3674.0	404.7	China (China)	IND
	310769	HU	0.064	5582.7	431.6	Eastern Europe (Hungary)	TEJ
	311123	HU	0.000*	7127.6	914.5	Western Europe (Italy)	IND
	310155	HU	0.136	5254.8	952.1	Subcontinent (Afghanistan)	ARO

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	311514	HU	0.000*	6245.9	440.6	China (China)	IND
	310517	HU	0.413	4714.9	622.4	China (Hong Kong)	IND
	Lemont	US check	0.330	3879.7	188.4	North America (U.S.A.)	TRJ
Zinc (Zn)	Combined selected (flooded)	HF	0.884	57.46	7.82	NA	NA
	Combined selected (unflooded)	HU	0.962	58.71	4.47	NA	NA
	Unselected	Not HF or HU	NA	59.04	2.98	NA	NA
	310266	HF	0.260	37.27	5.38	Central America (El Salvador)	TRJ-IND
	311643	HF, HU	0.745	50.17	3.47	South Pacific (Malaysia)	TRJ
	310823	HF, HU	0.042*	102.48	19.54	Mideast (Iraq)	AUS
	310355	HF, HU	0.430	43.80	5.15	South Pacific (Malaysia)	TRJ

Element[w]	Genotype	Grain selection[y]	p-value	Means	Std. errors	Origin	Sub-species[z]
	310769	HU	0.625	67.80	10.86	Eastern Europe (Hungary)	TEJ
	310197	HU	0.275	39.54	2.79	South America (Suriname)	TRJ-IND-AUS
	311661	HU	0.642	48.05	7.93	Subcontinent (Sri Lanka)	IND
	311536	HU	0.426	47.14	2.53	Subcontinent (India)	IND
	Lemont	US check	0.002[*L]	39.81	3.15	North America (U.S.A.)	TRJ

[w]For each element, every genotype was selected within the first 200th ranking except for Fe and S under flooded conditions for which genotypes were selected between 300th to 500th rank as there was none within 200th rank.
[x]Color codes: Green- significantly higher than the unselected means (p value ‹ 0.05); red- significantly lower than the unselected means.
[y]HF= High in flooded; HU = High in unflooded; LF = Low in flooded; LU = Low in unflooded; , na = not available, NA = Not applicable..
[z]TRJ= Tropical Japonica; TEJ = Temperate Japonica; IND = Indica; ARO = Aromatic; AUS = Aus subspecies; per Agrama et al. 2010 (Agrama *et al.*, 2010).
*p value ‹ 0.05, means are significantly different from the unselected means.
*[L] Mean leaf concentration of the high-grain selections was lower than the unselected.

Table 2. Shoot ionomics (µg/g) of Malaysian genotype GSOR 310356 at different pH regimes.

	pH		
Element	4.7	5.4	6.1
Na	710.1	881.7	710.6
Mg	1042.7	1015.3	1446.5
P	14466.2[x]	14893	12517
S	4475.1	4678.6	5173.2
K	29541	30568	29168
Ca	6115.2	5196.1	7364
Mn	144.9	171.4	1510.7
Fe	541.3	442.6	404.2
Ni	2.6	2.15	1.61
Cu	33.9	31.1	41.2
Zn	86.1	81	173.9
As	0.19	0.18	0.162
Se	3.41	3.73	3.15
Rb	7.95	6.62	6.19
Sr	6.77	4.97	6.19
Mo	8.23	8.93	16.6

[x]GSOR 310356 showed high shoot-P, -Fe and -As concentrations (dark yellow cells) and low shoot-Mn, -Cu, and -Zn concentrations (light yellow cells) at pH 4.7 and 5.4.

Table 3. Shoot ionomics (µg/g) of Iraqi genotypes GSOR 310823 at different pH regimes.

	pH		
Element	4.7	5.4	6.1
Na	473.6	589.3	459.1
Mg	1002.7	979.9	1354.5
P	15104.4[x]	13928	11679
S	6024.2	5596.9	6886.3
K	39951	38583	44745
Ca	5166.8	5499.8	5923
Mn	234	225	1805
Fe	627.3	668	459.3
Ni	2.99	1.76	2.07
Cu	39.9	38.5	52.2
Zn	126	119.7	286.4
As	0.171	0.192	0.151
Se	3.19	3.21	2.9
Rb	5.97	6.38	5.59
Sr	3.73	4.38	3.77
Mo	3.56	3.78	4.06

[x]GSOR 310823 showed high shoot-P, -Fe and -As concentrations (dark yellow cells) and low shoot-Mn, -Cu, and -Zn concentrations(light yellow cells) at pH 4.7 and 5.4.

Table 4. Shoot ionomics (µg/g) of US genotype Lemont at different pH regimes.

	pH		
Element	4.7	5.4	6.1
Na	850.1	760.1	810.4
Mg	1033.2	1060.4	1354.1
P	16745.7[x]	15739	13414
S	4191.2	4291.2	5221.1
K	33323	33794	37398
Ca	4701.1	5165.9	5941.6
Mn	104.2	158.3	1281.1
Fe	393.1	493.7	330.6
Ni	2.06	2.77	1.91
Cu	28.9	31.4	40.2
Zn	68.6	78.8	157.8
As	0.176	0.177	0.136
Se	3.92	3.67	3.5
Rb	8.67	8.73	6.86
Sr	4.73	5.31	3.83
Mo	4.1	3.18	4.29

[x]Lemont showed high shoot-P, -Fe and -As concentrations (dark yellow cells) and low shoot-Mn, -Cu, and -Zn concentrations (light yellow cells) at pH 4.7 and 5.4.

Table 5. Shoot traits of GSOR 310356, GSOR 310823 and Lemont at different pH regimes.

	pH	Genotype	GSOR 310356	GSOR 310823	Lemont
Leaf respiration ($\mu mol\ m^{-2}\ s^{-1}$)	4.7	Mean	-0.867	-0.931	-0.915
		S.E	0.063	0.067	0.065
	6.1	Mean	-1.102	-1.305	-1.402
		S.E	0.075	0.117	0.122
Leaf color	4.7	Mean	2.735	2.294	2.583
		S.E	0.076	0.096	0.093
	6.1	Mean	2.824	2.618	3.124
		S.E	0.06	0.068	0.105
Shoot fresh weight (g)	4.7	Mean	0.184[y]	0.268	0.16
		S.E[*]	0.017	0.021	0.011
	6.1	Mean	0.255	0.368	0.254
		S.E	0.012	0.018	0.012
Photosynthesis ($\mu mol\ m^{-2}\ s^{-1}$)	4.7	Mean	3.217	4.291	3.656
		S.E	0.351	0.673	0.371
	6.1	Mean	4.575	7.274	7.549
		S.E	0.368	0.644	0.768

[*] S.E. - Standard error.

[y] All genotypes showed less shoot fresh weight, lower rate of photosynthesis and leaf respiration at pH 4.7 (pink colored cells) when compared to 6.1 and GSOR 310823 and Lemont showed less leaf color than pH 6.1.

Table 6. Root traits of GSOR 310356, GSOR 310823 and Lemont at different pH regimes.

	pH	Genotype	GSOR 310356	GSOR 310823	Lemont
Root radius (mm)	4.7	Mean	0.163[y]	0.168	0.18
		S.E[*]	0.012	0.008	0.011
	6.1	Mean	0.178	0.182	0.189
		S.E	0.009	0.008	0.01
Root perimeter (cm)	4.7	Mean	69.14	81.9	81.7
		S.E	6.125	5.887	3.438
	6.1	Mean	87.12	97.4	84.83
		S.E	4.668	3.748	3.887
Specific root length (cm)	4.7	Mean	512.05	720	650.89
		S.E	62.408	85.743	43.027
	6.1	Mean	765.32	936.8	724.44
		S.E	73.58	71.01	75.02
Root fresh weight (g)	4.7	Mean	0.096	0.179	0.097
		S.E	0.013	0.022	0.008
	6.1	Mean	0.144	0.262	0.152
		S.E	0.01	0.024	0.014

[y]All genotypes showed less shoot fresh weight, lower rate of photosynthesis and leaf respiration at pH 4.7 (blue colored cells) when compared to 6.1 and GSOR 310823 and Lemont showed less leaf color than pH 6.1.

Table 7. Root traits of GSOR 310356, GSOR 310823 and Lemont at different pH regimes.

	pH	Genotype	GSOR 310356	GSOR 310823	Lemont
Root width (cm)	4.7	Mean	3.733[y]	5.017	5.323
		S.E[*]	0.296	0.352	0.538
	6.1	Mean	5.847	5.869	5.873
		S.E	0.426	0.431	0.431
Root depth (cm)	4.7	Mean	12.582	11.855	13.268
		S.E	1.609	1.814	1.078
	6.1	Mean	12.693	14.25	14.28
		S.E	1.694	1.943	1.945
Root dry weight (g)	4.7	Mean	0.012	0.02	0.014
		S.E	0.002	0.002	0.001
	6.1	Mean	0.015	0.023	0.023
		S.E	0.001	0.002	0.003
Root volume (cm^3)	4.7	Mean	0.325	0.374	0.364
		S.E	0.025	0.025	0.017
	6.1	Mean	0.365	0.389	0.395
		S.E	0.016	0.019	0.02

[*] S.E. - Standard error

[y]GSOR 310356 showed less root width, GSOR 310823 showed less root depth and Lemont showed less root dry weight and root volume at pH 4.7 (blue colored cells) when compared to pH 6.1

Table 8. Root traits of GSOR 310356, GSOR 310823 and Lemont at different pH regimes.

	pH	Genotype	GSOR 310356	GSOR 310823	Lemont
Median no. of roots	4.7	Mean	9.88	11.91	12.31
		S.E*	0.753	0.833	1.36
	6.1	Mean	13.73	13.92	12.57
		S.E	1.167	1.504	2.067
Maximum no. of roots	4.7	Mean	21.85	24.27	25.02
		S.E	0.833	1.444	1.738
	6.1	Mean	26.79	27.05	24.98
		S.E	1.843	2.293	3.276
Root surface area (cm^2)	4.7	Mean	19.995	23.682	23.62
		S.E	1.765	1.692	0.982
	6.1	Mean	25.203	28.173	24.532
		S.E	1.357	1.084	1.116
Root width/depth	4.7	Mean	0.831	0.909	0.91
		S.E	0.223	0.15	0.209
	6.1	Mean	1.088	1.237	0.976
		S.E	0.195	0.377	0.229

*S.E. - Standard error

[y]GSORs 310356 and 310823 showed less median and maximum numbers of roots, less root surface and root width/depth ratio at pH 4.7 (blue colored cells) when compared to pH 6.1.

Table 9. Shoot element concentrations and their correlation coefficients with shoot dry weight ($P < 0.05$).

Elements	Correlation
Shoot -Na[x]	-.467**
Shoot-Mg	-.184*
Shoot-P	.077
Shoot-S	.206*
Shoot-K	.326**
Shoot-Ca	.013
Shoot-Cr	-.315**
Shoot-Mn	.341**
Shoot-Fe	.139
Shoot-Co	.043
Shoot-Ni	-.023
Shoot-Cu	.412**
Shoot-Zn	.345**
Shoot-As	-.396**
Shoot-Se	-.695**
Shoot-Rb	-.476**
Shoot-Sr	-.460**
Shoot-Mo	-.103
Shoot-Cd	-.074

[x] Elements that showed positive association (green colored cells) and negative (orange colored cells) association with shoot dry weight.

Table 10. Variable loadings on the first two principal components.

	Components	
Variables	1	2
Plant height	0.778[x]	0.096
Leaf number	0.563	-0.112
Leaf color	0.180	0.223
Leaf area	0.812	0.176
Number of tillers	0.338	0.010
Photosynthesis	0.523	0.184
Root fresh weight	0.719	-0.152
Root dry weight	0.270	-0.047
Shoot dry weight	0.869	0.126
Soil pH	-0.149	-0.278
Soil redox 1WAG	-0.103	0.104
Soil redox 3WAG	0.003	-0.025
Total root length	0.824	-0.021
Root diameter	-0.020	0.029
Root-Na	0.230	0.688
Root-Mg	0.088	0.560
Root-P	0.065	-0.102
Root-S	0.040	0.237
Root-K	0.227	0.864
Root-Ca	0.044	-0.467
Root-Cr	0.291	-0.378
Root-Mn	-0.169	0.246
Root-Fe	0.025	-0.248

Root-Co	0.175	0.336
Root-Ni	0.062	0.416
Root-Cu	0.022	0.553
Root-Zn	-0.233	0.343
Root-As	0.103	-0.214
Root-Se	-0.222	0.051
Root-Rb	0.158	0.826
Root-Sr	-0.043	-0.124
Root-Mo	-0.058	0.610
Root-Cd	0.128	0.087
Shoot-Na	-0.504	-0.086
Shoot-Mg	0.210	0.066
Shoot-P	0.019	0.252
Shoot-S	-0.500	0.070
Shoot-K	0.761	0.178
Shoot-Ca	-0.559	-0.192
Shoot-Cr	-0.208	-0.070
Shoot-Mn	-0.092	-0.053
Shoot-Fe	-0.549	-0.020
Shoot-Co	-0.177	-0.001
Shoot-Ni	-0.255	0.086
Shoot-Cu	0.152	-0.053
Shoot-Ni	-0.290	0.076
Shoot-As	-0.516	-0.009
Shoot-Se	-0.834	-0.004
Shoot-Rb	0.225	0.176
Shoot-Sr	-0.487	-0.130

Shoot-Mo	0.069	0.330
Shoot-Cd	-0.646	0.142

[x]Highlighted cells indicate heavy loadings on principal components no. 1 and 2. Yellow cells indicate positive association while blue cells indicate negative association.