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Evaluation of Adaptive Traits in Rice for Low Phosphorus Environments

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Evaluation of Adaptive Traits in Rice for Low Phosphorus Environments

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SUMMARY

More than half of the world's population rely on rice (*Oryza sativa* L.) as the major principal staple cereal. Rice production is increasing in order to meet the current consumption needs of the expanding world population. Scarcity of agricultural land is exacerbated by abiotic stresses such as P (phosphorus) deficient soils, especially in areas where most subsistence farmers are affected by droughts. Strategies to increase stress tolerance for improved growing of rice under stress are needed in order to maximize crop production in upland areas with poor soil nutrients especially in drought prone areas. Adaptive stress tolerance traits in rice for increased root length and nutrient uptake and use are key criteria for genetic analysis.

In the main pot experiment with three different P treatment levels (T) and three upland varieties (Ashoka 200F, Ashoka 228 and Kalinga III) there were significant differences between genotypes for root length and chlorophyll content on day 45 (booting) and for plant height, tiller number and chlorophyll content on day 75 (flowering). While at day 120 (harvesting), there were significant differences between genotypes for plant height, tiller number, chlorophyll contents, plant biomass and P concentration in roots as well as the number of grains, total grain weight and single grain weight.

Meanwhile, the three levels of P nutrient treatments did not significantly influence the plant height and root length at day 45 and day 75 but there were significant differences for P concentration in shoots, P uptake in shoots and total Plant P uptake. Significant differences were found on day 120 with P treatment having significant effects on root length, dry shoot weight and P uptake in root. However, there were no significant interaction (GxT) effects for any of the measured traits at all three harvesting days (d45, d75 and d120). The main experiment does support the hypothesis that Ashoka 200F outperforms Kalinga III in tillering (day 75 and above), root length (day 45) and counted grain, but not for grain weight when grown under low P soil.

A separate experiment carried out using an aerated hydroponic system (AHS) found a significant genotypic effect on root length where Ashoka 200F had longer roots (9.53 ± 0.29 cm) compared to Kalinga III (7.70 ± 0.56 cm), PY 84 (7.13 ± 0.39 cm) and Ashoka 228 (6.88 ± 0.24 cm) when grown under 0 P for 7 days after germination. Under 0 P, genotypes PY 84 (10.13 ± 0.77 cm) and Ashoka 228 (9.95 ± 0.21 cm) grew taller shoots than Ashoka 200F (8.63 ± 0.17 cm) and Kalinga III (8.75 ± 0.25 cm). Gene expression of the inorganic pyrophosphatase (IPP) gene (LOC_Os05g02310) showed no significant difference in roots between genotypes across all treatments, but its expression was down regulated in shoots of Ashoka 228 under half P treatment. Gene expression of the ß-Glucosidase gene (09g31430_41), a candidate gene for a root length QTL in PY84, was upregulated in PY84 under half P treatment compared to

Kalinga III. On the other hand, no significant up regulation of this gene in PY 84 shoots was found compared to Kalinga III across different P treatments. This study depicts the complexity of P uptake, P use efficiency (PUE) and root traits associated with low P environments in rice genotypes. Further research is necessary to better understand the genetic and possible epigenetic variations acting in upland rice genotypes during their responses to low P environments.

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Abbreviations

ΔΔCt	Delta delta Ct
ΔCt	Delta Ct
AHS	Aerated hydroponic system
ANOVA	Analysis of variance
bp	Base pairs
Ca	Calcium
CRD	Complete randomize design
C_T	Cycle threshhold
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
Dro1	Deeper rooting 1gene
GxE	Genotype by environment
$H_2PO_4^-$	Phosphoric acid
IAEA	International Atomic Energy Agency
IRRI	International Rice Research Institute
JIRCAS	Japanese International Research Center for Agriculture
K	Potassium
K_2O	Muriate of Potash
kb	Kilo base
kg/ha	Kilogram per hectare
MARDI	Malaysian Agriculture Research and Development Institute
Mg	Magnesium
MIQE	Minimum information for publication of quantitative real-time PCR experiments
ml	Millilitre(s)
mm	Millimetre
MT	Million tonnes
N	Nitrogen
n	number
$NaH_2PO_4.H_2O$	Sodium phosphate monobasic
NaOCl	Sodium hypochlorite
NH_4NO_3	Ammonium nitrate
NIL	Near isogenic lines

P	Phosphorus
p	probability (significance)
P_2O_5	Triple super phosphate
PAE	Phosphorus absorption (uptake) efficiency
ppm	Parts per million
Pstol1	Phosphorus-starvation tolerance 1 gene
PUE	Phosphorus use efficiency
Pup1	Phosphorus uptake 1 gene
qRT9	QTL for root thickness and root length
qRT-PCR	Quantitative real-time PCR
QTL	Quantitative traits loci
RNA	Ribonucleic acid
RQ	Relative quantity
SE	Standard error
SEM	Standard error of the mean
SPAD	Chlorophyll meter
SSL	Self-sufficiency level
TE	Tris-EDTA
W	Watts

Contents

List of figures

List of Tables

CHAPTER 1

1 Introduction

1.1 Rice and food security

More than half of the world's population consider rice (*Oryza sativa* L.) as one of the major important principal staple cereal foods. It is estimated that as much as 3.5 billion people around the world consume rice as staple food and it contributes 30-80% of the daily nutrient intake of people in China, India and South East Asia (GRiSP, 2013). The demand for rice production for the population living mainly on rice-based diets increased and consumption in 2009 accounted for 78% of total production for rice (GRiSP, 2013). The figures are set to increase as the prediction by FAO suggests that rice consumption demand will increase from 395.4 metric million tonnes in the year 2000 to 533 million metric tonnes by 2030 (Abdullah *et al*., 2008; Alexandratos and Bruinsma, 2012). However, global rice production of rice grains have reduced due to the present challenges of adverse global climate trends and scarcity of agricultural land area. This is exacerbated by biotic, abiotic stresses and other factors (Lobell *et al*., 2011; Weller *et al*., 2016) causing large declines in grain yield by 10% for each 1°C increase associated with global warming (Peng *et al*., 2004).

Rice is commonly found cultivated in the majority of tropical and subtropical regions of the world such as Caribbean and Latin America (5.2%), Africa (2.8%) and others (1%) but is more predominantly grown in the majority of Asian countries (FAOSTAT database). Presently, rice production in China, India and South Eastern regions of Asia accounts for approximately 91% (Khush, 2004; Zeigler and Barclay, 2008; FAOSTAT database) of the total rice production in the world. Globally, rice is cultivated in different ecosystems from the flooded irrigated lowlands (approx. 60%), through rain fed lowland (approx.. 19%) to rain fed upland (approx. 15%) and other ecosystems included flooded lands (GRiSP, 2013).

The world population doubled in size since the 1970s from 3.76 billion to 6.91 billion people, and currently almost 2.4 billion people (34% of the global population) are recorded to be living in Asia. The threat of a food crisis in parts of Asia is severe due to increasing over-population, water scarcity, labour scarcity, global climate shifts, pest/diseases, limited agricultural land areas, industries, increasing incidence of drought, flood, urbanization, reduction in soil nutrient status as well as environmental issues associated with the increasing price of high-input agriculture (Sandhu and Kumar, 2017). In Asia, 40-46% of rice is produced under irrigated rain fed ecosystems (Gamuyao *et al*., 2012; GRiSP, 2013) and nearly 24% of all rice is grown on marginal land that often has poor and low nutrient soils. These problematic

lands are prone to a multitude of conditions of biotic and abiotic stresses. It is not surprising that most poverty is concentrated in areas with these particular problematic soil conditions leading to lack of resources and limited access to input such as nitrogen, phosphorus and potassium (N:P:K) fertilizers by the poor farmers. These factors perpetuate a vicious circle of low crop yields, which in turn make subsistence agriculture difficult in most areas of South Asia especially in the Eastern India and Nepal (Garrity and O'Toole, 1994; Babu *et al*., 2004).

The world's dependency on rice as one of the most important staple food crops has driven the need to increase crop yield which has always been the main objective for rice breeders. Prior to the Green Revolution, traditional rice landraces were selected and maintained by growers over the past centuries. This was mainly achieved with a limited understanding and knowledge of the factors governing the genetic variability that proved to be inefficient and time-consuming if applied today. The work of Norman Borlaug led to the development of high-yielding varieties (HYVs) of cereals, especially semi-dwarf wheat and rice, in association with expansion of irrigation infrastructure, modernization of cultivation methods and agronomic practices including higher use of N, P and K fertilisers and distribution of hybrid seeds to farmers. These advances led to the increase of agricultural yield production worldwide (Farmer, 1986). The increase in cereal production had a significant impact particularly in developing countries throughout Asia in the late 1960s and was later knows as the Green Revolution.

The *sd-1* gene was first identified from a Chinese genotype Dee-geo-woo-gen (DGWG) and crossed with Peta (tall), producing the semi dwarf cultivar IR8 (IRRI, 1996). The s*d-1* interferes with the production of gibberellin (GA) biosynthetic pathway causing for dwarfism trait in rice plants (Monna *et al*., 2002; Sasaki *et al*., 2002; Spielmeyer *et al*., 2002 and Hedden, 2003). The recessive character of shortened culm also resulted in improved lodging resistance and better harvest index. Since the introduction of *sd-1* in the 1960s the gene has been used extensively in modern varieties and has enabled rice production to satisfy the rising demand with the growing populations in many countries.

A "New Green Revolution" could deploy genetically superior cultivars produced via marker assisted selection, in combination with more sustainable practices such as agroecology and the system of rice intensification to allow growers to strive for the best possible combinations of agronomic conditions to increase yield output without continuous increases in fertiliser use. In addition, improved stress tolerance, such that in wheat and maize (Campos *et al*., 2004; and Duvick, 2005) should be developed in rice to achieve an optimum yield.

The current global climate change increases constraints and stresses on crops and today presents more challenges to crop growers than ever before. In order to secure a sustainable

level of food security and to meet the exponential demands of world population and its consumption breeders must recognise the stresses that limit the rice production in its various ecosystems. Progress in breeding for yield under stress will only happen if we improve the understanding of researchers, breeders and rice growers on how to respond to the stresses as the basis for improving existing varieties and landraces.

1.2 Malaysia rice farming industry MARDI perspective

Rice is the main staple food consumed in Malaysia with an estimated cultivated area of 689,700 ha in 2014 (FAOSTAT database). This includes irrigated lowland (approx. 66%), rain fed lowland (approx. 21%), rain fed upland (approx. 12%) and deep-water (1%) ecosystems (Almanac, 1993). The area of cultivated rice declined steeply towards the end of 1970s due to structural changes and the policy implementation of public irrigation for rice cultivation in Malaysia. These changes caused rapid escalation in the costs of labour, land, and construction which pushed the sector towards mechanized farming (Weaving, 1991). However, overall rice production continues to increase compared to cultivated area and yield rose steadily from 1.7 m tonnes to 2.6 m tonnes per annum from the 1980s to 2014 (**Figure 1.1**) (FAOSTAT database).

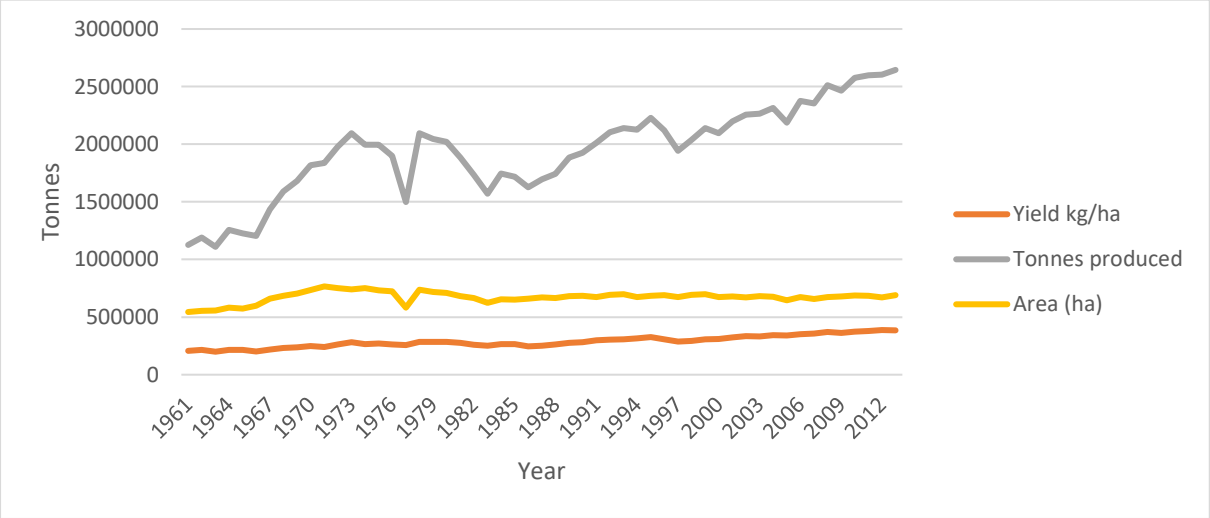


Figure 1.1 Rice yield, production and area cultivated in Malaysia, 1961-2013 (FAOSTAT database).

Malaysia has seen a steady increase of rice production since the 1990s yet the country has only achieved 60-70% self-sufficiency level (SSL) in rice production. To meet the deficit, Malaysia relies on importation of another 30-40% from neighbouring countries like Thailand, Indonesia and India to meet the demand of consumption 74 kg/capita/year (GRiSP, 2013). In

addition, the world rice crisis in 2008 affected Malaysia in terms of higher food import bills and increased the consumer price index (CPI). The Malaysian government implemented trade barriers on locally grown rice in a bid to protect domestic producers as well as trying to meet the national rice requirement (Arandez-Tanchuling, 2011). The issue of self-sufficiency in rice became more challenging due to climate change, limited production, increased input prices and labour costs and scarcity of arable land and water (MOA, 2011; Fatimah *et al*., 2011). Biotic (pests, bacteria, viruses and fungi) and abiotic stresses (low nutrient soils, high or low temperature, salinity, and drought) have also contributed to the shortfall in meeting the country's rice production and SSL target of 80% by 2017.

Since the 1970s the Malaysian Agriculture Research and Development Institute (MARDI) has thus far developed 37 new rice varieties for local industry that are better yielding, tolerant to pest and diseases. In addition MARDI provides comprehensive help to growers through consultation and field visits. These interventions have made a significant impact on the Malaysian rice industry and increased the use of MARDI developed rice varieties. Among MARDI's high impact research is the collection and development of rice germplasm in collaboration with International Rice Research Institute (IRRI), JIRCAS and IAEA. The new rice varieties with earlier maturity and more resistance/tolerance to pests and diseases include MR 253, MR 263 and MR 269 which are replacing existing varieties MR 219 and MR 220 that are more prone to rice blast disease. Rice varieties that are widely used by the majority farmers are Setanjung, Sekembang, MR 84, MR 211, MR 219 and MR 220. The latest varieties that have been released are MR 220CL1 and MR 220CL2, two pesticide (Imidazolinone) resistant varieties that are better suited for combating weeds and MR 253 that was developed to answer the farmers' problem of growing rice on peat, acidic and brackish soils in areas affected following the Tsunami in 2004.

During the past two decades, rice consumption has been on the decline as the Malaysian rising income per capita has led to dietary diversification. Malaysian people have been consuming more meat and vegetables and less cereals (GRiSP, 2013) although the demand for rice still increases with population size. The increase of wealth also means that consumers are able to afford more expensive types of rice such as aromatic rices which have risen in popularity. The Jasmine type (USD 1149/metric ton) now makes up 20% of the overall national import and Basmati type (USD 1310/metric ton) makes up around 10-15% (FAO Price update 2013). To reduce imports and for balance of trade MARDI has developed MRQ 74, a Basmati based aromatic rice, and MRQ 76 a Thai based aromatic genotype. Rice production will also need to be increased from the current 6 tonnes/ha to 7 tonnes/ha in order to meet the local consumption demand whilst reducing the import from neighbouring countries and achieving

the target of at least 80%-85% SSL by the year 2020. MARDI rice breeding strategies change with demand and the current objectives are:

i. Shorter maturity days, non-photosensitive to be planted twice a year
ii. Shorter height and erect stature
iii. Higher yielding
iv. Resistance to pest and diseases
v. Quality grain
vi. Suitable to be planted on problematic soils and environment specific

At present, MARDI has managed to address most objectives mentioned although objective (vi) needs more attention (Malaysian National Rice Conference Proceeding, 2013). Future objectives should also address improved yield under environmental stress (drought/water stress) and increasing productivity with lower levels of fertilizers (including phosphorus) which will become more crucial with climate change. Therefore, identifying genes for root growth and phosphate use efficiency in selected upland rice varieties will help MARDI to identify suitable breeding lines and develop strategies that can be applied to Malaysian rice varieties to boost yield productivity under drought and low phosphate (P) conditions, as well as key grain quality traits that are desired by end users.

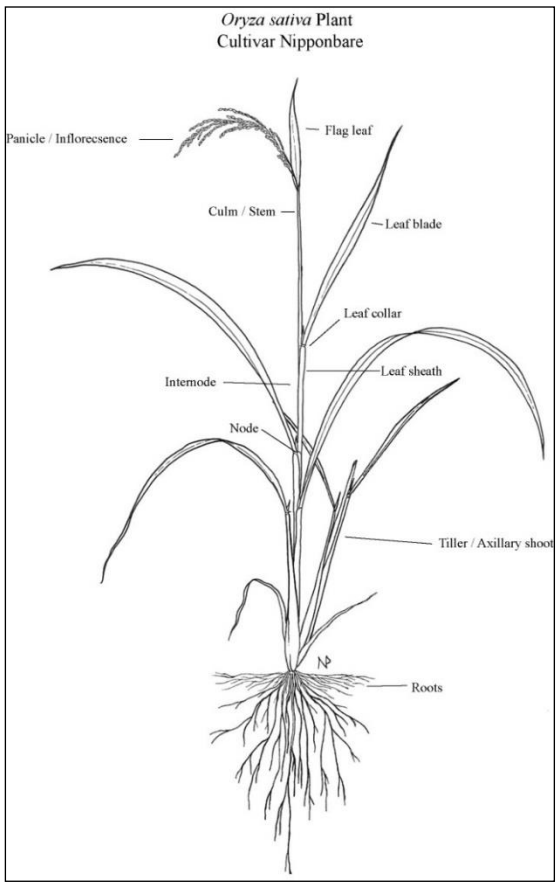
1.3 Literature

1.3.1 The rice plant

Rice is the world's largest cereal food crop, having been cultivated by early farmers originally from China. It is estimated to be more than 8,000 years and has a complex history of domestication (Sweeney and McCough, 2007). Taxonomically rice is a cereal crop of perennial grass in the Poaceae family (**Figure 1.2**), similar to wheat, oats and barley, which can be characterized as possessing erect column, long flat leaves and a fibrous root system (McDonald, 1979). Rice is placed under the genus *Oryza*, consisting of two distinctive cultivated sub-species (*japonica* and *indica*) and 21 other wild relatives (Vaughn *et al*., 2003). These two main sub-species are *Oryza sativa* and according to Brar and Khush (2002) the species *Oryza sativa* is the most widely cultivated species in the rice producing countries (in America, Asia and Europe) after wheat and maize. While the lesser popular rice species to the main stream rice industry, *Oryza glaberrima* is more popularly grown in regions of west tropical Africa.

1.3.1.1 General morphology

Biologically, rice is a monocot cereal crop that reaches its maturity ranging from 90 to 150 days starting from seeding to harvesting stage. These plants typically grow in a tuft (clump) of upright clumps (stems) in which a fully mature rice plant can reach a height of 0.4 to more than 5 meters tall with long flat leaf blades of 50-100 cm in length and 2-2.5 cm broad. These dimensions vary by genotype and environmental conditions. The branched panicle portion from each tiller of the crop arches to produce pendulous inflorescence (florets) of 30-50 cm in length containing many oblong spikelets; which after successful pollination develop into kernels filled with small edible grains (rice seeds) measured 5-12 mm in length and 2-3mm in thickness. The harvested kernel, which also known as rice, is enveloped by a hull or husk which is removed during milling. A single grain weighs on average around 10-45 milligrams at 0% moisture content. The grain length, width and thickness vary widely upon types of rice varieties (Ricepedia.org; GRiSP, 2013).



Kingdom: Plantae
Division: Angiosperms
Class: Monocots
Order: Poales
Family: Poaceae
Genus: *Oryza*
Species: *O. Sativa*
Binomial name: *Oryza sativa* L.

Figure 1.2. Illustration of *Oryza sativa* cv Nipponbare, drawing by Nicholas Polato (Source: http://archive.gramene.org/species/oryza/rice_illustrations.html)

1.3.1.2 Rice planting seasons in Asia

In the South Asian and Southeast Asian countries, rice is usually grown in annual two or three crop cycles with rice grown in the main season and one minor season (**Table 1.1)**. 60% of South Asian countries and 65% of East Asia grow rice by relying heavily on the rainy monsoon seasons as the main water source for irrigation management (FAOSTAT database)**.** This varies regionally for each country as great number of poor families in Asia still face poverty, food insecurity and malnutrition especially rice growing areas that are prone to drought.

Table 1.1 Main planting seasons for rice growers in South Asian and South East Asian countries. (Source: GRiSP, 2013).

South Asia	Main Planting Season	Harvesting
India	March - May	June - October
Bangladesh	April – May	July - August
Pakistan	May – July	October - November
Nepal	May – August	October - December
Sri Lanka	October – November	February - September
Southeast Asia		
Vietnam	May – August	September - December
Thailand	North: May/Dec – Jul/Jan South: Sep/Apr –Nov/May	North: Nov/May – Dec/Jun South: Mar/Aug – May/Sep
Cambodia	June - Jul	November - January
Phillipines	North: May – July South: October – December	October- December (North) March – May (South)
Myanmar	June – August	November - January
Malaysia: **-Peninsular** **-Sabah** **-Sarawak**	 September – October June – September January - May	 November – March December – April August - September
Indonesia	October – March	February - June

1.3.1.4 Rice growth

Morphologically, rice is a monocot cereal crop that reaches its maturity ranging from 90 to 120 days by going through a series of biological phases. These are divided into two stages. The first is the vegetative stage (germination, seedling and tillering) that can be characterized by active tillering, increase in plant height and leaf emergence. The second stage, known as reproductive stage (panicle initiation and heading), is characterized by culm elongation, decline in tiller number, emergence of flag leaf, booting, heading and flowering of spikelet. The period after the heading are known as the ripening period and can be subjected to harvesting depending on the rice varieties. Ripening can range from 30 days to 65 days and this length can be affected by environmental temperature. An example of the rice life growth can be simplified by the schematic diagram (**Figure 1.3**) in which a rice genotype goes through its vegetative stage (44-87 days), reproductive stage (19-25 days) and maturation period stage (30-45 days) (GRiSP, 2013).

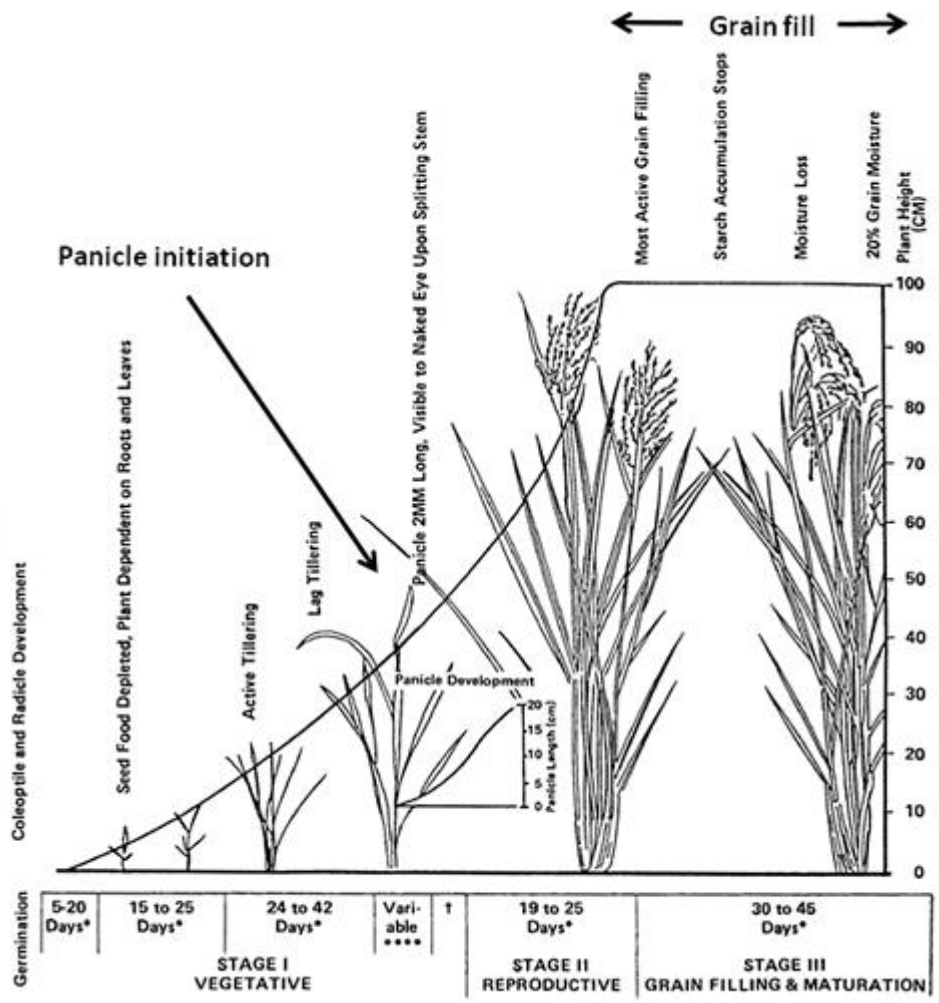


Figure 1.3 Schematic development stages of rice life growth phase. (Source:http://www.haifa-group.com/dutch/knowledge_center/crop_*guides*/rice/growing_rice/)

1.3.2 Rice Species

Genome wide studies on rice variation for phenotypic traits demonstrated that genetically distinct gene pools arose within a common wild ancestor of *Oryza rufipogon.* Two varietal groups are distinguished from the domesticated *Oryza sativa* L. species, namely the *indica* and *japonica*. These two main varieties of domesticated rice differs in various morpho-physiological traits (**Figure 1.4**).

Major sub species:

i) *Indica* rice

Indica is one of the major types of rice sub-species grown in the tropics and subtropics region of the world. In terms of eco-geographical terms, *indicas* are primarily found cultivated as lowland rice that are mostly grown throughout Asian countries like India, the Philippines, Pakistan, Indonesia, Malaysia, central and southern China and some parts of the African countries. *Indica* characteristics are distinguishable compared to *japonica* as the grains are typically four to five times longer than wide, slender and somewhat flat. While the spikelets are more likely to be awnless and the grains are more easily prone to shatter, the amylose contents is 23-31% and they are often low yielding. Two main well known *indica* types are Jasmine and Basmati.

ii) *Japonica* rice

It is one of a group of rice sub-species that is extensively cultivated in the northern and eastern China, Japan and Korea as well as in some areas of the world. *Japonica* genotype typically grows favourably more in the upland areas with cooler agro-climatic zones of the subtropics and in the temperate regions. The characteristics of *japonica* grains are short and roundish, while the spikelets are awnless to long-awned. Generally, the grains do not shatter easily, have a rather low amylose content of 0-20% and they are high yielding. Often *Japonica* is used in sushi due to its moist and glutinous (sticky) character. Two main *japonica* varieties are Akita Komachi and Koshihikari.

Minor sub species:

Molecular approaches through the usage of restriction fragment length polymorphism (RFLP), isozymes, simple sequence repeat (SSR) markers, and single nucleotide polymorphysms (SNPs) have provided a more precise resolution of the rice population structure. Through this, it has helped to hasten the rice research community to produce the first

rice genetic map (McCouch *et al*., 1988) and various DNA markers have been widely applied to explore the genetic diversity and its architecture. Molecular studies were used to identify subpopulations within *O. sativa* as stated by Garris *et al*. (2005) whereby 169 SSR markers were used on a set of 234 diverse accessions of *O. sativa* genotypes. Through this, five subpopulations have been clearly identified as *indica*, *aus*, *tropical japonica*, *temperate japonica* and *aromatic*. Caicedo *et al*. (2007) also successfully identified the same group via SNP markers derived from 111 randomly sequenced regions of the domesticated rice from a subset of 72 accessions. Both of the studies concur with the original study made by Glaszmann (1987) on the classification of the Asian rice varieties using isozymes markers on a set of 1700 diverse *O. sativa* genotypes. From these subpopulations, *indica* and *aus* can be varietally grouped under *indica* whereas *temperate japonica*, *tropical japonica* and *aromatic* falls more closely under the *japonica* varietal group.

Figure 1.4 Difference between *indica* and *japonica* rice grain. (Source: http://www.agromagazine.it/wp/etichettatura-e-clausola-salvaguardia-tavolo-verde-per-difendere-il-riso/)

1.3.3 Rice genotype classification

Generally it is accepted worldwide that rice grown by rice farmers can be commonly grouped into three classes based on the length and shape of the grain (**Figure 1.5**). These are:

i) Long grain

These group of rice varieties are usually characterized by their long slender grain that is about four or five times in length than it is width. The length measurements for a single grain typically vary between about 7 to 9 millimetres. The endosperm is hard, vitreous texture and relatively less sticky, as some long-grain rice contains less amylopectin than short-grain cultivars. Such example of these long grain types grown today were developed from rice type

Oryza sativa var. *indica*, which produced the famous Indian Basmati and Jasmine rice and they have the highest selling price on the market due to their characteristics and quality.

ii) Medium grain

The second group of rice is intermediate in its shape, size as well as texture. These rice varieties generally have a shorter, wider kernel which is around two to three times longer than long grain rice with average measurement of about 5 to 6 millimetres in length with an endosperm that is soft and chalky. The amylose contents is 12-19% in these medium grain varieties, cooked grains are more moist and tender, and have a greater tendency to cling together than long grain.

iii) Short grain

The third group of rice or usually referred to as pearl rice are characterized by the short, plump, almost round kernel of 4 to 5 millimetres with less than twice its length width ratio and contain a starchy substance called amylopectin, which causes stickiness (long-grain rice has much less of this starch). These types of rice sell for the lowest price in the market but are widely used in dishes where a creamy or sticky texture is wanted, such as risottos, puddings and sushi.

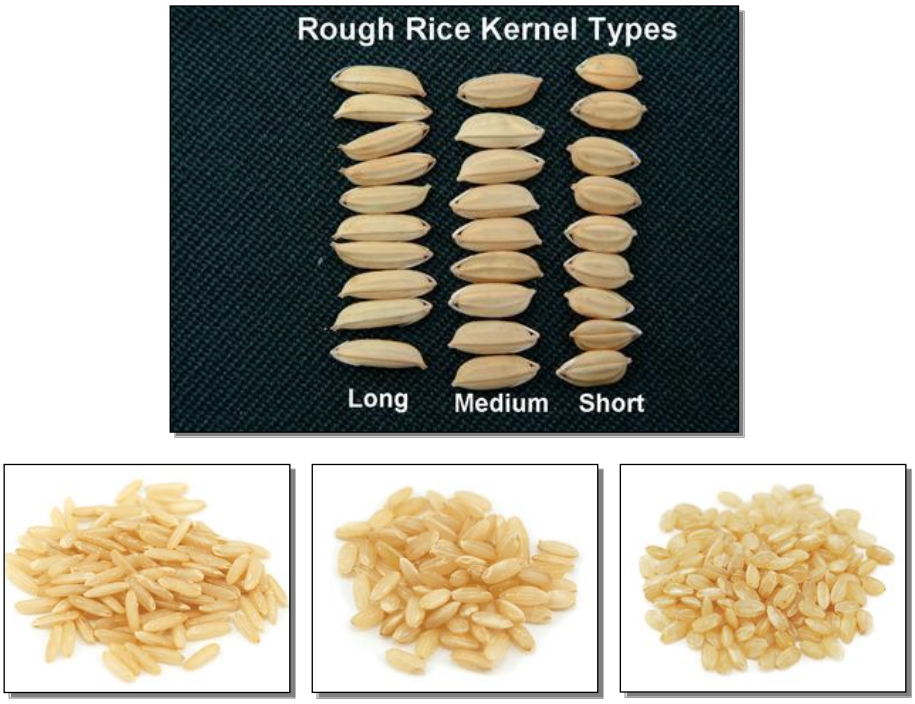


Figure 1.5. Three different types of rice classes according to their length and shape of grain. Unhusked rice grains (above) and kernels of brown rice from which husks have been shelled of long, medium and short grain (below). (Image credit: http://wholegrainscouncil.org/whole-grains-101/types-of-rice and Lundberg Family Farms)

1.3.4 Rice ecosystems and their hydrological status

Rice is considered to be exceptionally different from other domesticated cereal crops in that it is a tropical C_3 grass that has evolved and thrived growing in various range of ecosystems. It can tolerate submerged in waterlogged soil conditions for a lengthy period of time that would kill other crops, is moderately tolerant towards different salinity and soil acidity but highly susceptible to temperature related factors such as drought and cold (Lafitte *et al*., 2004). Rice cultivation and production systems that have been practiced by many farmers throughout the world differ greatly in terms of their cropping intensity and yield but the ecosystems in which the rice is cultivated play an important factor in determining the rice field management. Rice cultivated around the world are generally categorized based on hydrological conditions and ecosystems: irrigated, rainfed lowland, deep water and upland (Poehlman and Sleper, 1995; Halwart and Gupta, 2004) as illustrated in **Figure 1.6**.

i) Irrigated

Worldwide, irrigated ecosystem is by far the most common ecosystem found in rice grown countries with 93 million ha dedicated to irrigated lowland rice production (GRiSP, 2013). It is estimated that 56% of the global rice production is in Asia found in this ecosystem which have a favourable water balance, fertile soil and low risk towards drought or flooding due to controlled water systems. In addition, it is by far the most productive system by producing 75% of the total global rice production as the rice are generally fed with enough inputs such as fertilizers than any other ecosystems (Khush, 1997).

ii) Rainfed lowland

Rainfed lowland rice (RLR) is considered the second most important rice ecosystem as the rice grown in this area contributes 19% (about 52 million ha) of the total global rice production area as reported by IRRI previously. The crops grown are not irrigated but instead rely entirely on the rainfall from monsoon season and water flowing from a higher topography area collected in water catchment areas. The water sources obtained are used to flood the crop to a maximum sustained depth of less than 50 cm for at least a portion of the crop cycle (Banta and Mendoza, 1984). Although 75% of rainfed lowland rice grown in the Southeast Asian countries such as Cambodia, Philippines, Malaysia and Indonesia received an ample amount of rain throughout the year. More than 50% of the rainfed lowland rice areas are grown on soils with potentially major fertility constraints and severe P fixation problems mostly from the

South Asian countries such as Eastern India, Nepal, Bangladesh , Burma and Thailand and these areas are drought-prone or highly drought prone (Huke, 1982).

iii) Deep water

Deep water rice ecosystem are defined as a growing area that is naturally flooded in which the rice crops are immersed in water depths greater than 50 cm for an extended length of time due to the inundation period (two to six months) of the rainy Asian monsoon season that occurs during April to November as explained by Sakamoto *et al*., (2009). Initially, rice seeds grown in this ecosystem suffer from drought at its early stages of growth for a few weeks before flooding occurs and water levels remains high at a later stage of the plant growth which can last for several months until the end of the growing season. This type of ecosystems represents only a small fraction of about 8-9% from the global rice cultivation area as described by Khush (1997) but is of importance to many rice producing countries in South and Southeast Asia (India, Bangladesh, Myanmar, Vietnam and Thailand) that generally situated adjacent to rivers and deltas and near the coastal areas (Catling, 1993). A flood prone area is particularly complex environment due to various soil types and regions, water submergence depth and timing of flooding.

iv) Rainfed upland

Finally, the rainfed upland rice ecosystem is defined as an area where rice is grown in an uneven land surface due to its geographical and topographical location that leads to soil drainage and impossible surface water accumulation (Khush, 1997). It accounts of about 4% of the total global rice production area and ranked as the lowest yielding ecosystem as most upland rice growers are subsistence poor farmers and cannot afford to purchase inputs; therefore only little or no fertilizers are applied throughout the growing season. In addition, most rice varieties grown are of traditional landrace which yielded poorly and prone to lodging but are well adapted to the non flooded ecosystem.

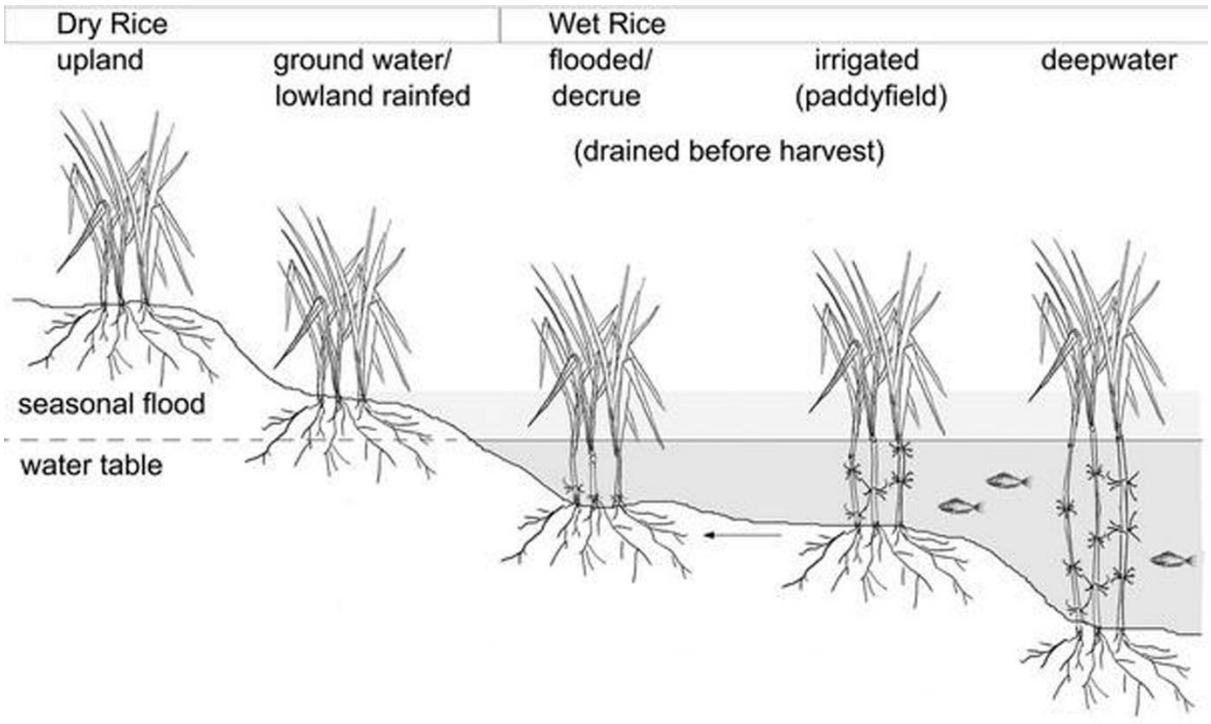


Figure 1.6 A diagrammatic representation of rice ecosystems characteristics in Asian region. (Image credit: https://sites.utexas.edu/mecc/2013/11/17/climate-smart-agriculture-more-rice-less-methane/)

1.3.5 Abiotic stress

Although biotic stresses (brown plant hopper, blast, tungro, gall midge and bacterial blight) affects the rice production in South Asia, Southeast Asia and Africa. Abiotic stresses adversely influence the survivability, biomass accumulation and crop yield in rice. The negative environmental stress presents a major challenge in the quest of producing sustainable food production as it reduces yield potential as high as 70% in major crop species and not just limited to rice such as corn, soyabean and maize (Al-Kaisi *et al*., 2013) which hold extreme importance in food production industries.

In the early stages of rice crop development, its vegetative growth are easily subjected to a wide range of environmental stresses that could limit the outcome of the crop productivity. Pertubances coming from abiotic factors such as water availability (drought and flood), soil problems (salinity, soil pH, nutrient deficiencies and toxicities), extreme temperature fluctuations (hot and cold) (Asraf and Fooland, 2007; Cao *et al*., 2006; Hasegawa *et al*., 2000 and Witcombe *et al*., 2008) contributes towards large scale crop loss annually. About 20% of the total global rice production that resides in the Asian regions with an estimated 23 million hectares of growing areas are readily prone to extreme drought stress (Pandey *et al*., 2007).

Furthermore, low temperature and photoperiod also contributed as one of the major factors impeding the cereal plant growth. Vernalisation and low temperature causes damage at anthesis while the length of photoperiod experienced by cereal crops will hasten or delay its booting and flowering period.

A single abiotic stress will decreases a plant's ability to withstand the second stress and usually crops are exposed to multiple combinations of abiotic stresses. For example, a high temperature condition will result in the exacerbation of subsoil mineral contents that leads to constraints on the root growth (Tester and Bacic, 2005). In terms of abiotic stress, the irrigated and deep water rice ecosystem rarely suffer from water shortage as it is located geographically nearer to water source, regularly receive abundant rainfall during the monsoon season and water management is structured (Bouman *et al*., 2006). Whereas in both the rainfed lowland and upland cultivation systems, limited water source and no accessibility to proper irrigation infrastructure leads to drought stress which are the most prevalent abiotic stress factors that limits the crop yield in most areas of South Asia especially Eastern India and Nepal (Garrity and O'Toole, 1994 and Babu *et al*., 2004). Upland rice is most prone to drought stress when compared to lowland as water does not accumulate in the fields due to the irregular, sloping topography and usually drains from the highest to the lowest elevation. These risks coupled with severe poverty, limited water source and no accessibility to proper irrigation infrastructure and old rice varieties, limits the scope for yield intensification in most areas of South Asia especially Eastern India and Nepal (Garrity and O'Toole, 1994 and Babu *et al*., 2004).

1.3.6 Drought stress on rice production regions

Among all the mentioned abiotic stresses, drought has been the main catalyst for major constraints to rice production in rain fed as well as upland rice ecosystems areas across rice producing countries in Asia and sub-Saharan Africa (Bimpong *et al*., 2011). Price *et al*., (2002) and Bernier *et al*. (2008) defined drought as 'a period of no rainfall or irrigation that affect crop production' for a period of time causing crop competition for water source that affects crop growth and yield production due to the interaction between precipitation, evapo-transpiration, irradiation, soil physical properties, soil nutrient availability. Areas affected by medium to large-scale natural disasters, drought alone accounted for 44% in crop and livestock losses in Asia and Africa (Sandhu and Kumar, 2017) as frequent occurrence of drought events in these regions and areas shown in **Table 1.2**.

Pandey *et al*., (2007) estimated that in the regions of rice production countries of Asia, a combined total of 23Mha of rice fields (10Mha in upland and 13Mha in lowland) are prone to drought. The range and severity of drought condition can be observed through SPEI Global

Drought Monitor in parts of leading rice producing countries such as China, India, Indonesia, Bangladesh, Vietnam and Thailand (**Figure 1.7**).

Most of the rice producing areas like Jharkhand, Orissa and Chhattisgarh in the eastern states of India suffers a 40 percent loss in total rice production estimated to be valued at $650 million (Pandey *et al*., 2007). The impacts of drought prone environments towards rice production affect the majority of poor farmers' economy in these areas causing them to reduce food consumption, withdrawing children from education, selling off their assets, and in some extreme cases migrate and change the farming professions for other type of employment in order to meet their immediate needs (Bernier *et al*., 2008). Farmers in these areas have not benefitted from high yielding modern/Green Revolution varieties, which need irrigation and high levels of fertilisers. Stress resistance is complex and abiotic stress requires either phenotypic plasticity so plant can respond phenotypically to stress and adapt it is encountered, or escape mechanisms due to more 'fixed' phenotype such as early flowering or growing long roots at an early stage before drought occurs. Although this second strategy does not allow plants to reach full yield potential in high input environments. The best advances have been made with selection carried out in the target environment proves to be the key and alternative options in improving rice production, food security and economic stability for 3 billion people in Asia.

Table 1.2 Most vulnerable drought-prone areas in Asia and Africa.

Region	Areas most vulnerable to drought	Drought events
Asia/Pacific	India, Nepal, Bangladesh, China, Laos, Cambodia, Pakistan, Afghanistan, Sri Lanka, Bhutan, Indonesia, Thailand, Myanmar, Vietnam, Malaysia	1876, 1878, 1896, 1902, 1907, 1928,1930, 1936, 1941, 1942, 1944, 1958,1961, 1964, 1972, 1973, 1974, 1983,1987, 1993, 1996, 2000, 2002, 2010
Africa	Ethiopia, Kenya, Eritrea, Somalia, Uganda, Djibouti, Mauritania, Angola, Zambia, Zimbabwe, Mozambique, Malawi, Lesotho, Swaziland	1888, 1972, 1973, 1983, 1985, 1991,1992, 1999, 2002, 2002, 2003, 2010,2011, 2012

Source: Modified from Spring 2015 global attributes survey. (Sandhu and Kumar, 2017)

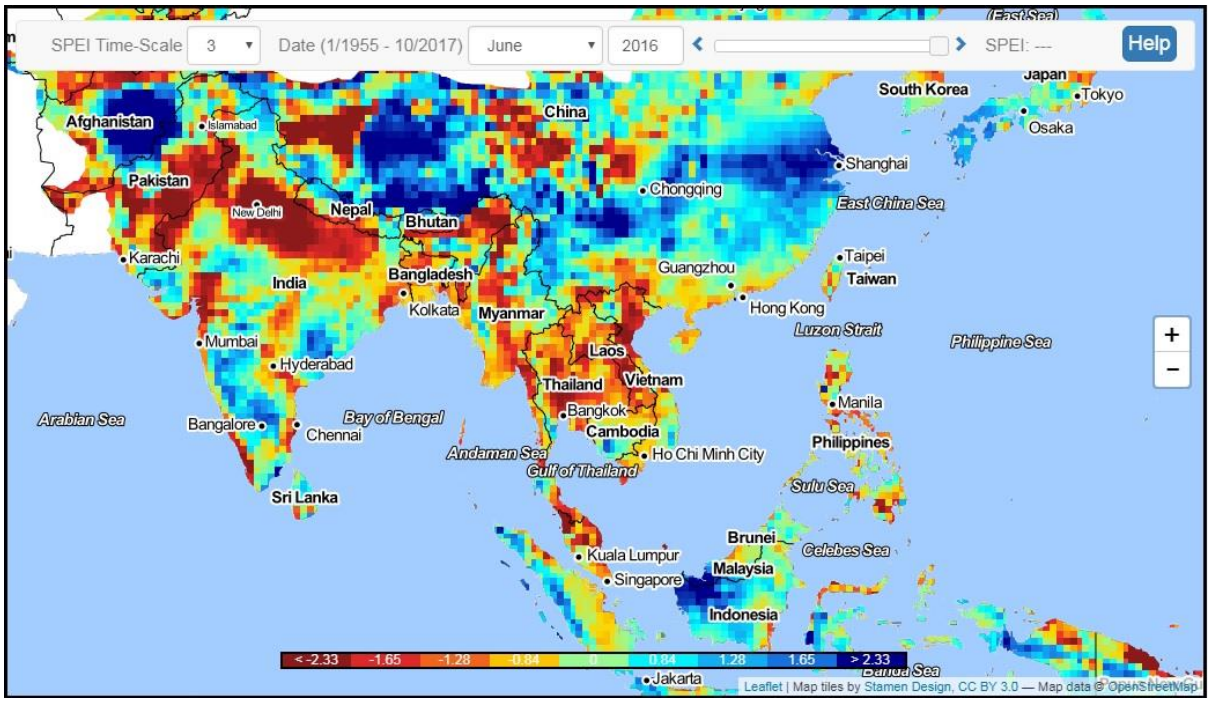


Figure 1.7 Drought conditions in Asia (SPEI Global Drought Monitor, 2016)
(Source: http://spei.csic.es/map/maps.html)

1.3.7 Phosphorus decline in crops

Global climatic change (i.e., rainfall and temperature) has greatly influenced crop production around the world and could potentially compromise food security globally and locally. In addition, acid soils occupying approximately 30% (3950 m ha) of the world's land area. Which from that, 67% of the acid soils supports woodlands and forests, 18% covered by praire, savanna and steppe vegetation with only 4.5% (179m ha) are available for arable crops (von Uexkull and Mutert, 1995). Potentially arable land worldwide lacks optimum levels of nutrients or is too acidic that inhibits plants growth correlated to an extreme reduction in crop yields. These low fertility acid soils can be attributed to myriads of combinations such as aluminium toxicity, iron toxicity, calcium deficiency, potassium deficiency including phosphorus deficiency.

Worldwide mineral P fertiliser (P_2O_5) consumption has been steadily increasing the total P content in agricultural soils from 34.5 m tonnes to 46.7 m tonnes since 2002-2014. As of 2014, Indonesia (0.77 MT), Bangladesh (0.56 MT), Malaysia (0.46 MT), Thailand (0.45 MT), Philippines (0.17 MT) and Nepal (0.04 MT) saw and increase in P fertilizer use. Comparatively, China (1.56 MT) and India (0.64 MT) (FAOSTAT database) are still by far the largest consumer of P fertilizer in the world used for various agriculture purposes including rice crop fertilization in order to compensate the lack of low P soil in parts of marginal land. Comparing to the UK where around 70% of the total UK area are utilised for arable and

horticultural crops, uncropped arable land, common rough grazing, temporary and permanent grassland and land used for outdoor pigs (**figure 1.8**). However, the estimated soil nutrient balances of phosphorus have declined by 47% since 2000 to 2015 indicating a reduction in the surpluses of nutrients that were potentially lost to the environment (Defra 2016). This are supported by data that the overall mineral phosphate application rates have been in decline between the year 1990 and 2016. The decline have levelled off in recent years with phosphate application currently standing at 18kg/ha on all crops and grass resulting in an average soil P index of ~ 2 (Defra 2016).

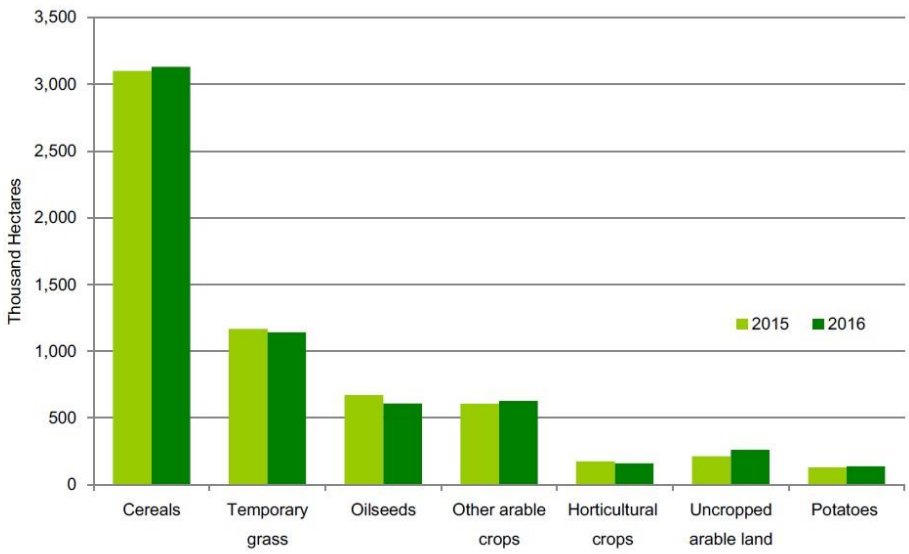


Figure 1.8 Total croppable land areas on agricultural holdings in the UK (Defra 2016)

In terms of how a soil is determined for plant-available P, extraction methods are employed to measure the plant-soil P availability via the Olsen extraction (Syers, Johnston & Curtin 2008). This are then indexed based on the value of P (mg l^{-1}) and classified into ranges representing P indices (**Table 1.3**). Olsen-P values of 10-15 mg kg^{-1} are considered in the band of low P fertility classed as P index 1. While soil with values greater than 46 mg kg^{-1} are classed as high P fertility or index 3+. England and Wales uses commercial laboratories for the soil P index system which reports results on a volumetric units (mg.L^{-1}) rather than the weight unit basis (mg.kg^{-1}), but the two reporting methods gives similar results for mineral soil investigation (Withers *et al*., 2017).

Table 1.3 Classification of Olsen's soil P (mg l^{-1}) into indices (Defra 2010)

Index	mgL^{-1}	Description
0	0-9	Very low
1	10-15	Low
2	16-25	Moderate
3	26-45	High
4	46-70	Very high
5	71-100	Very high
6	101-140	Very high
7	141-200	Very high
8	201-280	Very high
9	>280	Very high

1.3.8 Phosphorus deficiency

During rice crop vegetative growth, poor soil fertility due to the deficiency in macro nutrients such as potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P) will have a great impact in determining crop yield. These negative conditions are frequently observed on infertile soil especially in the tropics and subtropics regions where soils are highly weathered and leached causing soils to be more acidic leading to crop infertility. In terms of function in rice plants, phosphorus (P) is a vital nutrient component along with nitrogen (N) and potassium (K) needed in adequate supply particularly in early stages of growth, root development, tillering, early flowering and ripening that results in achieving maximum crop yield and grain quality in plants (Bennet,1993; Better Crops International, 1999; Hopkins *et al*., 2008).

The use of phosphorus is unsustainable in agriculture production system and natural phosphorus reserves are limited as mineral nutrients (Van Kawenbergh, 2010) due to plants acquire P from the rhizosphere solution as phosphate (Pi) in the form of $H_2PO_4^-$ (Hammond *et al*., 2004; White and Hammond, 2008) and often Pi in the soil are low (2-10µM) (Raghothma, 1999). Plants grown in low P soil can lead to yield loss as many as 5% to 15% of the maximum yield potential and are more critical especially in highly withered soils, calcareous and alkaline soil (Hinsinger, 2001). Severely P deficient plants will exhibit thin and spindly look with decreased overall growth development and delayed leaf emergence, reduced tiller number, delayed secondary root development and late maturity. In addition, symptoms also appear as a decrease in leaf numbers, leaf blade length, reduced in panicle per plant, reduced in seeds per panicle and reduced filled seeds per panicle. All these symptoms will reduce tillering capacity of a rice plant to potentially produce a higher yield when grown in a P impoverished soil.

Hence, affecting the biomass, dry matter between the roots and shoots. As a result, this will penalize the grain yield as well as seed production capabilities (Glass *et al.*, 1980; Elliot *et al* 1997 and Grant *et al*., 2001). Other symptoms such as the leaves being short, narrow, very erect with a blackish 'dirty' dark green to purplish in colour (Hoppo *et al*.,1999) while the number of leaves, panicles and grains per panicles are reduced (Better Crops International, 2002).

P deficiency were also seen to be significantly reducing the net photosynthesis rate in rice plants. Studies performed by Xu *et al*., (2007) showed that prolonged P deficiency more than 16 days, decreases the maximum efficiency of photosynthetic components such as PSII photochemistry, PSII quantum yield, the electron transport rate and photochemical quenching rate compared to the control plants. Therefore, it can also be said that the less amount of chlorophyll contents in rice leaf plants during vegetative growth could lead to the decrease in photosynthetic efficiency per unit of chlorophyll produced per leaf area (Marschner, 2011). Lopez- Canteraro *et al*., 1994 reported that total chlorophyll concentrations were correlated to the level of P fertilization in aubergine plants (*Solanum melongena* cv. Bonica) and influence the growth and root morphology of *Acer mono* seedlings (Razaq *et al*., 2017). Measuring chlorophyll content in rice can be time-consuming, destructive and the use of flammable chemicals and solvents methods of determining chlorophyll photochemically (Watanabe *et al*, 1980; Peng at al., 1999 and Gu *et al*., 2017). For this purpose, a simple and quick alternative method using the Soil Plant Analytical Division (SPAD) values of a handheld SPAD-502 chlorophyll meter that produces readings typically between 0.0 to 50.0 is preferred in determining the index of relative chlorophyll contents whereby the instrument calculates the amount of chlorophyll present per unit leaf area. SPAD meter were also used in other studies to measure the chlorophyll contents such as in turf grasses, *Arabidopsis thaliana* and leafy vegetables (Rodriguez and Miller, 2000 and Limantara *et al*., 2015).

Attempts of adding phosphorus-based fertilizers are becoming non-economical and can be ecologically unsafe especially in the areas where most are subsistence farmers and affected by droughts. Altieri and Nicholls (2017) reported that scientist and crop producers are mitigating these changes and minimising crop loss through agroecological strategies by practising a better soil management systems and utilising superior crop genotypes that can tolerate and survives the current drastic environmental climate. There have been concerted efforts over the past decade where rice breeders and industries alike have been developing breeding programmes and field evaluation for quantitative trait loci (QTLs) for traits associated with abiotic stress. Longer roots were found to be influenced by QTL 9 (Steele *et al*., 2006 and Steele *et al*., 2007) and it has been introduced in Kalinga III, an upland rice genotype with improved tolerance towards specific environmental and abiotic stress particularly in the regions

Eastern India which are prone to drought and also suffer from nutrient deficiency including low P.

According to Borlaug and Dowswell (2005), breeders constant selection of better genotypes that produces higher yield potential alongside the increased application of fertilizers particularly nitrogen (N) and phosphorus (P) have effectively increased the rice crop production worldwide over the last century. On the contrary, as the cost of agriculture inputs increased tremendously and became more expensive over the years, developing countries with low-input agriculture systems such as from Eastern India are not able to afford the application. Moreover, P deficiency in either low or high pH soil under drought condition is an added problem that can attribute as part of limiting factors for crop production and can present a more critical situation over the course of long-term effect for subsistence farmers in developing countries. Therefore, it is important for rice crop to be able to utilize the P efficiently in order to ensure the cost-effectiveness as well as sustainability of crop performance growing in extreme environments (Collins *et al*., 2008).

There is a need for a long-term strategies to address this problem and numerous researches have been carried out regarding crop productivity under low P such as barley (Huang, *et al*., 2011), common bean (Ho, *et al*., 2011), Canola (Yang, *et al*., 2011; Shi, *et al*., 2013), maize (Mendes, *et al*., 2014) and wheat (Bolland and Brenan, 2008; Ying, *et al*., 2011). By designing a more efficient breeding program that can synthesize new rice varieties with a higher drought tolerance and high yielding ability that are typically cultivated in regions where P availability is low, it will enhance food security in rice-dependant countries.

1.3.9 Rice genome sequencing

Molecular approaches through the usage of restriction fragment length polymorphism (RFLP), Isozymes, simple sequence repeat (SSR) markers, and single nucleotide polymorphisms (SNPs) have provided a more precise resolution of the rice population structure. Through this, it has helped to hasten the rice research community to produce the first rice genetic map (McCouch *et al*., 1988) and various DNA markers have been widely applied to explore the genetic diversity and its architecture.

There have been a hundred plant genome sequences published, but the map-based sequence of the rice genome, *Oryza sativa* L. ssp. *japonica* cv. Nipponbare was the first important monocot cereal crop that have been sequenced to a high-quality level by the International Rice Genome Sequencing Project (IRGSP) in 2002 (Matsumoto *et al*., 2005). Having the smallest of the major cereal crop genomes at an estimated 400 to 430 Mb (Eckardt, 2018). It has since become the reference sequence for understanding and identifying the

underlying traits, diversity and relationships of the rice genome's and gene mobility among thousands of rice cultivars and its wild relatives.

It has helped as a reference point for sequencing all-major cereal crops with larger genome sizes such as maize (Schnable *et al*., 2009), sorghum (Paterson *et al*., 2009), soybean (Schmutz *et al*., 2010), barley (International Barley Genome Sequencing Consortium 2012) and wheat (International Wheat Genome Sequencing Consortium 2014). To date, *Oryza sativa* L. ssp. *Indica* genome cultivar 93-11 (Yu *et al*., 2002; Zhao *et al*., 2004) and Shuhui498 (R498) (Du *et al*., 2017) as well as Koshihikari, an elite *japonica* rice cultivar which is closely related to Nipponbare (Yamamoto *et al*., 2010) was managed to be sequenced annotated since then. The sequenced rice genome have become a powerful tool in the agricultural industry allowing breeders to develop and enhance new varieties with desirable traits that can target specifically for yield, good eating quality and varieties that can be developed to adapt specific cultivation environments as well as resistance to biotic and abiotic stress due to the global climate change.

1.3.10 QTLs identified for abiotic stress phenes

Drought over the past decades have a significant impact on the agriculture on both developed and developing countries on majority of cash crops such as cereals that majority of the world's population depends on. It is identified to be the primary constrains and will continue to be the problem through the exacerbation of climate change incidence in many regions increasing the severity of reduced food production (Lynch *et al*., 2014; Lobell and Gourdji, 2012). Advances in molecular techniques over the past decades to rapidly screen and analyze quantitative traits loci (QTLs) from drought resistance rice (Courtois *et al*, 2009 and Price, Cairns *et al*., 2002) and the use of molecular assisted markers (MAS) in identifying genomic regions associated with specific traits have become powerful tools for selective breeding on genotype.

As a result, various crop improvement programmes focusing on the development of crop cultivars with drought adaptation and higher yield production have been directed to address the issue. Among the crop traits, roots plays a significant role in water acquisition as the crop's drought tolerance mechanism in a water deficit soil and environment for it fitness and growth. Genotyping crop cultivars possessing improved root traits towards water acquisition and increased yield have been reported by various papers such as variation in anatomical phenes and root architectural phenes (Lynch *et al*, 2014). The word 'phene' are used by Lynch *et al*., (2014) used to describe 'phenotype' as 'gene' to 'genotype'. The prospect of deploying these root traits phenes on rice crops will greatly facilitate the industry as the crop

will have the ability to improve water acquisition and optimizing water/nutrient exploration by reducing the metabolic cost in a deficit environment in return for growth and yield production. According to Lambers *et al*., (2002) plants spends 50% more on soil exploration than daily photosynthesis in terms of metabolic cost. The superiority of plant productivity lies on plant ability to reduce its metabolic expenditure acquiring a limited soil resource, leaving surplus metabolic availability for other acquisitions such as vegetative growth and reproduction.

A common bean (*Phaseolus vulgaris*) demonstrated a 75% increase in phosphorus (P) acquisition of roots in both P-efficient and P-inefficient genotypes under low P conditions (Nielsen *et al*., 1998, 2001). Although the P-efficient genotype portrayed a greater root growth per unit root respiration when compared to its counterparts. The more efficient genotypes are able to gain twice root biomass, suggesting the phenes are associated with its ability to explore the soil in a P stress environments while minimally utilizing its metabolic cost (Lynch and Ho, 2005). Studies by Postma and Lynch (2011) supported the importance of root cost for soil resource acquisition by proving that under a severe N and P deficiency, the growth of 40 day old maize plants are reduced by as much as 40% and 70% respectively. However the study were not performed in a field conditions which are subjected to biotic and biotic stress.

1.3.11 Root traits

Approaches in seeking quantitative trait loci (QTLs) that are stable in target environments that co-segregate with improved yield have been made over the years. Rice traits such as leaf rolling from Azucena/Bala (Lafitte *et al*., 2004) and grain yield with QTLs associated for maturity, panicle number and plant height in Vandana x Way Rarem population (Bernier *et al*., 2008) are considered as drought tolerance in rice. Further research and genetic analysis have been performed over the past 20 years to identify rice QTLs for traits that can be linked to drought resistance (Nguyen *et al*., 1997). Putative candidates that confers for osmotic adjustment (OA) and root physiological and morphological traits have been mapped between an upland (CT9993) and a lowland genotype (IR62266) (Nguyen *et al*., 2004; Yang *et al*, 2004).

Generally, rice plants known to have a shallow rooting system compared to other cereal crops. It is hypothesized that various root traits in rice such as having thicker and longer roots (deep root system) enhances its survivability in drought stress condition by postponing dehydration by penetrating into deeper soil profile to reach water and nutrient source (Yoshida and Hasegawa, 1982). Price *et al*. (1999) also reported that deep root system found in upland rice varieties enhances its water uptake and contributed towards drought resistance ability.

Root traits are governed by various genes through QTLs and it is also one of the important characteristics in maintaining plant stature. In addition, crops with a maximum root distribution are advantageous compared to others in terms of its ability to provide functions essential to plant fitness such as water and nutrient uptake (Lafitte *et al*., 2001). It was widely reported that root length, root density and soil penetrating ability translates into larger surface area are better at absorption of necessary nutrients needed for plant growth and reproduction and in turn enhances grain output (Fukai and Cooper 1995; Nguyen *et al*., 2004; Kano, *et al*., 2011). Such rooting characteristics are desirable traits in many crops as it concurs with drought avoidance ability growing in adverse environments.

Important phenotypic root traits in rice pertaining drought tolerance/resistance have been among the main focus for physiological and QTL mapping studies among researchers in order to breed rice in abiotic stress environment for a sustainable agriculture (Witcombe *et al*., 2008). It is documented that some of the many QTLs for roots are common across different genetic backgrounds (Li *et al*., 2005) such as QTL for root length and thickness on chromosome 9 that is expressed in many range of environments. Previous report indicated that it was among the four targeted root QTLs that had significantly increased the root length when introgressed into a novel genetic back ground (Steele *et al*., 2006).

Champoux *et al*. (1995) are the first to have successfully located genes directly responsible for rice root traits by using molecular markers, and over the years as many as 30 root morphological variables been identified to be related to root traits QTLs (Price *et al*., 2000; Steele *et al*, 2006; Steele *et al*, 2007; Uga *et al*, 2013). By mapping the QTLs for root traits in various rice populations related to rain fed lowland environments, Price *et al* (2000) and Zhang *et al*. (2001) have been able to identify QTLs for root architecture and penetration ability. Similarly, other QTLs associated with various root traits including seminal and lateral root length, root thickness, root penetration, lateral and adventitious root number have also been reported in various rice population located on chromosome 2,5,7,9 and 11 (Zheng *et al.*, 2000; Zheng *et al.*, 2003; Price *et al.*, 2000; Shen *et al*., 2001; Steele *et al*, 2006; Kanagaraj *et al*, 2010) (**Table 1.4**).

Table 1.4 QTLs and genes related to drought tolerance.

Associated QTL	Trait improved	Genes	References
1	Root-shoot growth, deep root growth, osmotic adjustment		Price,*et al*. (2002) Robin *et al*. (2003) Vikram *et al*. (2015)
2	Root length, thickness & penetration		Price *et al*. (1997)
7	Root length & mass; Deep rooting	*Dro1*	Price *et al*. (1997) Uga *et al*. (2013; 2015)
9	Root length, root thickness, straw yield, relative water content	qRT9	Courtois *et al*. (2000) Price *et al*. (1997;2002) Steele *et al*. (2006; 2007) Li *et al*. (2015)
11	Root length & penetration		Price, *et al*. (2002)
12	Biomass, panicle number, lateral root, panicle branching Phosphorus uptake	*Pup1* *Pstol1*	Bernier *et al*. (2009) Dixit *et al*. (2015) Heuer *et al*. (2009)

1.3.12 Breeding rice for drought resistance

Department for International Development (DFID), Plant Science Research Programme (PSP) and Centre for Arid Zone Studies (CAZS) carried out research to mitigate drought through research and development to breed rain fed rice for drought prone environments in South and Southeast Asia (Witcombe *et al*., 2002). Examples like Kalinga III; a local rice genotype was carefully identified as the parent by the farmers through participatory varietal selection (PVS) due to its suitability growing in upland water-limiting environments, earliness to flowering, harvesting days at 90 days, high grain and fodder, resistant to brown spot, tolerant to cold and produces an average yield of 2.5-3.0 t/ha. Kalinga III was originally released in 1983 with areas of adaptability in Bihar, Jharhand, Gujarat and Orissa. It is however had a limited adoption due to its poor rooting characteristics, weak in stems and non-aromatic. The genotype weakness was later improved by using Kalinga III as a parent in the DFID-PSP funded Participatory Plant Breeding (PPB) rice project in eastern India that were also collaboratively developed by the Gramin Vikas Trust Eastern India Rainfed Farming Project (GVT(E)), Birsa Agricultural University (BAU), Ranchi and the Centre for Arid Zone Studies (CAZS) from the University of Wales, Bangor, UK. The second parents were chosen to be crossed with a genotype that have a complementary traits and high yielding but genetically distant in order to get a diversified segregation targeted at a range of rice ecosystem (Witcombe,

2002; Virk, *et al*, 2002; Gyawali *et al*., 2003). As a result from crossing Kalinga III x IR64, progeny Ashoka 228 and one produced by farmers (Ashoka 200F) showed promising results when tested in the farmer's field in the state of Jharkhand, Eastern India (Virk *et al*., 2004).

Improving the rice genetics in drought related traits through conventional breeding is not an easy undertaking as seasonal and spatial variations of droughts severity varies annually, making it too difficult and time consuming to carry out screening of traits contributing towards drought resistance (Courtois *et al*., 2003). In addition, the major difficulty that usually slows the process of developing new drought resistant rice varieties with positive yields are due to the large amount of empirical selection and combination of differences as a result from the genotypic adaptation against the environment (GxE) interactions (Fukai and Cooper, 1995). An in depth knowledge and understanding of the morphological, physiological, biochemical and genetic control mechanism governing the traits of drought related stresses are needed; coupled with molecular approach as a tool to give new insights to breeders in drought tolerance to and in turn complementing the existing conventional breeding programs to hasten crop yield improvement.

1.3.13 Research questions

This research aims to describe and utilise genetic variation among selected upland rice genotypes from previous studies. The thesis aims are to address the root trait related questions in order to answer these hypotheses:

- Is root length associated with phosphate (P) use efficiency (PUE) in rice grown under different P levels?
- Do root length and PUE candidate genes show variation (expression or polymorphism) with traits for adaptation to low P growth medium?

CHAPTER 2

2 Growth performance of upland rice under different phosphorus conditions

2.1 Introduction

Phosphorus (P) is an essential plant nutrient component needed for every phase of plant growth and an integral component for achieving maximum crop yields (Bennet, 1993; Hopkins *et al*., 2008). In the early stages of vegetative growth (up to 45 days), proper P nutrition is required in substantial amounts to promote strong early growth and development of a strong rooting system. It also promotes tillering, early flowering, and ripening. In addition, P content in plant dry weight can range from 0.05 to 0.5% (Vance *et al*., 2003) and this element plays a vital role as part of the structural components of the DNA, RNA and phospholipids as a component for adenosine phosphates (AMP, ADP and ATP) (Schachtman *et al*., 1998). In C3 cereal crops, such as rice (*Oryza sativa*), wheat (*Triticum* spp.), barley (*Hordeum vulgare*), rye (*Secale cereale)* and oat (*Avena sativa*), inorganic phosphate are involved in converting light energy to chemical energy and respiration activities during photosynthesis. Modifying various enzyme activities by phosphorylation and cell signalling in order to synthesize nutrients into sugars and starches (Salisbury and Ross, 1991).

P is a non-renewable fertilizer that cannot be synthesized artificially like N. In soils, P often has relatively poor mobility resulting in small fraction of available P are being absorbed by plant crops (Castro *et al*., 2013; Arredondo *et al*., 2014). Inefficient acquisition and application of P limits potential crop growth and yield, particularly in poor soil deficient areas. Deficiencies in P nutrient area displayed as a stunted growth with yellowing leaves, fewer tillers, and the decrease in root length and mass. Due to scarcity of available phosphorus, demand for P in fertilizers is increasing as more agricultural crops are planted to feed the human population.

It is recognised that increasing the P fertilizer hoping to balance crop P offtake does not effectively increase the P available in soil to be utilized efficiently by a plant (P use efficiency), which is now seen as wasteful (Withers *et al*., 2017). It also presents itself as one of the hurdles in obtaining high grain yielding crops (Veneklaas *et al*., 2012; van de Wiel *et al*., 2016). Plant P utilization only accounts for around 0 to 30% of the P fertilizer applied so that usually results in growers overcompensating in the P fertilizer application (Syers *et al*, 2008). Current agricultural and agronomical practices aim to increase phosphorus use efficiency in crops such as *Solanum tuberosum, Oryza sativa* and *Triticum spp.* (Hopkins *et al*, 2008; Rose *et al*., 2013; Fageria; 2014; Shabnam and Iqbal 2016). Socio-economic factors such as poverty can contribute to less than optimum use of fertilizers. Other hindrances for growers to achieve

optimal crop growth can be physical factors such as poor and marginal soils that are low in nutrients and often acidic. The possible solution to mitigate this problem can be lessen by breeding and introducing P use efficient (PUE) genotypes with longer root traits to better suit the environment it is being grown, particularly in drought prone areas.

Identifying rice genotypes with high productivity under low P environment is the logical approach to further improve rice yield. For breeding successful P-efficient crops, selection and evaluation of optimal genotypes are needed especially when PUE traits are involved and can be scored easily. Two key factors that contribute to the phenotypic traits of crop performance and yield and P uptake which is measured by assessing the ability of a plant (grain + straw) to absorb available P from the soil; and the phosphate use efficiency (PUE) which is the ratio of efficiency at which P is taken up and converted into biomass to the harvestable parts.

Identifying genes that are expressed in plants that to respond positively when grown in prolonged P deficient soils assists in developing crops with improved P uptake and PUE. Hammond *et al*. (2009) conducted experiments on *Brassica oleracea* grown under different P levels and observed that significant QTLs associated with shoot-P and root traits correlated with the measure of PUE they were identified in chromosome 3 and chromosome 7 of *B. oleracea*. While Fageria *et al*., (2013) found that P concentration of corn, soya bean, dry bean and upland rice decreases while the P uptake in plant shoots increased significantly in a quadratic exponential trend as the plant age. It was also noted that P uptake was higher in grains when compared to shoots. Vandamme *et al*., (2016) found an increase of 40 - 60% in P uptake and 15% in PUE for rice with P-efficient genotypic traits grown in target specific environments. The culmination of all the research will allow crop yield improvement via QTL and MAS in developing high P uptake and PUE crops growing in P deficit soils and with little to no P fertilizer application.

At present the *phosphorus uptake 1* (*Pup1*) gene which was initially identified from a small small diversity study of 30 rice genotypes, found from two rice land races, Dular and Kasalath had the highest inorganic phosphate content (Wissuwa and Ae, 2001). The *Pup1* gene were also revealed to be present in may upland rice breeding lines and not in the irrigated lines (Heuer *et al*., 2009). Wissuwa *et al*, (2002) had successfully mapped *Pup1* gene to rice chromosome 12 and a smaller effect QTL was also identified on chromosome 6 as a result from Kasalath x Nipponbare population study. It is the only identified major quantitative trait locus (QTL) for P uptake and enhanced the PUE in rice with an identified gene and hence it is most well studied for use in breeding for tolerance to phosphorus deficiency ecosystem (Heuer *et al*., 2009). Recent trials and development of *Pup1* breeding lines (Batur, Situ Bagendit and

Dodokan) using marker assisted breeding have proven effective using Kasalath as the *Pup1* donor genotype (Chin *et al*, 2011). It was noted that *Pup1* presence in the irrigated *indica* NILs of IR64-*Pup1* and IR74-*Pup1* breeding lines also have a phenotypic effect on the lignification of roots and producing longer root hairs under drought and P-stress (Heuer *et al*., 2009; Pariasca-Tanaka *et al*., 2009).

Previous unpublished study by Bangor University MSc. student Boon Fei Chin (2013) of PUE in rice showed that Ashoka 228 had a better growth performance (66%) compared to Kalinga III (55%) in P-deficient soil by producing more tillers and higher mean percentages of yield in all low P treatments under glasshouse conditions. It was also noted that experiemnt Ashoka 228 had a higher P concentration in leaves (19.74 mg/L) compared to Kalinga III (17.33 mg/L). While mean root length of Ashoka 228 (9.3 cm) were also longer than Kalinga III (8.8 cm) in P deficient soil. It was documented from field studies that Ashoka 228 and Ashoka 200F perform better in productivity with taller height, earlier maturity stage, higher yielding, more tolerant to drought and lodging compared to other local upland ecosystem rice genotypes (Virk *et al*., 2003, Steele *et al*., 2004). Even though no *Pup1* QTL have been associated in Ashoka 228 (Heuer *et al*., 2009), it will be interesting to compare the performance of both Ashoka genotypes for their P uptake and PUE performance under low P condition compared to its upland parent Kalinga III as it might controlled by different QTL. The experiment in this chapter were designed to test the research question do upland rice varieties show different uptakes responses to low and high phosphate?

The aim of this chapter is to investigate the relationship of plant growth response in different P nutrient concentrations administered to a low P soil with the objective of evaluating the performance of selected upland rice genotypes growth in relation to:

- Agro morphological data (plant height, root length, tiller numbers, dry weights, chlorophyll count and yield) throughout the vegetative and reproductive stages of Ashoka 228 and Ashoka 200F comparing to Kalinga III. The hypothesis was that the Ashoka varieties would perform better for the traits mentioned under low P nutrient condition.

- Plant P uptake and phosphorus use efficiency (PUE) converted to biomass grown in low phosphorus soil and can be further explored by linking by the rice plant morphological variables. The hypothesis for this would be the Ashoka varieties have different genetically determined mechanisms when compared to Kalinga III.

2.2 Materials and Methods

2.2.1 Soil analysis and low phosphorus soil

All the experiments were carried out in a glasshouse and the experiment was located at Henfaes Research Centre, Bangor University, Abergwyngregyn, Gwynedd (henceforth "Henfaes") of the north Wales coast at 53° 14'N, 4° 01'W. The soil used for this experiment was a eutric cambisol obtained from the lowland part of the farm known as Morfa (**Figure 2.1**). Pre analysis of soil samples were made in April 2016 from the location and was sent to Lancorp Laboratories (York, UK) to evaluate the soil P contents before carrying out the actual experiment and found to contain 26 mg/L^{-1} pH 6.5 (Index 3). The analytical methods used by Lancorp Laboratories are as described in DEFRA Reference Book 427 and the index values are determined from the DEFRA Fertiliser Recommendations RB209 8th edition. Therefore, in order to lower the P contents, four combinations of eutric cambisol : silver sand dilutions were made and sent for analysis to NRM Laboratories (Bracknell, UK). The sands with a 1:3 ratio was found to be the best combination for low P so it was used for the experiment (**Table 2.1**).

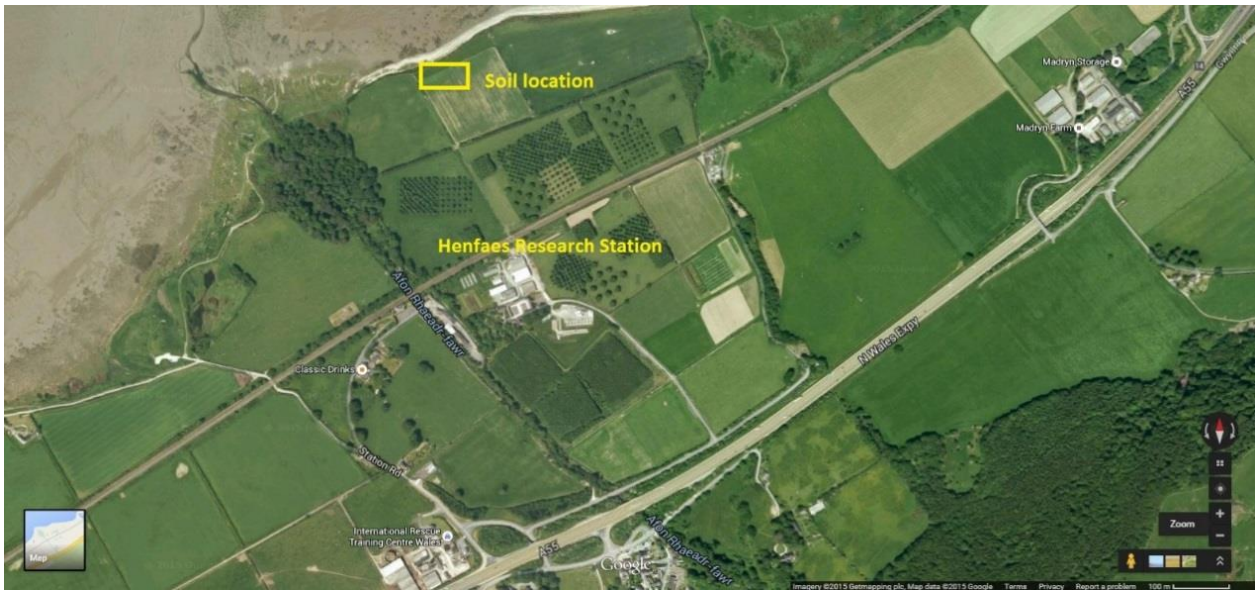


Figure 2.1 Satellite image-showing location of soil used at Henfaes Research Centre, Bangor University, Abergwyngregyn. (source: https://earth.google.com).

Table 2.1 Soil analysis report from NRM Laboratories (Bracknell, UK) of eutric cambisol soil and silver sand ratio combinations made to simulate low P soil.

Soil Composition ratio		Soil pH	Index			mg/L (Available)		
Eutric cambisol soil	Silver sand		P	K	Mg	P	K	Mg
1	0	6.3	2	0	1	17.2	58	32
3	1	5.9	1	0	1	13.6	50	35
1	1	6.2	1	0	1	11.6	36	28
1	3	6.5	0	0	0	8.2	17	25

Pilot study (July – Nov 2015)

A pilot experiment was carried out on a smaller scale for Ashoka 200F, Ashoka 228 and Kalinga III. The seed germination, planting and P nutrient treatments follows the same methods as **section 2.2.2** and **2.2.4**. There were several differences in the pilot (2015) compared to the main study (2016) experiment, these were:

- Only undiluted eutric cambisol soil were used.
- Three pre-germinated seedlings of the same genotype were planted in the same pot.
- Destructive analysis was only performed on day 45 (one plant removed per pot) and day 126 (one plant removed per pot) while data collection excluded root length, root dry weight and chlorophyll (SPAD) data.
- The pilot experiment consisted of 36 pots (3 genotypes x 3 phosphorus treatments x 4 replications) and did not have equal numbers of plants in every pot due to some not surviving.

Main experiment (May-Sept 2016)

2.2.2 Plant pre-germination and growth in contrasting soil P treatments

Germination of Ashoka 228, Ashoka 200F and Kalinga III rice seeds was carried out before sowing into contrasting soil P treatments in order to check the seed viability. The seeds were germinated in batches and transplanted according to each variety's growing periods. The variety with the longest harvesting dates were transplanted first followed by variety with the shortest harvesting dates so that sampling time can synchronised and be collected

simultaneously during vegetative, booting, flowering and harvesting days. The seeds were first soaked in distilled water for 5 minutes and then submerged in 1% sodium hypochlorite (Sauer and Burroughs, 1986; Mewm *et al*., 1994) for 30 minutes with constant aeration with a Tetra APS 50 aquarium pump (power 2W and airflow 50 l/h). The seeds were then rinsed in distilled water to remove NaOCl and later dried using paper towel. The sterilized seeds were then immersed in 250 ml deionized water and aerated with a small pump for 24 hours at room temperature. This serves to prime the seeds by softening the hard seed coat and to leach out any chemical inhibitors that may prevent germination. The seeds were then placed in a plastic container (9.5 x 15.0 x 4.5 cm) lined with moistened filter papers, incubated at 26°C and 12 hours of good fluorescent lab lighting exposure. Seeds should germinate within 72 hours or until the seed sprouted roots approximately 5 mm in length.

By using a forceps, the 3-day-old germinated seedlings were selected for uniformity and carefully transplanted into a 4-litre pot (dimension: 17.5 cm x 23 cm) at a depth of 2.0 cm (hole punched with a plastic dig seedling tool). Each pot containing approximately 4.5 kg of eutric cambisol: silver sand (1:3) obtained from Henfaes Research Station (Abergwngregyn). Prior to filling pots with soil, each pot were lined with cut out muslin cloth (15 x 15 cm) covering the bottom of the pots as to prevent soil loss throughout the experiment. The pots were then placed on a plastic saucer (13.5 cm) and watered with tap water three times a week. The plants were kept in a greenhouse (28°C ± 2°C; 12 hrs day/12 hrs night cycle period) for 120 days until all of the three rice genotypes treated with different nutrient treatments had reached maturity.

2.2.3 Pot experimental design and sampling

Modified method similar to Fageria *et al*. (1988) was implemented in carrying out the experiment. A factorial design was used with 3 genotypes x 4 replications x 3 P treatments x 3 harvest in a complete randomized design (**Table 2.2 and Figure 2.2**). All pots were fed nutrient treatments on day 0 (sowing day), day 40 (active tillering) and day 60 (panicle initiation) as shown in **Table 2.3, 2.4 and 2.5**. Data collections of plant height (cm), tiller numbers and chlorophyll (SPAD) were recorded at every sampling dates. Three destructive sampling harvests were performed and data recorded for the root length (cm), shoot dry weight (g), root dry weight (g), grain number, total grain weight (g) and single grain weight (mg) on day 45 during tillering (Harvesting 1), day 75 during flowering (Harvesting 2) and day 120 after grain ripening (Harvesting 3) after sowing respectively (**appendix 2.1** and **2.2**).

At each harvest, all three genotypes from the four replications of the three treatments totalling to 36 pots were sampled and removed from the experiment. The destructive analysis

process were carried out by the removal of plants from pots and carefully breaking, shaking and removing as much soil as possible by hand. Care was taken to not hold the plants by the shoot during soil removal to avoid breakage between shoot and root. The root system was then further washed with pipe water on a pavement, cleaned and the muslin cloth carefully removed to reduce root tearing and loss. Once the task completed, the plants were laid flat on a dry surface and lengths of the shoot and the longest root were measured using a ruler and recorded.

Afterwards, the grains were extracted by hand threshing and grain number determined using Tripette & Renaud Numigral seed counter, weighing total grain weight with Ohaus Adventurer electronic balance and determining single grain weights by dividing the total grain number /total grain weight. The plants shoot, root systems and grains samples (harvested at d 45, d 75 and d 120) were weighed with Ohaus Adventurer electronic balance for fresh weight and later dried to a constant weight in a Sanyo MOV-212F convection oven at 85 °C 48 hours and the dry weight were recorded.

Table 2.2 Complete randomize design (CRD) design of PUE experiment.

89	20	43	12	52	13	79	5	102	30	90	103	64	94	4	9	63	23	62	92	100	50	49	59	77	16	24
95	7	38	54	10	101	32	74	33	22	80	88	107	84	86	36	76	46	8	93	45	48	85	28	98	44	57
66	69	26	70	78	40	34	61	14	53	99	39	91	11	3	82	37	81	106	21	58	1	15	56	71	97	47
60	75	72	67	108	17	2	65	35	51	73	6	83	55	41	87	27	18	104	68	29	96	31	42	19	25	105

Legend: Full P Half P 0 P

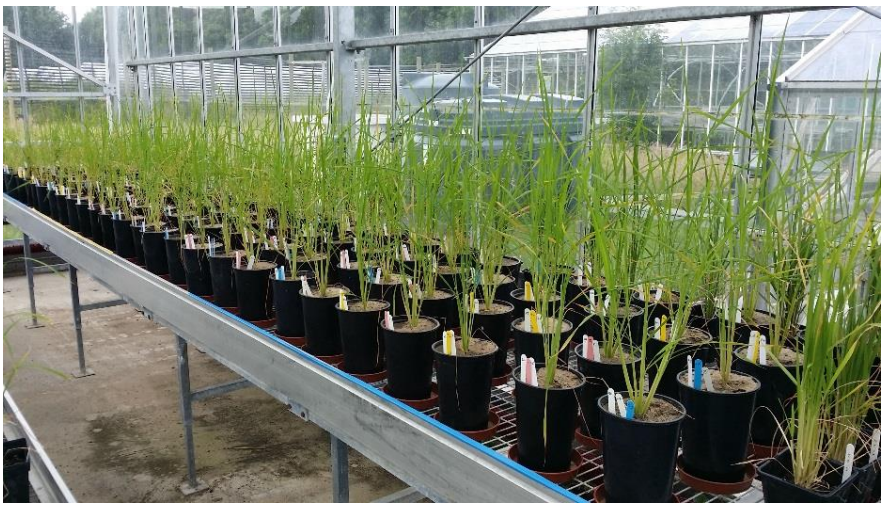


Figure 2.2 Experimental layout of 108 (4 litre) pots containing Ashoka 228, Ashoka 200F and Kalinga III in low phosphorus soil (Eutric Cambisol + silver sand) treated with three levels of

phosphorus nutrient treatments (none, half and full) in complete randomize design (CRD). Pots labelled with corresponding colours refers to treatments given to plants. Red = 0 P, Yellow = half P and Blue = full P.

2.2.4 P Nutrient preparation

The rate of application of fertilizer was be adapted from Steele *et al.* (2007) where a total of 40N: 20P: 20K kg/ha had been applied in the rice field. A 20 kg/ha N and all the P and K as the basal dose, then 10 kg/ha at the booting stage and 10 kg/ha N at flowering (Steele *et al.*, 2007). The dosage were calculated and scaled down to fit the experiment and the nutrients were diluted in water and administered to each 4-litre pot (**Tables 2.3**, **2.4** and **2.5**).

Table 2.3 Treatments of rice seeds in low phosphorus soil.

Ammonium Nitrate , NH_4NO_3 (Fertilizer = 34.5% N)					
Application	**Rate per ha (kg/ha)**	**Rate per ha (g/m^2)**	**Surface area of 4 litre pot (m^2)**	**Amount applied per pot (g)**	**Remarks**
On sowing day	58.82	5.82	0.0226	0.132	1.58g/6 litres for 12 pots (500ml/pot)
40 days after (active tillering)	29.41	2.94	0.0226	0.066	0.792g/6 litres for 12 pots (500ml/pot)
60 days after sowing (panicle initiation)	29.41	2.94	0.0226	0.066	0.792g/6 litres for 12 pots (500ml/pot)
Muriate of Potash , K_2O (Fertilizer = 60% K)					
Application	**Rate per ha (kg/ha)**	**Rate per ha (g/m^2)**	**Area of 4 litre pot (m^2)**	**Amount applied per pot (g)**	**Remarks**
On sowing day	33.40	3.34	0.0226	0.075	0.9g/6 litres for 12 pots (500ml/pot)
Triple super phosphate , P_2O_5 (Fertilizer = 46% P)					
Application	**Rate per ha (kg/ha)**	**Rate per ha (g/m^2)**	**Area of 4 litre pot (m^2)**	**Amount applied per pot (g)**	**Remarks**
On sowing day	43.43	4.343	0.0226	0.049 (half strength)	0.588g/6 litres for 12 pots (half strength)
				0.098 (full strength)	1.176g/6 litres for 12 pots (full strength)

Table 2.4 The fertilizer rate application of rice plant per hectare (Steele *et al*, 2007) and adapted rate of fertilizer application in one pot from sowing date to panicle initiation.

Element	**Rate of N:P:K (%) application per hectare**	**Total rate of application (g/pot)**
Ammonium Nitrate	40	0.264
Muriate of Potash	20	0.075
Triple super phosphate	20	0.098

Table 2.5 Three different treatments of total nutrient application for nitrogen (ammonium nitrate), potassium (muriate of potash) and phosphorus (triple super phosphate) applied to pots in from sowing date to panicle initiation.

Nutrient	**Ammonium Nitrate (g)**	**Muriate of Potash (g)**	**Triple super phosphate (g)**
Treatment 1 (No P)	Full strength - 0.264	Full strength - 0.075	Nil
Treatment 2 (Half P)	Full strength - 0.264	Full strength - 0.075	Half strength - 0.049
Treatment 3 (Full P)	Full strength - 0.264	Full strength - 0.075	Full strength - 0.098

2.2.5 Chlorophyll measurements

A hand held chlorophyll SPAD-502 Plus meter (Konica-Minolta Optics Inc., Japan) that measures a leaf transmittance in the red (650nm; the measuring wavelength) and infrared (940 nm; the reference wavelength used to adjust for non-specific differences between samples) regions of the electromagnetic spectrum was used to measure the index of relative leaf chlorophyll contents. The uppermost fully expanded leaves were selected and readings were taken around the midpoint from each leaf blade. Three separate SPAD readings from one leaf/tiller/plant were recorded and the mean SPAD value calculated for each pot.

2.2.6 Analysis of plant and grain P contents

The dried shoot, root and grain samples (**section 2.2.3**) were ground into a fine powder using Foss CT 193 Cylotec^{TM} miller before proceeding to analysis of P contents. Ground shoots and grains are then weighed (200 mg) and placed in acid-washed 20 ml glass vessels before dry ashing in Carbolite CWF 1200 muffle furnace (550°C, 16 h). The ash was checked for extent of destruction by appearing as clean white, otherwise the ashing process are repeated for a further 4 - 8 hours. Total P content measurement was determined via ascorbate/molybdate blue method (Murphy and Riley, 1962; Benton Jones, 2001 and Hepell *et al*, 2015) by fully digesting the ash with 1 ml of 6 M HCL acid on a heat plate (Cole Parmer) for 30 minutes.

Care was taken by not letting the acid digestion to boil and evaporate by heating the samples to a medium setting. Afterwards, the samples were diluted by adding 9 ml of deionized water, homogenized and left overnight for the mixture to settle. Then, 80 µl of samples and standard controls of potassium dihydrogen orthophosphate (0-50 mg/L) were added to the wells required in 96 well plates. Next, by using a multichannel pipette 180 µl of AMES Reagent (2.5 M Sulphuric acid, 4% ammonium molybdate, 10% ascorbic acid and 0.3% of potassium antimonyl tartrate) was added to each well. Afterwards, 30 µl of 10% Ascorbic acid was added to each well and the plate was left at room temperature for 20 minutes until a visible blue colour change developed in the wells. The absorbance was measured by reading the optical density of each well at 820 nm using the Epoch absorbance microplate reader (Biotek Instruments Ltd, UK). A calibration graph was plotted using the absorbance values from serial dilutions of standard phosphorus solution (absorption vs. concentration). The results were calculated as follows and recorded (**appendix 2.3**):

$$PO_4\ \text{P in tissue sample } (\mu g\ g^{-1}) = \frac{PO_4\ \text{P in sample } (\mu g\ ml^{-1}) \text{ x volume of extractant (ml)}}{\text{weight of ashed sample (g)}}$$

2.2.7 Phosphorus use efficiency

The phosphorus uptake (P uptake) and phosphorus use efficiency (PUE) in all samples were calculated for each time of harvesting stage by using similar formula used by Fageria, *et al*., (1988) and Syers *et al*., (2008) and according to Withers, P (pers. comm. 26 April 2018) :

Shoot P uptake = [P] concentration in shoot (mg/g) x dry weight of shoot (g)

Root P uptake = [P] concentration in root (mg/g) x dry weight of root (g)

Grain P uptake = [P] concentration in grain (mg/g) x dry weight of grain (g)

Total Plant P uptake = ([P] concentration in shoot+root) x (dry weight of shoot+root)

$$\text{P use efficiency (PUE)} = \frac{\text{Total P uptake in plant with P treatment} - \text{Total P uptake in plant without P treatment}}{\text{Amount of P applied in treatment x } 100}$$

2.2.8 Statistical analysis

Data were analysed by analysis of varience (ANOVA) and means were compared with Tukey's HSD test at the 5% probability level. The relationship between different levels of P concentrations treatments administered to the upland rice to the growth performance, P content and P uptake were analysed using SPSS statistics software package version 22.0 for Windows (SPSS Inc, IBM Corporation, Illinois, USA). The independent variables were the three plant genotypes (Ashoka 200F, Ashoka 228 and Kalinga III) and three different P nutrient levels (No P, ½ P and Full P). The dependant variables were the plant height (cm), root length (cm), tiller numbers, dry shoot weight (g/plant), dry root weight (g/plant), dry grain weight (g/plant), counted grain numbers (grain/pot), SPAD values, P concentration in shoot (mg/g), P concentration in root (mg/g), P concentration in grain (mg/g), P uptake in shoot (mg/plant), P uptake in root (mg/plant) and P uptake in grain (mg/plant).

A general linear model (GLM) of univariate analysis of variance was carried out to analyse all of the data variables mentioned above with Tukey's HSD as a post-hoc test, where the statistical significance was determined at $p < 0.05$. All of the values analysed are presented as means ± the standard error of the mean (± SEM).

2.3 Results and Analysis

2.3.1 Pilot study results

2.3.1.1 Day 45

ANOVA on plant height, tiller number and plant phosphorus contents in Ashoka 200F, Ashoka 228 and Kalinga III found no significant difference between either the genotypes, phosphorus treatment or interactions between genotypes and treatments for all the plant variables.

No significant differences between treatments or genotypes were found for plant dry weights. However there were significant interactions between treatment and genotype plant dry weight [$F(4, 27) = 3.35$, $p < 0.05$] (**Figure 2.3**). At full P, the combined genotypes mean dry weight (10.7 ± 1.23 g) accumulated more biomass weight than the combined genotypes mean dry weight in 0 P (8.5 ± 0.92 g) by a difference of 2.2 g.

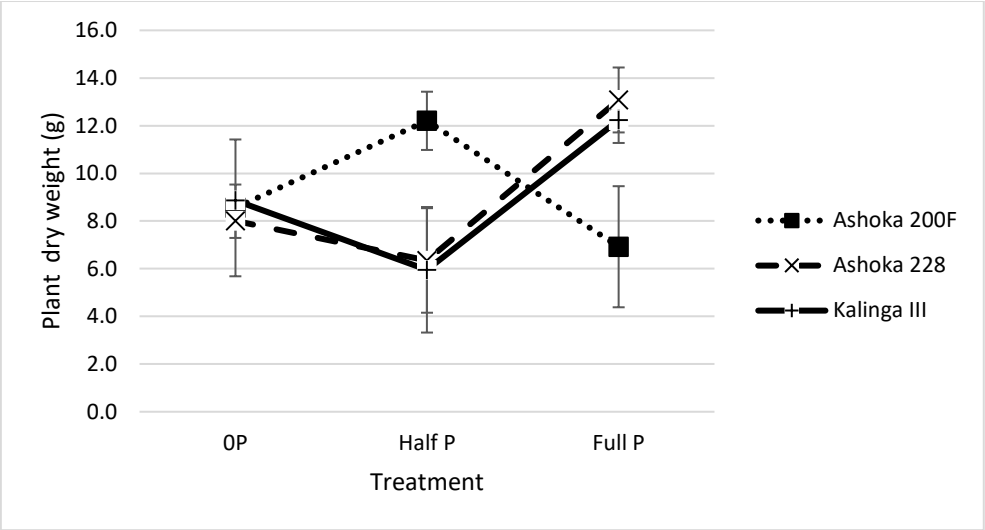


Figure 2.3 Effect of different P treatments on plant dry weight (g) at 45 days after sowing. Values represent means (n = 4). Vertical bars indicate the standard error of the mean (± SEM).

2.3.1.2 Day 126: Treatment effects

When data for all three genotypes were pooled, differences between treatments were found and summarized in **Table 2.6.** Genotypes treated with different phosphorus nutrient levels [F (2, 27) = 4.25 $p < 0.025$] grew taller in full P (105.0 ± 1.54 cm) than those treated in 0 P (98.3 ± 1.56 cm). Genotypes treated in full P produced higher grain dry weight than 0 P treatment by a difference of 1.7 g ($p < 0.05$). The number of grains produced per plant were also higher in full P (480 ± 67.53) compared to 0 P (286.5 ± 58.78) although there were no significant difference detected. There were no significant difference in interactions between the genotypes and treatments for all of these variables.

Table 2.6 Pilot experiment effect of different P treatments on plant variables (mean for all varieties) at 126 days after sowing.

126 day	**Treatments**			
Variable	**0 P** **(0 mg.kg-^{1})**	**Half P** **(49 mg.kg^{-1})**	**Full P** **(98 mg.kg^{-1})**	***p* value**
Plant height (cm)	98.3 ± 1.56^{a}	103.3 ± 2.03^{ab}	105.0 ± 1.54^{b}	< 0.05
Grain dry weight (g)	2.6 ± 0.43^{a}	4.1 ± 0.43^{ab}	4.3 ± 0.56^{b}	< 0.05
Number of grains/plant	286.5 ± 58.78^{a}	444.9 ± 54.62^{a}	480.8 ± 67.53^{a}	0.068

Value represent means (n = 12) and standard error of the mean (± SEM). Means with different letters across a row of variable are significantly different ($p < 0.05$).

2.3.1.3 Day 126: Genotype effects

The significantly different variables for pooled data across treatments are summarized in **Table 2.7**. The variables tiller number, plant dry weight and seed phosphorus content were not significantly different between any of the genotypes tested. Grain dry weights are significantly heavier in Ashoka 200F than Ashoka 228 by 1.8 g ($p < 0.05$). This too reflected in the number of grains produced per plant where Ashoka 200F (570.6 ± 73.20) significantly produced more grains than Ashoka 228 (292.7 ± 44.73). However, individual grains were much lighter in Ashoka 200F (12.6 ± 0.56 mg) when compared to Kalinga III (17.0 ± 0.48 mg) by a significant 4.4 mg ($p < 0.05$). Phosphorus content in plant biomass [$F(2, 27) = 3.75$, $p < 0.05$] of Kalinga III (7.5 ± 0.52 mg/g) was significantly higher than Ashoka 200F (6.0 ± 0.42 mg/g) and Ashoka 228 (6.0 ± 0.45 mg/g) by 1.5 mg/g. No significant difference were found in all of the variables mentioned for genotype and treatment interactions.

Table 2.7 Pilot experiment effect of different genotype variables (mean for all varieties) after at 126 days after sowing.

126 day	**Genotype**			
Variable	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	***p* value**
Grain dry weight (g)	4.7 ± 0.58^{b}	2.9 ± 0.43^{a}	3.4 ± 0.38^{ab}	< 0.05
Number of grains/plant	570.6 ± 73.20^{b}	292.7 ± 44.73^{a}	349 ± 40.73^{a}	<0.05
Single grain weight (mg)	12.6 ± 0.56^{a}	16.2 ± 0.50^{b}	17.0 ± 0.48^{b}	<0.001
Shoot P content (mg/g)	6.0 ± 0.42^{a}	6.0 ± 0.45^{a}	7.5 ± 0.52^{b}	<0.05

Value represent means ($n = 12$) and standard error of the mean (± SEM). Means with different letters across a row of variable are significantly different ($p < 0.05$).

2.3.2 Main experiment (non-destructive phenotyping)

2.3.2.1 Plant height measured over the course of experiment

There were no significant differences in terms of the main effect of phosphorus nutrient treatments and the interaction between treatments and genotypes on all three rice genotypes for height but there were genotype differences for overall plant height (**figure 2.4)**. At the early vegetative growth stage of day 7 – 21, Ashoka 228 had a significantly higher overall height compared to Ashoka 200F and Kalinga III. There were no significant differences between any of the genotypes during active tillering stage from day 28 – 63. However, there was a statistically high significant difference in the main effect for rice genotype effect at day 70 for

plant height effect F (2, 27) = 16.8, $p < 0.001$. Ashoka 228 (71.2 ± 0.91cm) was 4.4 cm taller than Kalinga III (66.8 ± 1.42 cm) and 8.1 cm taller than Ashoka 200F (63.1 ± 0.96 cm). At day 75, there was a high significant differences in genotypes tested [F (2, 27) = 34.7, $p < 0.001$] with Ashoka 228 height (85.2 ± 0.88 cm) outgrew Ashoka 200F (70.6 ± 1.51 cm) by 14.6 cm and Kalinga III (74.6 ± 1.44 cm) by 10.6 cm. During the early reproductive stage at day 84, Ashoka 228 (94.8 ± 1.36 cm) had the tallest height compared to Kalinga III (88.8 ± 2.28 cm) and Ashoka 200F (77.8 ± 1.30 cm) with differences of 6.0 cm and 17.0 cm, respectively. However at day 98, Ashoka 200F (102.8 ± 2.36 cm) outgrew Kalinga III (101.2 ± 1.03 cm) by only 1.6 cm but have a taller height than Ashoka 228 (95.1 ± 1.47 cm) by 7.7 cm until harvesting day (**figure 2.4**).

2.3.2.2 Tiller numbers measured over the course of experiment

There was no significant difference found between the three P nutrient treatments given for tiller numbers for all three varieties tested at early vegetative stage, dayS 7 – 56. However, there was a significant difference in genotype main effect at day 63 with F (2, 27) = 4.3, $p < 0.05$. It was recorded that Ashoka 200F had a higher tiller number (12.8 ± 0.64) when compared to Ashoka 228 (11.1 ± 0.43) and Kalinga III (11.0 ± 0.35). This significant difference trend carried throughout the active tillering, reproductive to harvesting day 112 with Ashoka 200F (13.0 ± 0.62) produced 1.6 more tillers than Ashoka 228 (11.4 ± 0.47) and 1.7 tillers more than Kalinga III (11.3 ± 0.30). The overall tiller numbers produced by all three genotypes are presented in **Figure 2.5**.

Figure 2.4 Plant height for Ashoka 200F, Ashoka 228 and Kalinga III from day 0 until day 112. Values represent means (*n*=36 from day 0 to 45, *n*=24 from day 46 to 75, *n*=12 from day 76 to 120). Vertical bars indicate the standard error of the mean (± SEM). Three red vertical dotted lines represents destructive harvests at day 45, day 75 and day 120 respectively.

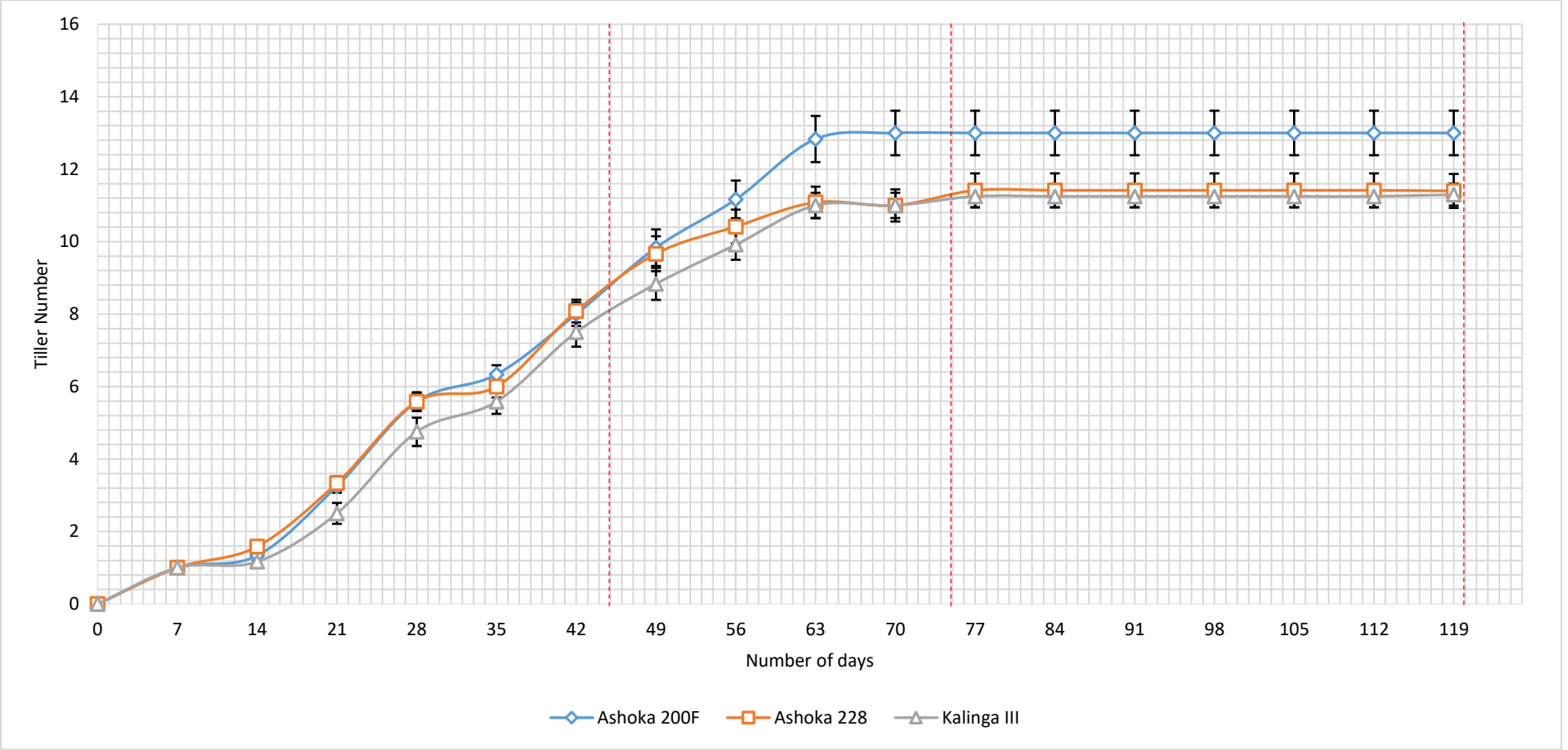


Figure 2.5 Plant tiller number for Ashoka 200F, Ashoka 228 and Kalinga III from day 0 until day 112. Values represent means (*n*=36 from day 0 to 45, *n*=24 from day 46 to 75, *n*=12 from day 76 to 120). Vertical bars indicate the standard error of the mean (± SEM). Three dotted lines represents destructive harvests at day 45, day 75 and day 120 respectively.

2.3.3 Main experiment (Destructive harvest)

Summary of the experiment showing significance of genotype, phosphorus treatment and genotype x treatment interaction for all measured variables at day 45 (tillering), day 75 (flowering) and day 120 (grain filling) are presented in **Appendix 2.4**

2.3.3 Observation of upland rice varieties at 45 day.

2.3.3.1 Root length

There was a significance difference in the mean root length at for the genotypes tested [F (2, 27) = 3.95, p = 0.031]. At 0 P treatment, Ashoka 200F (31.1 ± 1.36 cm) had slightly longer root length than Kalinga III (24.9 ± 1.20 cm) and Ashoka 228 (24.0 ± 0.54 cm) by 6.2 cm and 7.1 cm respectively ($p < 0.05$). This was also observed in half P treatments, where Ashoka 200F (30.0 ±1.90 cm) was longer than Ashoka 228 (25.4 ± 0.83 cm) and Kalinga III (23.9 ± 0.75 cm) by 4.6 and 6.1 cm respectively ($p < 0.05$). While at full P, there were no significant difference of root length between the three genotypes tested (**Appendix 2.5)** and **figure 2.6**). However, a combined ANOVA from all treatments does not seem to show any significant differences between the genotypes. There were also no significant difference found between the treatments and no significant interaction between genotypes and treatments on day 45. Root length of Ashoka 200F reduces as P availability increases, but root length of Kalinga III and Ashoka 228 increase at higher P levels.

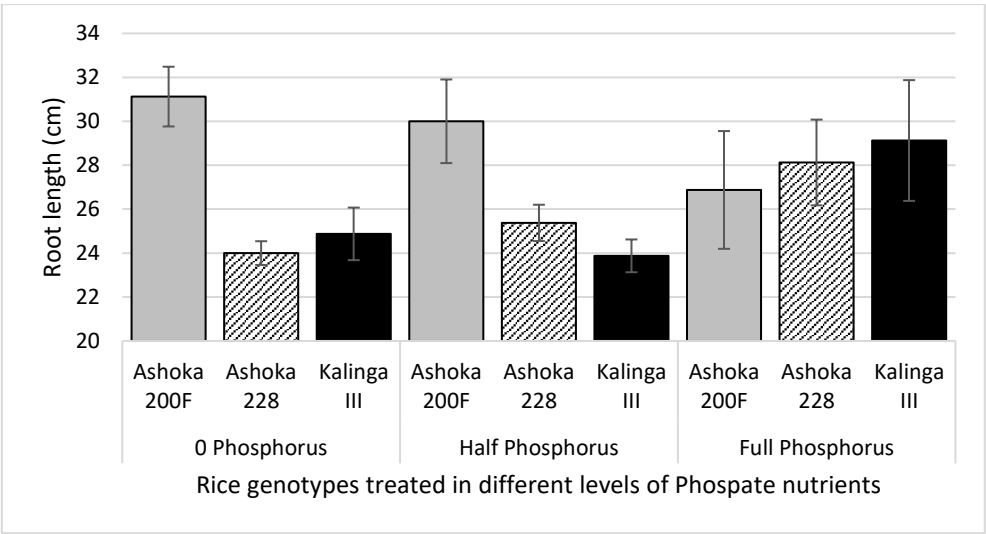


Figure 2.6 Effect of different P treatments on plant root length (cm) at 45 days after sowing. Values represent means (n=4). Vertical bars indicate the standard error of the mean (± SEM).

2.3.3.2 Dry weights

Dry weights of plant shoots and roots showed no significant differences in all the three genotypes across all three treatments.

2.3.3.3 SPAD Reading

No significant differences were found for treatments or interaction between genotypes and treatments. Analysis for mean SPAD values for genotypes across all treatments on day 45 (**Figure 2.7**) indicated that there were significant differences for genotypes tested [F (2, 27) = 9.00, p = 0.001]. Ashoka 228 (34.2 ± 1.19) had a significantly higher value as compared to Kalinga III (29.8 ± 0.60) and Ashoka 200F (29.8 ± 1.31).

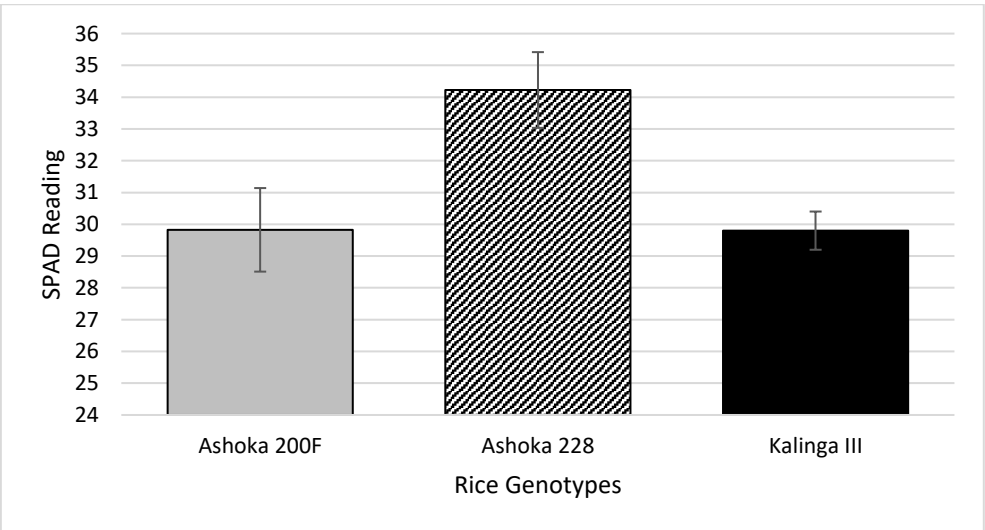


Figure 2.7 Plant SPAD readings at 45 days after sowing in different genotypes combined for all treatments. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.3.4 P content and P uptake

All the treated rice genotypes were analysed for phosphate contents at day 45. There were no significant differences found across all of the three rice genotypes or genotype and treatment interactions for shoot and root P contents. Although there are highly significant difference for treatments F (2,27)= 32.28 , p < 0.001 in shoot P contents between 0 P (3.3 ± 0.13 mg/g), Half P (4.1 ± 0.11 mg/g) and High P (4.7 ± 0.12 mg/g) (**figure 2.8**).

Total plant P uptake were also to found to be highly significant in for P treatments [F (2, 27) = 39.64, p < 0.001] in shoot at full P (13.3 ± 0.32 mg/plant), half P (11.7 ± 0.29 mg/plant) and 0 P (8.8 ± 0.41mg/plant) (**figure 2.9**). There were no significant differences in genotypes or interactions between genotypes and treatments. Analysis of the P uptake in roots

at day 45 also found no significance difference in genotype, treatments and genotype and treatment interactions.

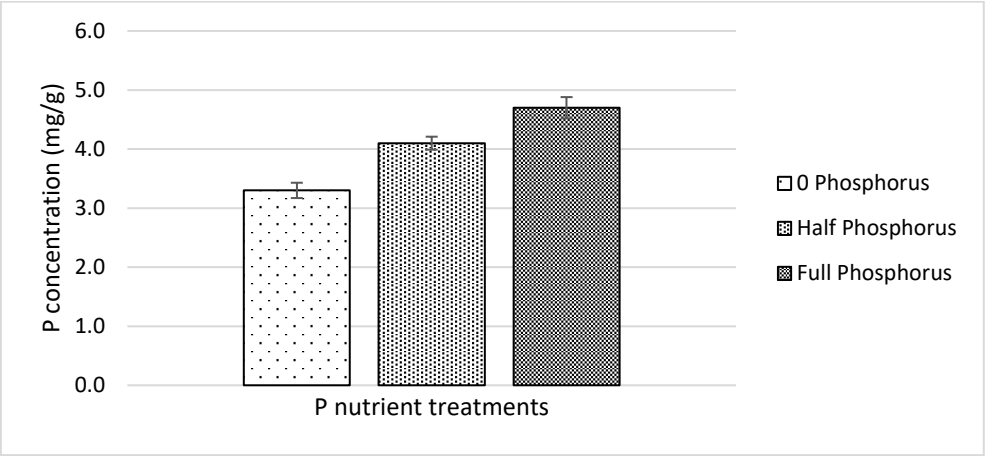


Figure 2.8 Effect of different P treatments on plant shoot P contents (mg/g) combined for all genotypes at 45 days after sowing. Values represent means (*n*=12). Vertical bars indicate the standard error of the mean (± SEM).

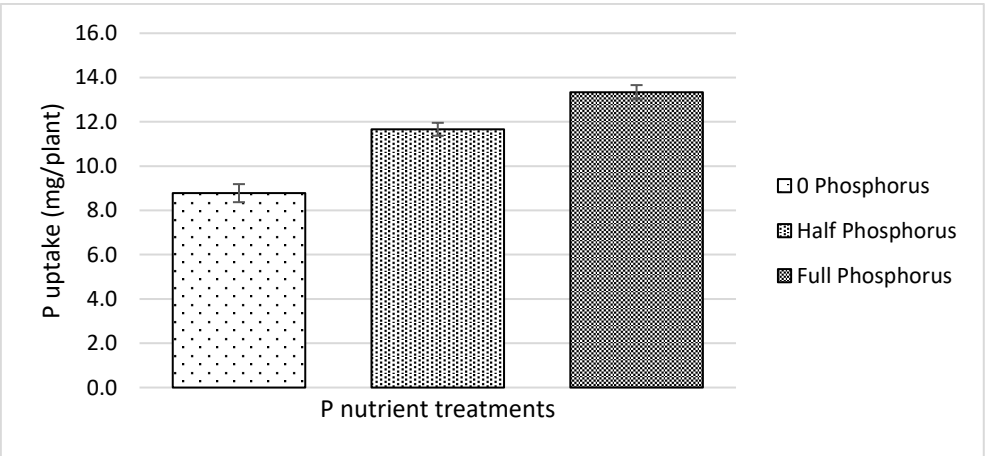


Figure 2.9 Effect of different P treatments on plant shoot P uptake (mg/plant) combined for all genotypes at 45 days after sowing. Values represent means (*n*=12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.4 Observation of upland rice varieties at 75 day. (Appendix 2.6)

2.3.4.1 Dry weights

In terms of shoot dry weights, there were no overall significant differences of dry weights for treatments with F (2, 27) = 3.19, $p = 0.057$. The mean dry weights in shoots treated with full P (9.4 ± 0.14 g) seemed to have a slightly heavier weights by only 0.7 g differences compared to varieties treated in 0 P (8.7 ± 0.17 g) (**figure 2.10**) and shoot dry weight was significantly different between 0 P and half P ($p = 0.046$). ANOVA analysis on root dry weight found no significant differences in genotypes, treatments and genotype and treatment interactions at this harvest.

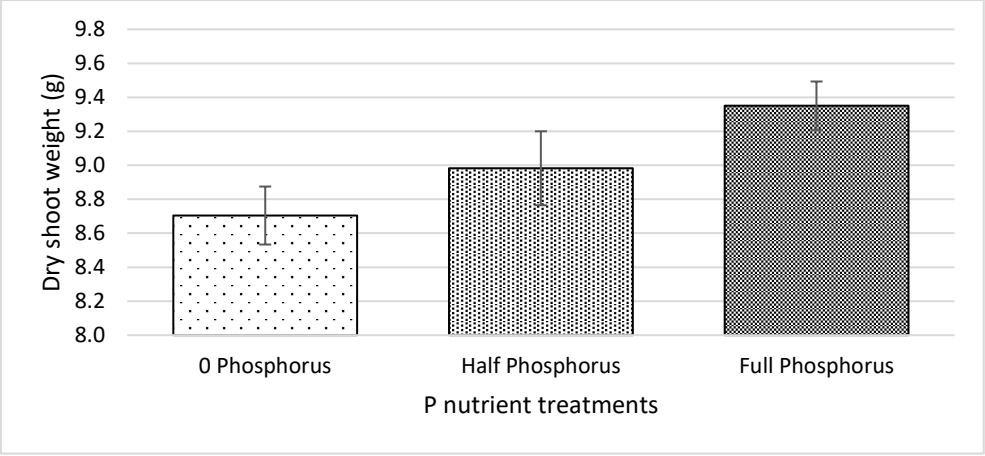


Figure 2.10 Effect of different P treatments on plant shoot dry weight (g) combined across all genotypes at 75 days after sowing. Values represent means ($n = 12$). Vertical bars indicate the standard error of the mean (± SEM).

2.3.4.2 SPAD Reading

There were a significant difference in the genotypes [F (2, 27) = 12.8, $p < 0.001$] with Kalinga III (39.1 ± 0.42) produced significantly higher SPAD values in all of the treatments than Ashoka 228 (37.1 ± 0.47) while Ashoka 200F (36.3 ± 0.36) had the lowest values (**figure 2.11**). No significant differences were found on the main effect for treatments or interaction between genotypes and treatments interactions.

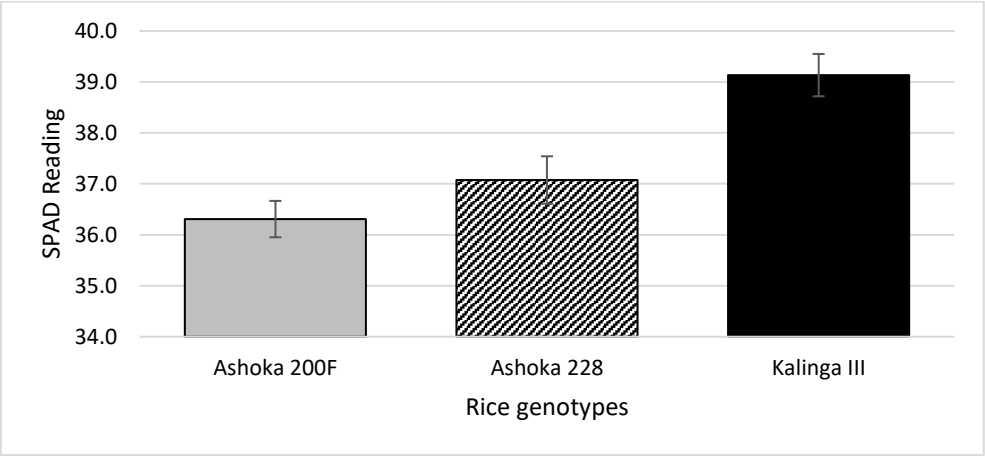


Figure 2.11 Effect of different P treatments on plant shoot SPAD values combined across all genotypes at 75 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.4.3 P contents and P uptake

Phosphate content for shoots in all of the three rice genotypes treated with different levels of P nutrient concentration showed a significant difference between treatments [$F(2, 27) = 11.95$, $p < 0.001$]. Full P treatment (3.5 ± 0.15 mg/g) having the obvious higher overall P contents in all of the varieties tested as compared to half P (3.0 ± 0.13 mg/g) and 0 P (2.6 ± 0.07 mg/g) as shown in **figure 2.12**.

Similarly, total plant P uptake in the genotypes tested was also to found to be highly significantly different between treatments [$F (2, 27) = 22.0$, $p < 0.001$] with Full P uptake in shoot (32.2 ± 1.25 mg/plant) when compared to half P (26.9 ± 1.02 mg/plant) and 0 P (22.3 ± 0.55 mg/plant) as shown in **figure 2.13**. There were no significant differences found in roots between genotypes or genotype and treatment interactions for both P contents and P uptake.

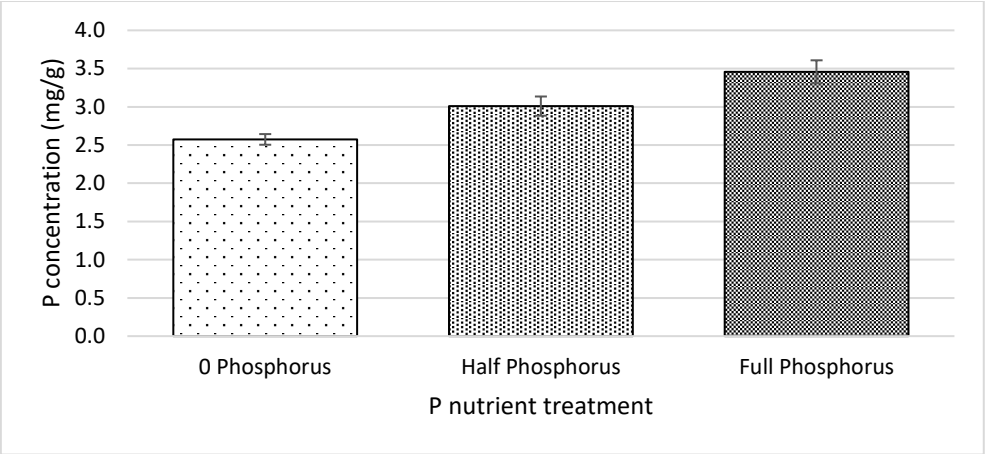


Figure 2.12 Plant shoot P content (mg/g) combined across all genotypes at 75 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

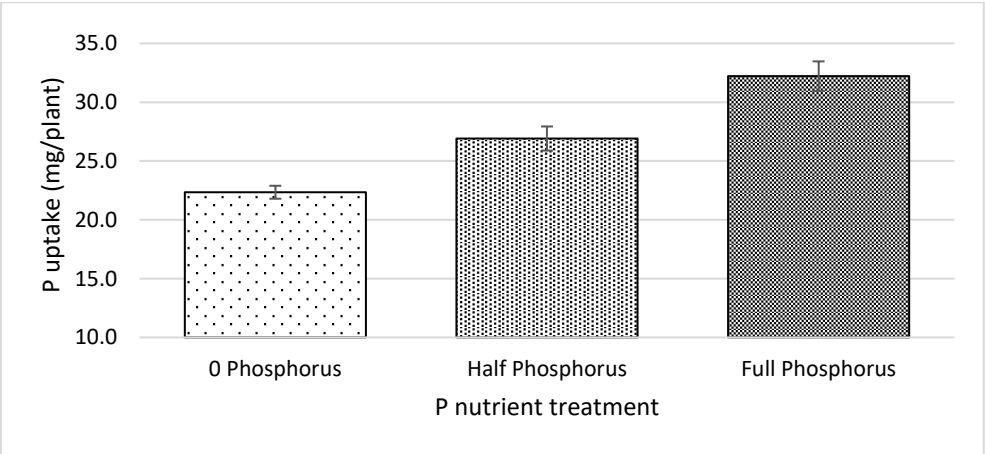


Figure 2.13 Plant P uptake (mg/plant) combined across all genotypes at 75 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5 Observation of upland rice varieties at 120 day (Appendix 2.7)

2.3.5.1 Plant height

Plant height analysis at harvesting day showed there is a significant difference in height for the genotypes tested [F (2, 27) = 6.07, p = 0.007]. Ashoka 200F (102.8 ± 2.36 cm) grew taller by 7.7 cm compared to Ashoka 228 (95.1 ± 1.47 cm) but only a marginal 1.7 cm when compared to Kalinga III (101.1 ± 1.03 cm) (**figure 2.14**). There were no significant differences found for treatment effect on all three genotypes or genotype and treatment interactions.

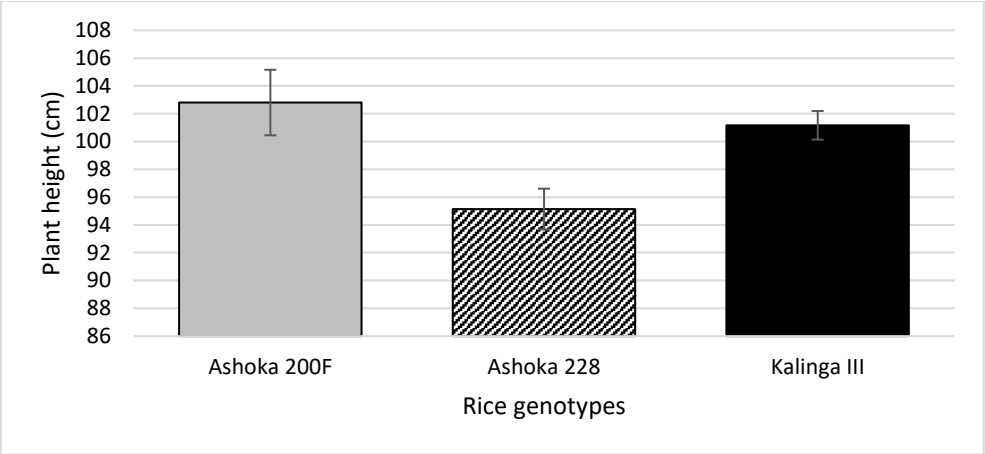


Figure 2.14 Plant height (cm) combined across all treatments at 120 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5.2 Root length

Root length analysis showed that there were a slight significance on the P nutrient treatments [F (2, 27) = 3.35, p = 0.05]. A combined ANOVA for treatment showed that all three genotypes have longer roots when treated at full P (32.8 ± 1.77 cm) than at half P (28.0 ± 4.99 cm) or 0 P (26.4 ± 1.36 cm) (**figure 2.15**). There were no significant difference between the three genotypes or genotype and treatment interactions.

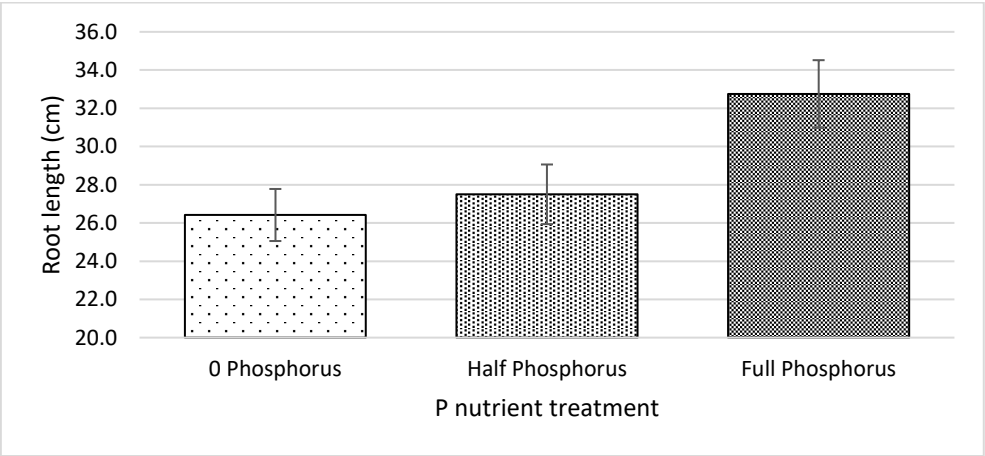


Figure 2.15 Plant root length (cm) combined across all genotypes at 120 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5.3 Grain number

Analysis showed a significance difference in number of grains per plant for combined genotype tested [F (2, 27) = 5.50, p = 0.01]. This holds true as Ashoka 200F (617.1 ± 10.90) produced a higher grain numbers compared to Kalinga III (557.4 ± 12.43) while Ashoka 228 (533.0 ± 29.24) had the lowest grain count in all of the combined treatments (**figure 2.16**).

There were no significant differences found in treatments or genotype and treatment interactions.

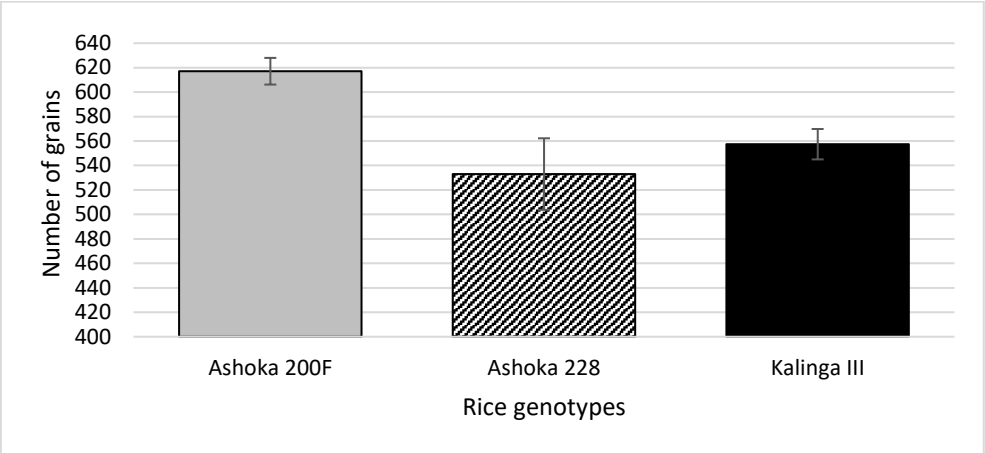


Figure 2.16 Counted grains per plant combined across all treatments at 120 days after sowing. Values represent means (*n*=12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5.4 Dry shoot weight

The dry shoot weight analysis of the shoot tissue showed a high significant difference for treatment given [F (2. 27) = 27.7, $p < 0.001$] with full P (12.6 ± 0.26 g), half P (11.6 ± 0.27 g) and 0 P (10.7 ± 0.18 g). It is also observed that there were significant difference for genotypes [F (2, 27) = 12.7, $p < 0.001$], over all treatments Ashoka 200F (12.2 ± 0.28 g) mean shoot dry weights were higher compared to Ashoka 228 (10.9 ± 0.26 g). In full P treatment, Kalinga III shoots (13.0 ± 0.34 g) weighed 1.1g more when compared to Ashoka 228 (11.9 ± 0.31 g). While in 0 P, Ashoka 200F (11.3 ± 0.21 g) weighed 0.7g more than Kalinga III (10.7 ± 0.18 g). However, there were no significant difference found in genotype and treatment interactions (**figure 2.17**).

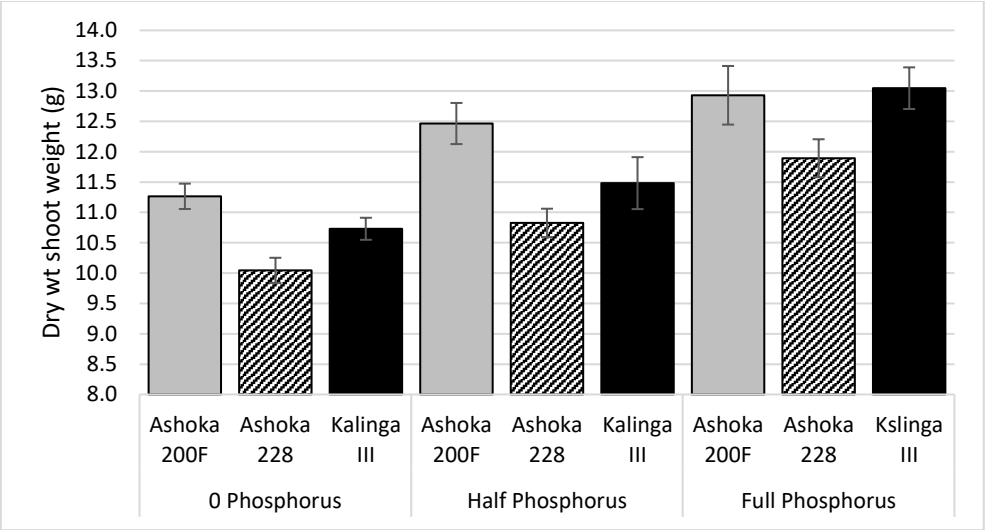


Figure 2.17 Effect of different P treatments on plant shoot dry weight (g) at 120 days after sowing. Values represent means (n = 4). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5.5 Dry root weight

Roots analysis only showed that there were significant differences in genotypes tested [F (2, 27) = 5.5, $p < 0.05$]. A combined ANOVA for each genotype from all three treatments showed that Ashoka 200F (11.60 ± 1.78 g) had almost double the mass in the root weights when compared to Kalinga III (6.7 ± 1.13 g) and Ashoka 228 (6.1 ± 0.66 g), with 4.9 g and 5.5 g more, respectively (**figure 2.18**). There were no significant differences found for the treatments or genotype and treatment interactions.

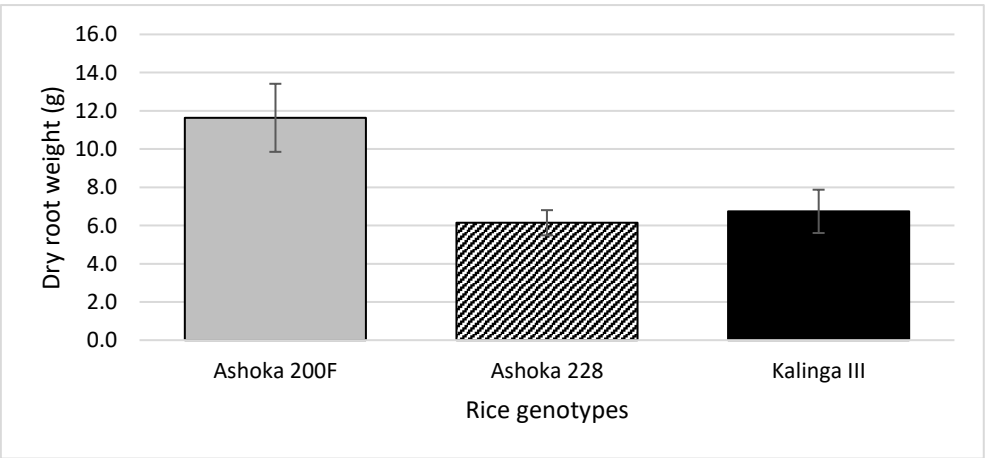


Figure 2.18 Plant root dry weight (g) combined across all treatments at 120 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5.6 Total grain weight

Ashoka 200F produced a higher tiller numbers and grain counts in all of the treatments (**Appendix 2.7**). The total grains weight per plant showed there were significant differences in genotypes tested [F (2, 27) = 17.8, $p < 0.001$). However, it is interesting to see that Kalinga III produced more yield weight (12.0 ± 0.29 g/plant) followed by Ashoka 200F (10.9 ± 0.18 g/plant) and Ashoka 228 (10.2 ± 0.20 g/plant) with the lowest yield (**figure 2.19**). There were no significant difference in the treatment given or genotype and treatment interactions.

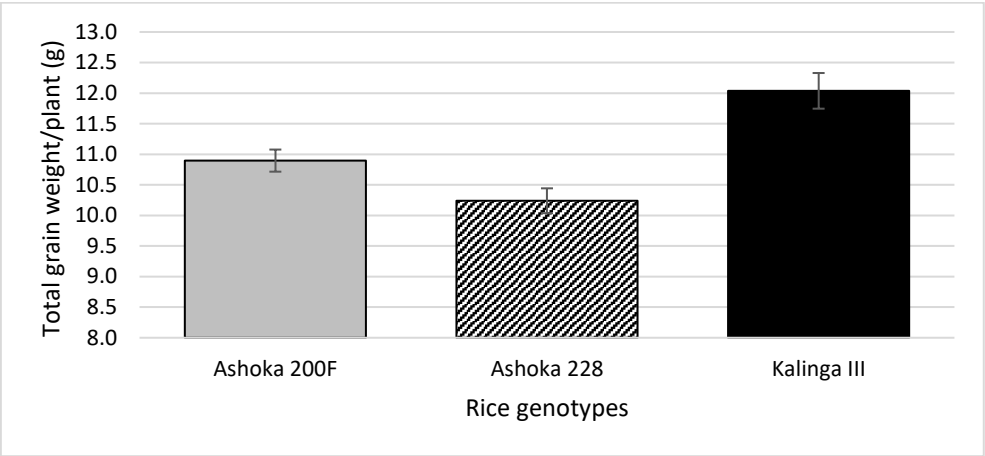


Figure 2.19 Total grain weight (g) per plant combined for all treatments at 120 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5.7 Single grain weight

No significant difference was found in all of the treatments or genotype and treatment interactions. Looking at a single grain weight analysis, it revealed a significant difference between the three genotypes [F (2, 27) = 16.85, p < 0.001] with Kalinga III (20.0 ± 0.18 mg) and Ashoka 228 (18.7 ± 0.77 mg) producing significantly heavier grain weight than Ashoka 200F (14.7 ± 0.87 mg), by 1.3 mg and 5.3 mg, respectively as shown in **figure 2.20.**

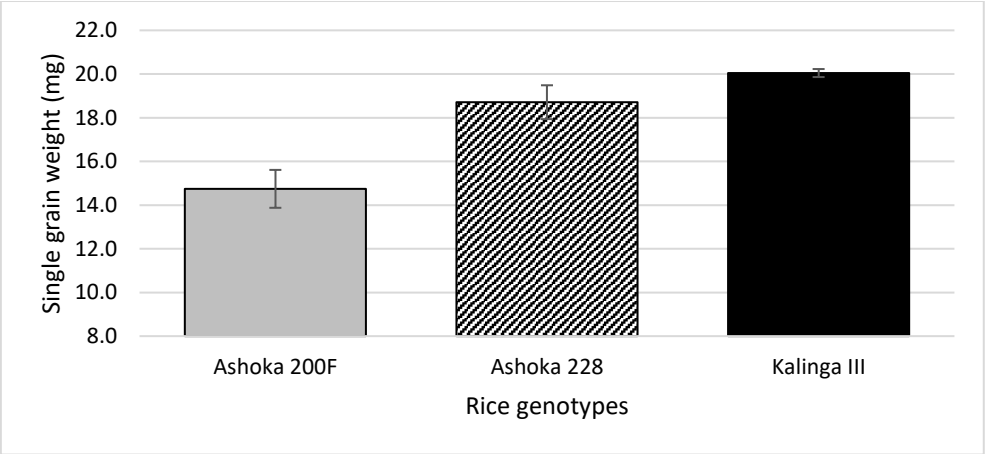


Figure 2.20 Single grain weight (g) combined for all treatments at 120 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5.8 SPAD Reading

No significance difference was found for the treatments or interactions between genotypes*treatments, but analysis showed that there is a high significance difference in genotypes tested [F (2, 27) = 29.9, $p < 0.001$]. The combined ANOVA for genotype showed that Ashoka 200F (33.7 ± 0.78) produced the highest SPAD values followed by Kalinga III (29.8 ± 1.29) while Ashoka 228 (22.4 ± 1.02) had the lowest SPAD values in all treatments given as shown in **figure 2.21**. However, there were no significant differences found for treatments or genotype and treatment interactions.

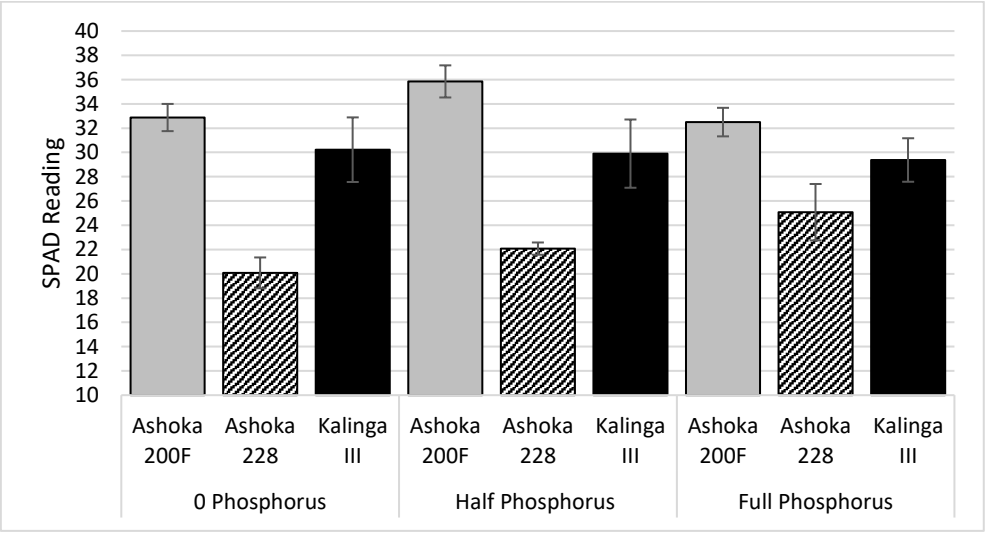


Figure 2.21 Effect of different P treatments on SPAD values at 120 days after sowing. Values represent means (n = 4). Vertical bars indicate the standard error of the mean (± SEM).

2.3.5.9 P contents and P uptake

The analysis of the effect of different phosphorus concentration administered to all three test genotypes yielded a high significance difference in the P contents in shoot tissues between treatments [$F(2, 27) = 47.2$, $p < 0.001$]. Overall, phosphate contents increased in all genotypes treated with higher levels: 0 P (0.4 ± 0.06 mg/g), half P (1.3 ± 0.08 mg/g) and full P (1.7 ± 0.13 mg/g). There were no significance difference found in genotypes or genotype and treatment interactions (**figure 2.22**).

However, analysis of P contents in the roots found that there were no significance difference for treatments but there were significant differences in the combined genotypes tested [$F(2, 27) = 9.2$, $p = 0.001$]. Overall, Kalinga III (0.8 ± 0.05 mg/g) had more P content across all three treatments compared to Ashoka 228 (0.7 ± 0.06 mg/g) while Ashoka 200F (0.5 ± 0.05 mg/g) had the lowest phosphate contents (mg/g) in the roots (**figure 2.23**). No significant difference was found in P content for treatments or genotype and treatment interactions for all three genotypes tested in all three treatments.

Meanwhile, P uptake in plant shoots were highly significant for treatments [$F (2, 27) = 53.8$, $p < 0.001$]. The combined genotype level of P uptake in 0 P (4.6 ± 0.66 mg/plant) was significantly lower compared to half P (14.7 ± 0.90 mg/plant) and full P (21.4 ± 1.60 mg/plant) (**figure 2.24**). While in root, there were significant difference in the P uptake (mg/plant) in root tissues for combined genotypes for each treatments applied [$F (2, 27) = 3.5$, $p = 0.046$]. The three genotypes absorbed more phosphorus at full P (5.0 ± 0.43 mg/plant) as compared to half P (4.1 ± 0.38 mg/plant) and 0P (3.7 ± 0.27 mg/plant). Similarly, there were no significant difference found in the genotypes tested or the genotype and treatment interactions in the experiment.

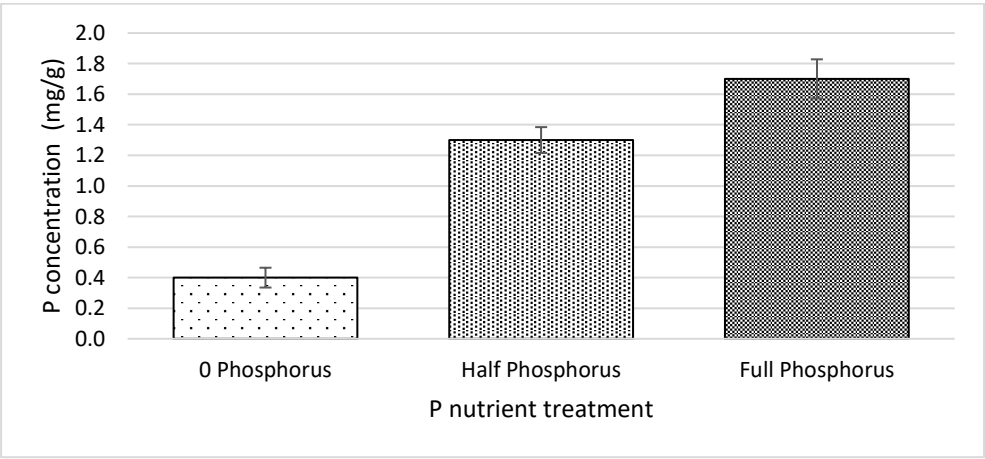


Figure 2.22 Effect of different P treatments on plant shoot P contents (mg/g) for combined genotypes at 120 days after sowing. Values represent means ($n = 12$). Vertical bars indicate the standard error of the mean (± SEM).

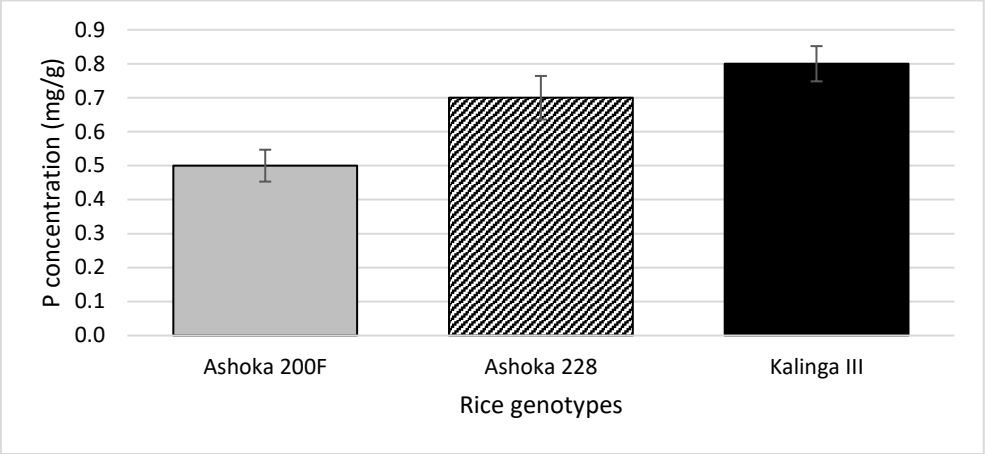


Figure 2.23 Plant root P contents (mg/g) for combined treatments at 120 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

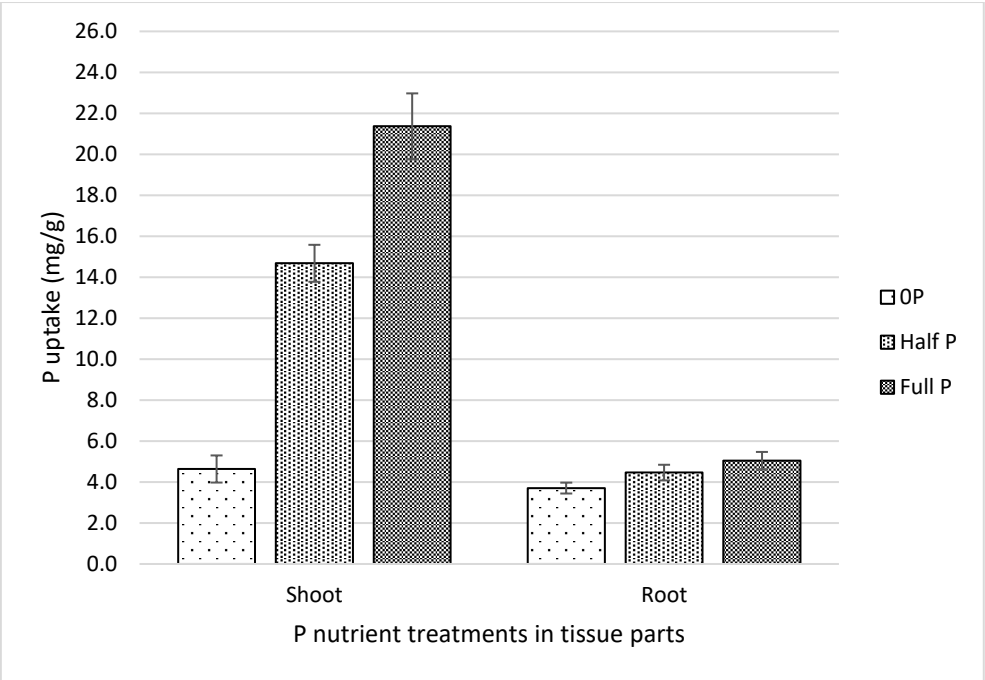


Figure 2.24 Effect of different P treatments on plant shoot and root P uptake (mg/g) combined across genotypes at 120 days after sowing. Values represent means (n = 12). Vertical bars indicate the standard error of the mean (± SEM).

2.3.6 Phosphorus utilisation efficiency

At 120 days ANOVA revealed a highly significant difference in tests of between subject effects of the plant genotypes across all treatments [$F(2, 27) = 22.56$, $p < 0.001$] and between treatments [$F(2, 27) = 36.78$, $p < 0.001$] for plant and grains phosphorus efficiency ratio. There

was a high significance in the interactions between genotype and treatment interactions [F (4, 27) = 6.96, p = 0.001]. In each treatment, P utilisation for the total plant biomass (shoots + roots) of each genotypes was highest at the lowest level of P (0) nutrient treatment with Ashoka 200F (35.1 ± 5.26) having higher P utilisation compared to Ashoka 228 (14.5 ± 2.17) in terms of mg dry matter/mg P absorbed. This pattern of genotype difference was also seen for half P and full P treatments where Ashoka 200F was significantly higher than the other two genotypes (**Table 2.8**).

For P utilisation measured in grains, a highly significant difference could only be seen between the genotypes tested [F (2, 27) = 13.93, $p < 0.001$] where Kalinga III had a higher P utilisation at half P (3.1 ± 0.22) than Ashoka 228, and full P (3.3 ± 0.11) than both Ashokas. There were no significant difference at 0 P treatment. Ashoka 228 had the lowest P utilisation of the three genotypes tested at half P. No significant difference was detected in all of the genotypes and treatments at harvesting day 45 and 75.

2.3.7 Phosphorus Use Efficiency (PUE)

A significant difference in the plant genotypes was detected [F (2, 27) = 5.86, $p < 0.05$] at 120 days for total plant biomass and grains phosphorus use efficiency (**Table 2.9)**. In each treatment, Ashoka 200F's total plant biomass (shoots and roots) comparatively had the highest PUE in terms of mg P absorbed at 0 P (661.8 ± 276.56 mg/g), half P (421.4 ± 44.96 mg/g) and full P treatments (725.2 ± 66.75 mg/g) compared to Ashoka 228 (302.1 ± 36.24 mg/g) with $p < 0.05$.

PUE for grains was also highly significant for the genotypes tested [F (2, 27) = 18.13, $p < 0.001$]. However, this was due to Kalinga III which had significantly higher PUE at half P (149.7 ± 16.08 mg/g) $p < 0.05$ and full P (162.9 ± 5.42 mg/g) with $p < 0.001$. Ashoka 228 had the lowest PUE in all of the treatments. No significance found for treatments or genotype and treatment interactions.

Table 2.8 Phosphorus utilisation in total plant biomass (shoot + root) and grains of Ashoka 200F, Ashoka 228 and Kalinga III in different P treatments at 45, 75 and 120 days after sowing at maturity.

	P uptake efficiency ratio (mg dry wt/ mg P absorbed)									
	No P			Half P			Full P			Remarks
Harvesting day	Ashoka 200F	Ashoka 228	Kalinga III	Ashoka 200F	Ashoka 228	Kalinga III	Ashoka 200F	Ashoka 228	Kalinga III	
45	1.4 ± 0.11[a]	1.3 ± 0.49[a]	0.9 ± 0.15[a]	0.8 ± 0.13[a]	1.2 ± 0.15[a]	1.2 ± 0.19[a]	1.4 ± 0.32[a]	1.1 ± 0.12[a]	1.0 ± 0.14[a]	ns
75	5.6 ± 1.31[a]	3.8 ± 0.06[a]	4.3 ± 0.84[a]	3.4 ± 0.31[a]	3.2 ± 0.25[a]	3.4 ± 0.16[a]	4.7 ± 0.49[a]	3.5 ± 0.48[a]	5.0 ± 1.10[a]	ns
120	35.1 ± 5.26[b]	14.5 ± 2.17[a]	15.6 ± 0.53[a]	12.6 ± 0.78[b]	8.5 ± 0.72[a]	8.2 ± 0.64[a]	12.2 ± 0.64[b]	7.1 ± 0.75[a]	8.9 ± 0.64[a]	**
Grains	2.5 ± 0.27[a]	2.2 ± 0.25[a]	2.6 ± 0.21[a]	2.5 ± 0.19[ab]	2.2 ± 0.09[a]	3.1 ± 0.22[b]	2.2 ± 0.07[a]	2.3 ± 0.15[a]	3.3 ± 0.11[b]	**

Values represent means (n=4). Means within the same harvesting day followed by different letters within same row are significantly different between genotypes within treatments ($p < 0.001$) by Tukeys HSD test.

Table 2.9 Phosphorus use efficiency (PUE) in plant biomass (shoot + root) of Ashoka 200F, Ashoka 228 and Kalinga III in different P treatments at 45, 75 and 120 days after sowing and in grains at maturity.

Phosphorus use efficiency (mg/g)										
	No P			Half P			Full P			
Harvesting day	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Remarks**
45	45.9 ± 6.18^{a}	37.8 ± 19.03^{a}	22.5 ± 4.38^{a}	25.8 ± 5.76^{a}	45.8 ± 11.39^{a}	49.0 ± 14.48^{a}	67.0 ± 22.31^{a}	49.4 ± 10.67^{a}	38.5 ± 7.94^{a}	ns
75	437.6 ± 157.04^{a}	203.9 ± 12.45^{a}	255.9 ± 72.29^{a}	268.1 ± 67.13^{a}	193.2 ± 19.62^{a}	231.3 ± 30.23^{a}	457.1 ± 105.35^{a}	309.7 ± 70.28^{a}	575.7 ± 232.06^{a}	ns
120	661.8 ± 276.56^{b}	262.0 ± 32.53^{a}	284.1 ± 24.12^{a}	421.4 ± 44.96^{b}	327.4 ± 69.57^{ab}	291.8 ± 31.83^{a}	725.2 ± 66.75^{b}	302.1 ± 36.24^{a}	503.5 ± 167.94^{ab}	*
Grains	122.6 ± 8.29^{a}	108.3 ± 7.94^{a}	124.9 ± 5.17^{a}	112.4 ± 7.35^{a}	101.7 ± 8.28^{a}	149.7 ± 16.08^{b}	122.3 ± 2.03^{a}	105.9 ± 6.16^{a}	162.9 ± 5.42^{b}	**

Values represent means (n=4). Means within the same harvesting day followed by different letters within same row are significantly different between genotypes within treatments; * ($p < 0.05$) and ** ($p < 0.001$) by Tukeys HSD test.

Table 2.10 Ashoka 200F, Ashoka 228 and Kalinga III SPAD readings over different growing stages and yield components at harvest (day 120)

Genotype	SPAD Reading			Total counted grain/plant	Single grain wt. (mg)	Grain yield (g/plant)
	Day 45 (Tillering stage)	Day 75 (Flowering stage)	Day 120 (Harvesting stage)			
Ashoka 200F	28.8 ± 0.68[a]	36.3 ± 0.36[a]	33.7 ± 0.78[c]	617.1 ± 10.90[b]	14.7 ± 0.87[a]	10.9 ± 0.18[a]
Ashoka 228	32.7 ± 0.75[b]	37.1 ± 0.47[a]	22.4 ± 1.02[a]	533.0 ± 29.24[a]	18.7 ± 0.77[b]	10.2 ± 0.20[a]
Kalinga III	30.2 ± 0.51[a]	39.1 ± 0.42[b]	29.8 ± 1.29[b]	557.4 ± 12.43[ab]	20.0 ± 0.18[b]	12.0 ± 0.29[b]

Values represent means (n=12) and ± standard error of the mean (± SEM). Means with different letters within same column are significantly different between genotypes within treatments ($p < 0.05$).

2.4 Discussions

Phosphorus is a crucial nutrient for plant growth development (Vance *et al*., 2003; Hopkins *et al*., 2008). The rice morphological characteristics can be affected when grown in low P soil. Data were collected at three different stages of the plant growth to ascertain if low phosphorus conditions had any effects on the growth variables of the Ashoka varieties compared to Kalinga III. In addition, the chosen upland rice genotypes were also used to assess growth response, specifically the P uptake and the efficiency of phosphorus utilisation, grains produced and biomass production in experiments simulating rice grown on nutrient deficient soil that is often found and drought prone environments. The main experiment does support the hypothesis that Ashoka 200F outperforms Kalinga III in tillering (day 75 and above), root length (day 45) and counted grain produced, but not for grain weight when grown under low P soil. The work undertook a comprehensive assessment of plant growth variables (**Appendix Table 2.4**, **Appendix Table 2.5**, **Appendix Table 2.6** and **Appendix 2.7**), achieving the first complete comparison of the two Ashoka rice varieties with Kalinga III in contrasting P deficient soils that has not been done in the previous literature.

2.4.1 Limitation of measurement

There are several factors to be considered in evaluating the measurements made in this experiment. The pilot study (2015) was of limited value because there were different numbers of plants in the same pot, which mean that some plants had access to more P than others did. While the main experiment (2016), the root length measurements during the destructive analysis at 45, 75 and 120 days after sowing date were limited by some amount of roots loss during cleaning and separating of soil from the roots, which stuck to the cloth mesh, placed at bottom of pot. The amount of roots recorded may be affected due to this loss during pot extraction and washing and may not represent the true length of each sample even though efforts to minimize root loss were taken as much as possible.

2.4.2 Influence of P concentrations on growth variables/characteristic

The variations in plant growth variables particularly for tillering between the pilot and main experiment was due to the number of plants planted per pot. The growth variables were only significant because of either pooled treatment across all genotypes or pooled genotypes across all treatments. There were no significant differences found between the genotypes within each different treatments in the pilot experiment. The main experiment supports the hypothesis

that Ashoka 200F outperforms Kalinga III in tillering, root length and grain number, but not for grain weight when grown under low P soil.

2.4.2.1 Plant height

According to Fageria *et al*. (1988), the rice plant shoots were found to be sensitive towards P nutrients in soil and their P can be used a criterion and indicator for plant health and PUE. Since P is crucial for plant initial growth, this means that plants grown from seed in P deficient soils will be expected to produce a shorter shoots regardless of growth stages (Alam *et al*., 2009). But in this chapter we did not find any statistical significant differences ($p < 0.05$) between the three treatments for either overall plant height or genotypes tested in both pilot and main experiment at vegetative stage (day 45). However, the height difference could be seen at harvesting stage (day 126) in the pilot experiment where total mean height of all three genotypes grew taller by 6.7 cm ($p < 0.05$) when treated with high P nutrient (105.0 ± 1.54 cm) as compared to low P (98.3 ± 1.56 cm). However, there were no statistical differences in mean heights between the treatments given (**Figure 2.4**).

Regardless of the non-significance difference between the treatments, the experiment did show that phosphorus fertilizer influences the height of plant genotypes. Prior to flowering stage (day 75), Ashoka 228 grew taller than Ashoka 200F and Kalinga III ($p < 0.05$). Ashoka 200F had the maximum height (108 ± 2.14 cm) and Ashoka 228 was shortest (91.7 ± 3.14 cm) in half P treatment ($p < 0.05$). This trend changed upon reaching harvesting stage (day 120), when total mean height of Ashoka 200F was taller than Ashoka 228 and Kalinga III ($p < 0.05$). Ashoka 200F was slower to grow in height before booting, but became taller after booting. The application of phosphorus does suggest that it increases the genotypes metabolism and growth (Syers *et al*., 2008).

2.4.2.2 Root length

The second criterion that is most observed in rice and sensitive to P concentration in soil is root length (Fageria *et al*., 1988). Fitter (1985) and Hill *et al*. (2006) stated that plant species adjust accordingly to low P availability by increasing root length and branching of roots per unit of root mass in order to better absorb nutrients in soil. The chapter results are in agreement by Kirk & Du (1997) study where it was concluded adventitious elongation and lateral root development were enhanced under low P conditions. The roots of all three

genotypes were longer under full P than half P, and longer under half P than 0 P at both vegetative and maturity stages.

Interestingly, Ashoka 200F roots (31.1 ± 1.36 cm) were highly significant as compared to Ashoka 228 (24.0 ± 0.54 cm) having the shortest root length in no P treatment at day 45 ($p < 0.05$). Ashoka 200F seems to interact differently to low P than the other genotypes. It has longer roots at 0 P and half P than at full P, the opposite to what was found in Ashoka 228 and Kalinga III. Similar significance difference were also detected at day 75 with Ashoka 200F (31.5 ± 0.98 cm) produced the longest root compared to Ashoka 228 (23.4 ± 0.94 cm) under half P treatment ($p < 0.05$) as shown in **figure 2.6**. This indicated that Ashoka 200F could have developed a more adaptive root system to suit its nutrient requirements under low P conditions. While at day 120, no significant difference found in between genotypes in each treatment detected but overall combined mean length does show a significant difference where all genotypes treated with full P treatment produced a longer root than those treated under no P ($p < 0.05$) (**Figure 2.15**). However, lack of significance in root length between genotypes within each treatments was clearly due to limitations mentioned earlier (**section 2.4.1**). This indicated that Ashoka 200F could have developed a more adaptive root system to suit it's nutrient requirements under low P conditions.

2.4.2.3 Tillering

The results also revealed that plant tillers were found to be significant in the main experiment at day 75 and day 120 between genotypes where Ashoka 200F bore 1.7 more tiller numbers per plant than Kalinga III ($p < 0.05$) (**Figure 2.5**) but not significant in between treatments. Similar results were also observed in the pilot experiment where the genotypes tested were not affected by different levels of P given. However, it was noted that the mean number of tillers produced from all three genotypes was slightly higher at low P than that of tiller numbers in high P. The findings is somewhat different from Alam *et al*. (2009) tested on three irrigated rice varieties (BRRI dhan 29, Aloron and Hira-2) and Rodriguez *et al*. (1999) in wheat, where they concluded that the rate of rice and wheat tillers emergence were correlated with concentration of P nutrients available. Therefore, the genotypes tested in our experiment may play a role in the non-significant result, where no such correlation was observed.

A higher tiller mean number of tillers in Ashoka 200F at day 45 onwards translates to having a potentially higher amount of panicles and rice grains per plant produced compared to Ashoka 228 and Kalinga III. This could indicate a higher phosphate use efficiency in Ashoka 200F and is one very useful criterion in examining the PUE of rice genotypes. A similar result

was found by Fageria *et al.* (1988) where all growth variables increased significantly with increasing levels of soil P in 25 upland rice genotypes tested. Our results add evidence to suggest that there are a highly significant differences between different genotypes since they responded differently to nutrient environments.

2.4.2.4 Grain weight and count

Pilot experiment showed that at harvesting stage (day 126) (**Table 2.7**), overall mean grain dry weight of Ashoka 200F (4.7 ± 0.58 g) produced the more weight compared to Ashoka 288 (2.9 ± 0.43 g) ($p < 0.05$). However, the total mean dry grain weight of all three genotypes when treated under 0 P (2.6 ± 0.43 g) were less than the total grain dry weight treated with full P (4.3 ± 0.56 g) ($p < 0.05$) (**Table 2.6**). This could be due to the fact that Ashoka 200F produced total more number grains per plant compared to other genotypes but all genotypes produced poorly under 0 P treatment compared to full P treatment ($p < 0.05$).

Similarly, observation for the main experiment (**Figure 2.16**) showed that there were no significant differences between all three treatments but genotype comparison revealed that Ashoka 200F (617.1 ± 10.90) significantly produced more grain count than Kalinga III (557.4 ± 12.43) and Ashoka 228 (533.0 ±29.24) ($p < 0.05$). However, Kalinga III (12.0 ± 0.29 g/plant) produced significantly heavier overall grain weight compared to Ashoka 200F (10.9 ± 0.18 g/plant) and Ashoka 228 (10.2 ± 0.20 g/plant). This was due to the fact that the Ashoka 200F (14.7 mg) and Ashoka 228 (14.7 mg) single grain weight was significantly lighter than that of Kalinga III (20.0 mg) ($p < 0.05$) as shown in **figure 2.19** and **figure 2.20**.

2.4.2.5 Shoot and root dry weights

The dry weights of shoot and root of the three genotypes did not show any significant effect when different concentration of P were given in the pilot experiment. Meanwhile, the main experiment showed that there were significant overall effects on all of the three rice genotypes tested with full P (9.4 ± 0.14 g/plant) produced a heavier combined shoot weight than in no P (8.7 ± 0.17 g/plant) ($p < 0.05$) at day 75. Similarly, this is also true for harvesting stage (day 120) as full P (12.6 ± 0.26 g/plant) accumulates a heavier shoot weight than no P (10.7 ± 0.18 g/plant) ($p < 0.05$). While post hoc comparison between the three genotypes showed that Ashoka 200F gained significant ($p < 0.05$), overall dry weights for shoot (12.2 ± 0.28 g/plant) and root (11.6 ± 1.78 g/plant) than Ashoka 228 and Kalinga III. However, the findings did not show any significant effect between treatment and genotype. This also

suggests that the trend of shoot and root tissue mass increases for all of the varieties tested with increasing P nutrient input given (Fageria and Gheyi, 1999; Fageria and Barbosa Filho, 2007).

It was also noted that Ashoka 200F grown under low P, had more root biomass (13.0 ± 4.93 g/plant) than shoot biomass (11.3 ± 0.21 g/plant) leading to a higher root-to-shoot ratio compared to Ashoka 228 and Kalinga III. The results are similar to Kim & Li (2016) study where the effect of phosphorus on shoot growth saw an increase in the number of leaves, which subsequently leads to a disproportionate increase in shoot biomass than root biomass regardless of the plant growth stage, which agrees with the results in this chapter.

2.4.2.6 SPAD readings

In order to assess the chlorophyll status and correlating it with the rice genotypes treated with different levels of P, measurements of leaf chlorophyll is an important variable as an indicator of the plant general health. Influence of different phosphorus concentrations treatment on three upland rice genotypes (Ashoka 200F, Ashoka 228 and Kalinga III) were measured against chlorophyll contents at different stages of rice development. The SPAD readings gives the best indicator of photosynthetic activity in rice the relationship between the chlorophyll contents and the P content available to the plant.

Chlorophyll (SPAD) meter readings and P treatment at day 45, day 75 and day 120 responded significantly between the genotypes tested but not between the treatments given. The results showed that during the early stages of active vegetation day 45, Ashoka 200F (28.8 ± 0.68) had a significantly ($p < 0.05$) lower SPAD value than Ashoka 228 (32.7 ± 0.75) and Kalinga III (30.2 ± 0.51). Meanwhile prior to panicle initiation at day 75, Kalinga III (39.1 ± 0.42) was shown to have a higher SPAD value than Ashoka 200F (36.3 ± 0.36) or Ashoka 228 (37.1 ± 0.47) with ($p < 0.05$). In both stages, Ashoka 200F had the lowest SPAD readings compared to the other two genotypes tested. However, this changes during the harvesting stage at day 120 where Ashoka 200F (33.7 ± 0.78) was significantly had a higher SPAD value than Kalinga III (29.8 ± 1.29) and Ashoka 228 (22.4 ± 1.02) (**Table 2.10**).

This finding is similar to Peng *et al*. (1999) where SPAD values were relatively small and only 1 to 2 units greater between no P treated and P treated plants. This chapter also proved that Phosphorus deficiency does not reduce the SPAD values as the chlorophyll contents were not affected by the leaf P contents, since treatment of P does not have any significant effect. This is because phosphorus is not a constituent of chlorophyll, which means the concentration of chlorophyll under low P deficient rice becomes high (Fageria *et al*., 2003). It does however show that chlorophyll contents increases significantly at day 75 as rice plants are more active

heading towards flowering stage. While at harvesting stage on day 120, chlorophyll contents in rice plants reduced compared to flowering stage (**Table 2.10)**. Even though Ashoka 200F produced a significantly higher number of grains per plant, it also produced lesser grain yield (g/plant) as compared to Kalinga III. This was due to reasons that Ashoka single seeds weigh lesser than Kalinga III. The findings were different from Virk *et al*. (2003) and unpublished study by Bangor University MSc. student Boon Fei Chin who found and concluded that Ashoka 228 is more robust genotype with a higher yielding seed count than Kalinga III. This trend was also similarly observed for pilot experiments. Although no correlation and multiple regression calculations were made, it may suggest that maintenance of optimum chlorophyll content could lead to higher yield as similar to Ramesh *et al*. (2002) findings where it demonstrated importance of chlorophyll content in determining rice yield.

2.4.3 Plant growth and phosphorus analysis of shoot and root

The three levels of P nutrient treatments on the rice genotypes investigated did not significantly influence the plant height and root length at day 45 and day 75. Although at harvesting day 120, Ashoka 200F and Kalinga III were significantly taller than Ashoka 228 ($p < 0.05$). The lack of response across all three treatments and genotypes differs from Fageria *et al*. (1988) where all 25 rice genotypes had a highly significant differences ($p = 0.01$) in the growth variables with increasing levels of soil P. A closer look at the individual components of P contents, P uptake, Phosphorus efficiency ratio and PUE is explained to why this could occur.

2.4.3.1 P content

The results of the pilot study (**Table 2.7**) indicated that genotypes were significant different at $p < 0.05$ where Kalinga III (7.5 ± 0.52 mg/g) had a 1.5 mg/g higher P contents in the shoot tissue than Ashoka varieties. This could suggest that the Ashoka varieties differ in their P requirements. However in the main experiment, genotypes did not show any significant difference at any stages of growth except at day 120 where Kalinga III (0.8 ± 0.05 mg/g) had significantly higher P content in the root tissue than Ashoka 228 (0.7 ± 0.06 mg/g) and Ashoka 200F (0.5 ± 0.05 mg/g). Cumulative mean P content in shoot tissue from all three genotypes were significantly higher in between treatments as expected in full P treatment (4.7 ± 0.12 mg/g) compared to half P (4.1 ± 0.11 mg/g) or no P (3.3 ± 0.13 mg/g) at day 45. The same trend was recorded on day 75 and day 120 (**Table 2.11**).

Table 2.11 Phosphorus contents (mg/g) in shoot over different growing stages

P conc. in shoot (mg.g^{-1})	**Treatments**			
Variable	**0 P**	**Half P**	**Full P**	**remarks**
Day 45	3.3 ± 0.13a	4.1 ± 0.11b	4.7 ± 0.12c	F>H>0
Day 75	2.6 ± 0.07a	3.0 ± 0.13b	3.5 ± 0.15c	F>H>0
Day 120	0.4 ± 0.06a	1.3 ± 0.08b	1.7 ± 0.13c	F>H>0

Values represent means (n = 12) and ± standard error of the mean (± SEM). Means with different letters within same row are significantly different between genotypes within treatments ($p < 0.05$).

Total mean P content of shoot in each treatment does agree with what Veneklaas *et al*. (2012) who reviewed studies reporting that rice (Rose *et al*., 2010), wheat (*Triticum aestivum*) (Rose *et al*., 2007), canola (*Brassica napus*) (Rose *et al*., 2008; Rose *et al*., 2010) and sunflower (*Heliantus annuus*) (Hockling and Steer, 1983) displayed similar patterns of P accumulation and reduction of P content over vegetative growth period. As plants near grain production and harvesting stage, much of the P absorbed is remobilized and redistributed into the grains as seed P reserve needed for early growth cycle especially where soil P availability is low.

2.4.3.2 Total plant P uptake

P uptake is defined as the total P in the tissue parts (shoot, root or grain) per unit of P applied to the soil (mg P/ plant) which means that it gives indication of the efficiency of the plant to absorb available P from soil. The mean value for P uptake was not found to be significant in between genotypes or treatments at day 45 and day 75 but was highly significant on day 120 for plant and grain as shown in **Table 2.12.** Ashoka 200F (35.1 ± 5.26) exhibited a highly significantly ($p < 0.01$) value of plant P uptake (shoot + root) under all three P treatments compared to other genotypes. In contrast, P uptake for grains seems to be not significant for no P treatment but highly significant ($p < 0.01$) in Kalinga III grains treated under both half P (3.1 ± 0.22) and high P (3.3 ± 0.11) compared to Ashoka series. This tells us that even though Ashoka 200F is highly efficient in absorbing phosphorus from soil under low P availability, it was not efficiently redistributed into the grains like Kalinga III and Ashoka 228 as much of the P is used to increase tiller numbers, plant biomass and grain count.

Table 2.12 Total P uptake for genotype plants (shoot + root) and grains at harvesting stage (day 120).

	P uptake (mg dry wt./ mg P absorbed)									Remarks
	No P			Half P			Full P			
	Ashoka 200F	Ashoka 228	Kalinga III	Ashoka 200F	Ashoka 228	Kalinga III	Ashoka 200F	Ashoka 228	Kalinga III	
Plant	35.1 ± 5.26^{b}	14.5 ± 2.17^{a}	15.6 ± 0.53^{a}	12.6 ± 0.78^{b}	8.5 ± 0.72^{a}	8.2 ± 0.64^{a}	12.2 ± 0.64^{b}	7.1 ± 0.75^{a}	8.9 ± 0.64^{a}	**
Grain	2.5 ± 0.27^{a}	2.2 ± 0.25^{a}	2.6 ± 0.21^{a}	2.5 ± 0.19^{ab}	2.2 ± 0.09^{a}	3.1 ± 0.22^{b}	2.2 ± 0.07^{a}	2.3 ± 0.15^{a}	3.3 ± 0.11^{b}	**

Values represent means (n = 4) and ± standard error of the mean (± SEM). Means with different letters within same row are significantly different between genotypes within treatments (p < 0.01).

2.4.3.3 Phosphorus Use Efficiency (PUE)

Table 2.13 P use efficiency for genotype plants and grains at harvesting stage (day 120).

	Phosphorus use efficiency (mg/plant)									Remarks
	No P			Half P			Full P			
	Ashoka 200F	Ashoka 228	Kalinga III	Ashoka 200F	Ashoka 228	Kalinga III	Ashoka 200F	Ashoka 228	Kalinga III	
Plant	661.8 ± 276.56^{b}	262.0 ± 32.53^{a}	284.1 ± 24.12^{a}	421.4 ± 44.96^{b}	327.4 ± 69.57^{ab}	291.8 ± 31.83^{a}	725.2 ± 66.75^{b}	302.1 ± 36.24^{a}	503.5 ± 167.94^{ab}	*
Grain	122.6 ± 8.29^{a}	108.3 ± 7.94^{a}	124.9 ± 5.17^{a}	112.4 ± 7.35^{a}	101.7 ± 8.28^{a}	149.7 ± 16.08^{b}	122.3 ± 2.03^{a}	105.9 ± 6.16^{a}	162.9 ± 5.42^{b}	* *

Values represent means (n = 4) and ± standard error of the mean (± SEM). Means with different letters within same row are significantly different between genotypes within treatments * (p< 0.05); ** (p < 0.01).

P use efficiency is the product of total plant P uptake whereby the amount of biomass produced (mg/plant) per unit of P applied to the soil (mg/plant) are measured (see Section 2.2.7). The mean value for PUE plant biomass at full P treatment for Ashoka 200F was significantly higher comparatively to Ashoka 228 and Kalinga III in all of the three treatments.

It is also can be seen that PUE in Ashoka 200F (725.5 ± 66.75) was found to be significantly higher compared to Ashoka 228 (302.1 ± 36.24) under high P treatment. Similarly, this can also be seen in 0 P and half P treatment. On the contrary, Kalinga III had significantly higher PUE for grains in half and full P treatments compared to both of the Ashokas

This indicated that for each mg of available P in the soil at high P treatment, the Ashoka 200F produced a mean of 122.3 mg of grain. A similar trend can also be seen for 0 P and half P treatments where Ashoka 200F produced a higher PUE value in plant biomass but a lower value in grains as compared to Kalinga III (**Table 2.13**). The findings are similar to what Sattelmacher *et al*., (1994) stated that genotypes differ in the nutrient efficiency and how it was utilized throughout its growth stages up to producing yield was due to genetic variation that resulted in effectiveness of P uptake efficiency.

2.5 Conclusion

In a phosphorus-limiting environment, plants produce less tillers, less grain yield, less biomass accumulation and conserve energy loss by limiting the surface area of leaf produced. Plants will also induces photo inhibition and damage to PSII that includes the alteration to the biochemical metabolic pathways (Chaudary *et al*., 2008) which in turn affects photosynthesis process and decreases the photosynthetic efficiency (Xu *et al*., 2007). The growth of C3 plants such as rice are more affected under low inorganic phosphorus (Pi) supply and photosynthetic rates are affected by the concentration of Pi in the leaf. This was reported by Foyer and Spencer (1986) and Rodriguez *et al*. (1998) where P deficiency reduces the photosynthetic efficiency of soyabean, barley, spinach plants as well as in wheat.

With the rising cost of P fertilizers, it is desirable to have a genotype that responds and produces higher grains in low P conditions. The aim of this study was to provide insights of morphological screening on selected upland rice genotypes that may show indications of having enhanced PUE traits. In general, full P treatment did give higher readings for plant variables such as the shoot biomass and P concentration accumulated compared to no P treatments during active vegetative stage. However, there was no evidence of an interaction between the genotypes and treatments given at day 45, day 75 or day 120. Results in this study also indicated that Ashoka 200F differs significantly from Ashoka 228 and Kalinga III for the majority of the variables tested and in their P requirements. Under 0 P treatment, there was no significant difference in the P contents and P uptake, but Ashoka 200F did accumulated higher biomass due to more tillers leading to more grains being produced than in Ashoka 228 or Kalinga III. In addition, P use efficiency (PUE) also pointed out that Ashoka 200F had a higher

efficiency value of absorbing phosphorus but was not efficient in redistributing the phosphorus back into the grains. However, Ashoka 200F did not have a higher grain yield by weight compared to Kalinga III.

Furthermore, results of preliminary glasshouse experiments gave similar outcomes and support confidence in the main experiment. The combined evidence gives some support to the hypothesis that Ashoka varieties (particularly Ashoka 200F) perhaps differs at the genetic level from Kalinga III in determining the phosphate uptake and use efficiency compared to Kalinga III. This could be related to root and PUE QTLs and this will be explored in the next chapter where the candidate genes for PUE and root QTL are tested for the gene expression during the early vegetative stage of rice development.

CHAPTER 3

3 Expression of candidate genes in rice roots for PUE grown in hydroponics

3.1 Introduction

In parts of Asian areas such of Eastern India, Nepal and Bangladesh, most resource-poor farmers have limited access to fertilizer and can only afford to apply N to their crops rather the more expensive P (or K). In addition, not having suitable high yielding rice varieties for cultivating rice crops in upland areas that are prone to prolonged droughts adds to the farmers' problems(O'Toole, 2004). Moreover, depletion of non-renewable rock phosphate and the current rising prices of phosphorus reserves (Ashley *et al*., 2011) are likely to turn phosphate fertilizers to become more of a limited commodity. Rising prices are making resource-poor farmers in developing countries to less likely apply adequate levels of P to crops, as phosphorus is not as abundant as N or K. Hence, renewed interest in producing rice with low water and P inputs are the major objectives for upland rice breeding programmes. This target and sustainable agriculture are more realistic in the long term as the world's rock phosphate resources could be exhausted towards the end of 21st century with its increasing rate of use (Vaccari, 2009 and Van Kauwenbergh, 2010).

Drought and lack of phosphate (P) are two of the main important abiotic stresses to agriculture and they both act via the roots of plants. Phosphate is the only form of phosphorus that can be taken up by roots. Rice does not respond well to limited water particularly if it lacks in P nutrient, and neither can it respond well to improve P uptake if water is not available. Root architectural trade offs have been observed when rice is grown under combined drought and low P stress (Ho, *et al*., 2005). Therefore, in order to improve yield stability, a synergistic relationship between traits that improve water and P uptake and use efficiency (PUE) must be the goal of rice improvement. An improved nutrient status will allow crops faster initial root growth so they develop deeper roots and that translates into improved nutrient and water capture. Ho *et al*. (2005) stated that to acquire P early is essential as it produces a better chance of survival in later drought season. While Collins *et al.* (2008) called for the genetic dissection of traits for P-use efficiency as an urgent priority.

3.1.2 Candidate genes for PUE

Phosphorus efficiency has been split into two components. The first is P acquisition efficiency (PAE) which relates to the capability of a plant to uptake available P from the soil.

This could vary significantly in different genotypes due to differences of root size or root architecture that improves its P bioavaibility. While the other component is P use efficiency (PUE), that measures the internal efficiency of the genotype acquiring and remobilizing P via the accumulation in either grain yield or its vegetative tissues (Wang *et al*., 2010).

Much is known about interactions with P deficiency for the rice QTL for phosphorus uptake 1 gene (*Pup1*) (Hueur *et al*., 2009; Chin *et al*., 2010; and Sarkar *et al*., 2011) which was identified in an *aus*-type genotype Kasalath through quantitative traits loci (QTL) mapping for P uptake and enhanced the PUE traits in rice from low-P soils. A population study from Kasalath x Nipponbare by Wissuwa *et al*, (2002) had successfully mapped the *Pup1* gene to rice chromosome 12 while a smaller effect QTL was identified on chromosome 6. Their findings also stated that low P tolerance in rice was largely due to genotypic differences in P uptake whereas internal P-use efficiency was negligible to the overall effect of low P tolerance. Meanwhile, Gamuyao *et al*., (2012) identified a locus that confers tolerance towards phosphorus deficiency in Asian and African rice (Pariasca-Tanaka, *et al*., 2014) located downstream of a *Pup1*-specific protein kinase gene in Kasalath, later named as phosphorus-starvation tolerance 1 (*Pstol1*). Overexpression of *Pstol1* significantly enhanced grain yield in phosphorus deficient soil as well as enhancing early root growth allowing plants to acquire phosphorus and other nutrients (Gamuyao *et al*., 2012). The assumption of one QTL, one gene and one function associating *Pup1* and *Pstol1* gene to root length may not be entirely correct and has not been proven, since QTLs could involve a group of genes influencing the up and down regulation of certain mechanisms due to the external abiotic responses. Loci associated with PUE in chromosomes 1 (root), 4 (shoot), 5 (root), 11 (total) and 12 (shoot) were also detected in *indica* rice by Wissuwa *et al*. (2015).

Another study (Huang *et al*., 2011) investigated the expression of candidate genes for low-affinity Phosphate Transporters in Barley (paralogues of HvPHT). That study found that there was no clear pattern in their expression among four barley genotypes, but the expression of two of these genes was correlated with the genotypes that had different rates of PUE as well as with the expression of HvIPS1 (P starvation inducible noncoding RNA). In their experiments high PUE was found to be associated with high root-shoot ratios in low-P treatments.

Virk *et al*. (2003) carried out a participatory plant breeding programme to improve Kalinga III for yield under upland conditions. It was crossed with with IR64, a modern high yielding genotype adopted for irrigated ecosystems. Two varieties (Ashoka 228 and Ashoka 200F) were selected that both gave significantly higher yields compared to Kalinga III when grown under P deficient soils by farmers in India. The Ashoka varieties were the first modern

upland rice varieties to be widely adopted in India (DFID, 2011). Extensive phenotyping in experimental conditions and in farmers' fields have established the superiority of Ashoka over Kalinga III and Vandana beyond doubt - the yield advantage of Ashoka is substantial (mean of 20% higher yield compared to local genotype, and 30% greater than Kalinga III) and stable across sites and years. Ashokas are more resistant to abiotic stresses than local varieties as they produce more grain under difficult conditions – on poor quality soils - without relying on fertilizer inputs and they have better quality grain. The intensive selection pressure during the breeding programme resulted in a high level of Kalinga III alleles in Ashoka 200F (Steele *et al*., 2004).

A preliminary microarray screen (Steele and Price, unpublished) to compare gene expression between Ashoka 228 and its parents detected six genes with two-fold differences in gene expression between Kalinga III and both Ashoka 228 and IR64. The microarray work was carried out by A.H. Price at Aberdeen University, following the same protocol as described by Norton *et al*., (2008) for an experiment examining the root growth of Azucena and Bala in arsonate, but without arsonate and the varieties used were Kalinga III, IR64 and Ashoka 228 grown in half strength Yoshida nutrient growth solution, including phosphorus. One of these was Os05g02310, a gene for inorganic pyrophosphatase (IPP) and it had a two-fold difference in expression between Ashoka 228 and Kalinga III. An experiment with the aim of confirming the expression differences found in the microarray experiments was conducted using three replicates of four plants of the genotypes Ashoka 228, Ashoka 200F, Kalinga III and IR64 which were grown for 7 days in mini-hydroponics with half strength Yoshida solution following the same method for mini-hydroponics as described here (pages 96- 100) and total RNA was extracted from the roots for replicated gene expression analysis against two control genes (Sk1 and Ubi). Subsequent qRT-PCR analysis (Steele, unpublished) confirmed two-fold higher expression of Os05g02310 in Ashoka 228 and also in Ashoka 200F compared with Kalinga III. Recent study by Blair *et al*. (2011) and Hernandez-Domiguez *et al*. (2012) has shown that the locus played a role for low P adaptations in root tissues of common beans (*Phaseolus vulgaris* L.). This provides supporting evidence that the gene for inorganic pyrophosphatase (IPP) acts to improve PUE and plays a role in the plant adaptation to phosphorus deficiency in legumes. The legume gene is homologous with Os05g02310. This evidence led to a hypothesis that Os05g02310 may be responsible for improved PUE in rice. Experiments in this chapter were designed to test this hypothesis by comparing expression of this gene for IPP in the upland rice varieties Ashoka 228, Ashoka 200F and Kalinga III when they were grown under three different phosphate treatments.

3.1.3 Candidate genes for Rice root growth

Numerous studies report QTLs that effect root architecture and P uptake in plants, including: Arabidopsis (Lopez-Arredondo *et al*., 2014), barley (Huang et al., 2011) common bean (Ho *et al*., 2005), canola (Yang *et al*., 2011; Shi, *et al*., 2013), maize (Mendes, *et al*., 2014) and wheat (Bolland and Brenan, 2008). In all of the crops studied, greater root surface is found to be directly associated with improved PUE as a result of root hair gaining mass either in length or density.

Studies by Uga *et al*. (2013; 2015) and Arai-Sanoh *et al*. (2014) involved Dro1-NIL a near isogenic rice line carries a QTL on chromosome 7 that enhances rice yield under drought conditions. They have demonstrated that the homozygous allele from Kindang Patong (deep rooting cultivar) in the IR64 (shallow rooting) genetic background has enhanced root system architecture. This discovery was achieved through cloning and characterization of the deeper rooting 1 gene (*DRO1*) from Kindang Patong that was associated with the control of root growth angle. The *DRO1* gene is negatively controlled by auxin and is responsible in the elongation of the root tip cells causing lower root angle growth. An overexpression of *DRO1* causes downward direction of root growth increasing the root length in the soil profile in additional response to gravitropism. It is a good example of the fine mapping of a root QTL in rice and identification of the underlying candidate gene.

Similarly, another QTL, qRT9, which is responsible for controlling root length and root thickness in upland rice was detected in hydroponic culture with a population derived from Yuefu and IL392 (Li *et al*., 2015). A qRT-PCR analysis showed that qRT9 was strongly expressed especially in IL392 in the presence of salt stress. A number of QTL mapping studies in the population derived from Azucena (deep rooting japonica) and Bala (indica) identified QTLs that increased root length, penetration and thickness (Price *et al*., 2000 and Price, Steele*et al*., 2002). These experiments screened the Bala x Azucena mapping population in two ways; Price *et al*., (2000) recorded the ability of the roots to penetrate a simulated hard layer of wax in a growth room and Price, Cairns *et al*., (2002) used a greenhouse where the plants were grown for six weeks in soil in glass-sided chambers so that root length could be measured weekly, and at the end of the experiment the whole root system could be removed and sectioned for dry weight analysis. A subsequent breeding programme, through a participatory varietal selection (PVS) used different lines to asses different four root QTLs (QTL2, QTL7, QTL 9 and QTL11) which also includes maturity, yield and grain shape that were pyramided via marker assisted selection into Kalinga III. These breeding programme efforts have resulted in

their successful introgression into line Pyramid 84 (PY 84) (Steele *et al*., 2007) which later released as Birsa Vikas Dhan 111 in Jharkhand, India in 2009 (Steele *et al*., 2013). All four QTLs in combination in PY 84 enhanced the yield output significantly under abiotic stress (Steele *et al*., 2013) and this is hypothesized to be through its deeper roots increasing water and nutrient acquisition from the soil. QTL9, on Chromosome 9, was found to be the most highly significant of the QTLs, increasing root length by 10 cm in NILs carrying QTL9 compared to control Kalinga III (Steele *et al*., 2007). It is acting under both drought and well-watered conditions and contributes to improved grain yield (Steele *et al*., 2013). The region was also introgressed in the Kalinga III background and found to be associated with improved root penetration (Clark *et al*., 2008). Analysis by Khowaja *et al*. (2009) indicated that there are three individual QTLs in the region related to root traits at the QTL9 region using the Bala x Azucena mapping population. Although the uppermost QTL region (15.97- 16.58 Mbp) corresponds to the QTL *Dro1* (Uga *et al*., 2011), it is not in the same region that was introgressed into PY 84 (18.49 - 21.04 Mbp). The gene underlying the root QTL9 that is acting in PY 84 has not yet been identified.

Previous collaborations between Bangor University and UAS Bangalore have developed a fine mapping population that segregates for Azucena's root QTL9 (used in PY 84) in the Kalinga III genetic background. This included lines having different alleles across the 15 cM region of QTL9. The fine mapping studies narrowed down the effective loci to be between RM242 (18.8 Mbp) and RM24579 (19.02 Mbp). There are 51 genes within the confidence interval. Metabolic network analysis (Mohanty *et al*., 2016) of gene expression values in root and leaves at different developmental stages of a drought-tolerant rice line-DK151 (*Indica* genotype) revealed that two (Os09g31310 and Os09g31430) out of the 51 genes could be candidate genes for longer roots. This is because they are related to drought resistance (Lakshmanan *et al*., 2013). The gene Os09g31310 is annotated as an acetyltransferase (source: http://pfam.xfam.org/family/PF00583/). The gene Os09g31430 is a gene for β-glucosidase. There are over 30 β-glucosidase genes in rice and this enzyme is known to be involved in the regulation of cytokinin biosynthesis in roots, the accumulation and relative ratio of auxin and cytokinin phytohormones highly influences the root morphogenesis (Morte *et al*., 2014). At tillering under drought conditions it is upregulated in DK151 roots and down regulated in shoots. This led to the hypothesis tested here that the β-glucosidase gene (Os09g31430) shows different levels of expression in PY 84 compared to Kalinga III and that there are differences in expression patterns in roots and shoots.

A previous qRT-PCR unpublished study by Bangor University MSc. student, Prabhu Manickam (2016) tested five primer pairs for LOC_Os09g31430 which all amplified in DNA of Kalinga III and Azucena and three primer pairs for Os09g31310, which did not amplify PCR products. He then tested the LOC_Os09g31430 primers in Kalinga III and Nipponbare roots and found it was upregulated in Kalinga III compared to Nipponbare. Hence, this study used the successful primer pairs to test the above hypothesis in PY 84 and Kalinga III roots and shoots grown under different P concentrations.

3.1.4 Two candidate genes

The two genes (**Figure 3.1** and **Figure 3.2**) were selected for expression analysis in this project because there is evidence that they are acting in the upland lines bred for Eastern India and they have not been studied for expression in contrasting P treatments. Further knowledge of the activity of these known genes that are good candidates for either P uptake or longer root growth QTLs will strengthen understanding of the genetic basis for abiotic stress avoidance/tolerance. They have previously been identified in rice cultivars adapted to problematic drought prone areas, and could be deployed in more breeding programmes targeting yield under stress.

3.1.4.1 Candidate gene for inorganic pyrophosphatase for PUE (LOC_Os05g02310)

The candidate gene for PUE (LOC_Os05g02310) was chosen to test the hypothesis that it might be responsible for different levels of phosphate use efficiency or uptake and show different levels of expression in Ashoka 200F and Ashoka 228 compared to Kalinga III. It is located on rice chromosome 5 at 739646-742966 (Figure 3.1).

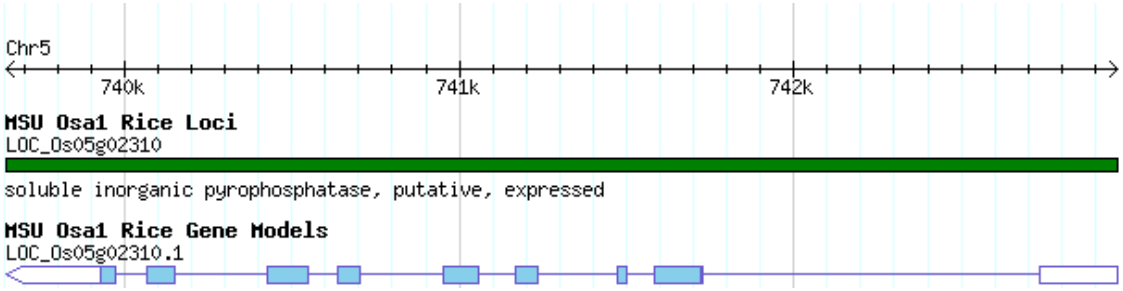


Figure 3.1 Location and predicted gene model of candidate inorganic pyrophosphatase gene Os05g02310. Image from Rice Genome Browser (http://rice.plantbiology.msu.edu).

3.1.4.2 Candidate gene for root length ß-Glucosidase (LOC_Os09g31430)

LOC OS09g31430 is a gene encoding ß-Glucosidase located on rice chromosome 9 at 18905662-18908766 (Figure 3.2). It is associated with physiologically important processes in plant especially response towards abiotic stresses and lignification and hydrolysis of cell wall oligosaccharides (Opassiri *et al.*, 2006). It is also probably in involved in the regulation cytokinin biosynthesis in roots. Here we test the hypothesis that this gene might be potentially responsible for deeper rooting phenotyping with different expression in PY 84 compared to Kalinga III.

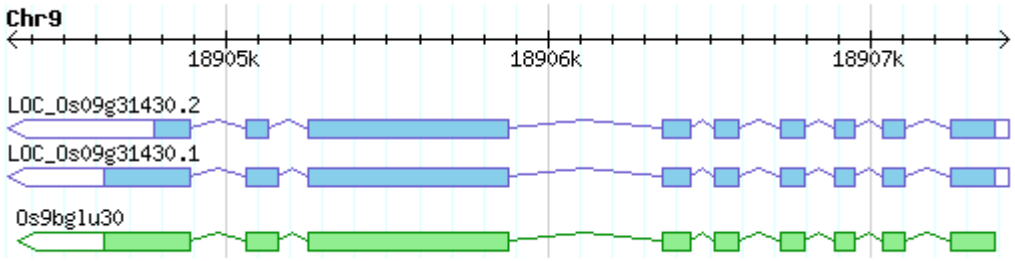


Figure 3.2 Location and two predicted splice variants (blue) of candidate root length gene Os09g31430. A rice paralogue is shown in blue. Image from Rice Genome Browser (http://rice.plantbiology.msu.edu).

3.1.5 Research questions

This chapter aims to study gene expression analysis in selected upland rice varieties of two candidate genes, one associated with Phosphate Use Efficiency (PUE) (LOC_Os05g02310) and one associated with roots at QTL9 (LOC_Os09g31430) (Lakshmanan *et al.*, 2013; Mohanty *et al.*, 2016). The work used plants at an early stage of growth, 7 days after seed germination, which were treated with different P nutrient concentrations. This set of experiments was designed to detect any significant relationship between the growth responses towards different levels of (P) and test for differences in gene expression of these candidate genes among selected upland rice varieties:

- Do Ashoka 228, Ashoka 200F and PY 84 have longer roots under low P nutrient condition than Kalinga III?
- Do Ashoka 228 and Ashoka 200F differ for expression of IPP (LOC_Os05g02310) compared to Kalinga III under different P concentrations?
- Does PY 84 differ for expression of ß-Glucosidase (LOC_Os09g31430) compared to Kalinga III under different P concentrations.

3.2 Materials and Methods

3.2.1 Preparation of Yoshida' Macro and Micronutrients stock solutions

The composition of nutrient solutions was adapted from Yoshida *et al*. (1976) and the component preparation of the stock solutions and the nutrient solution was used continuously for a period of 7 days experiment. Stocks were prepared fresh prior to carrying out the experiment. The macronutrients stock solutions were prepared by weighing in the required amount of reagents (**Table 3.1**) and transferred into respective 1 L Scott bottles, dissolved and topped-up to 100 ml with distilled water.

Preparation of micronutrients stocks used the micronutrients listed **Table 3.1** and they needed to be dissolved separately. 50 ml of distilled water were used to dissolve each reagent while 100 ml of distilled water needed to dissolve ferric chloride. All solutions are mixed and poured into 1 L Scott bottle. The ferric chloride solution was added to the mixture just before the citric acid and the solution was homogenized by stirring for 15 minutes with a magnetic stirrer. Finally, 50 ml of 1M sulfuric acid was added to the mixture and made up to 1 L and stirred for another 10 minutes. The final colour of the micronutrient solution was yellowish brown and it was stored in dark place.

3.2.2 Preparation and management of working nutrient solutions

Preparation of working nutrient solutions (**Table 3.1 and Table 3.2**) were given careful attention to make sure that the final nutrient solutions prepared were at pH 5.0, as any significant deviation (±1.0) will result in making some nutrients toxic and others deficient. Different concentrations for low P treatments (No phosphorus (0 P) and medium phosphorus) were prepared by not adding any sodium phosphate monobasic ($NaH_2PO_4.H_2O$) stock solution and adding only half the amount of the stock solution respectively for each treatment during the preparation of nutrient treatments prior to experiments.

3.2.3 Hydroponics system

An aerated hydroponic system (AHS) (**Figure 3.3**) was assembled from easily available laboratory components using a similar setup to modified hydroponics systems performed by Negi *et al*. (2016). This set up was used to run plant growth experiment for studying the effects of different levels of P on the responses root and shoot growth and candidate gene expression

of selected rice genotypes and classify their efficiency in utilizing P uptake necessary for growth.

Thirty 0.5 ml PCR tubes with the bottom portion cut out and stuffed with small amount of cotton wool were used to support seeds, and a Gilson 200 µl tip trays containing a styrofoam (length x width x height = 12 cm x 8 cm x 0.7 cm) with 30 punched holes (5 x 6) were used to hold in the PCR tubes on top of the Gilson pipette trays. Each Gilson pipette tray supported a rectangular plastic tray 13.5 x 8.5 x 6.5 cm in size and with 600 mL capacity as (**Figure 3.3)**. The hydroponics systems were fitted with a Tetra APS 50 aquarium pump (power 2W and airflow 50 l/h), flexible airline tubing (3mm diameter), a tee connector and an aquarium aerator stone in order to aerate and homogenise the nutrient solution treatments throughout the experimental period.

3.2.4 Experimental design

The design is arranged in a 7 x 4 factorial (varieties x replication) in a randomized block design with three treatments in each tray container. The nutrient treatments consisted of Yoshida nutrient solutions (**Table 3.1** and **3.2**) with different Phosphorus strengths (0 P, ½ P, and full P). The full strength P nutrient treatment was prepared as described earlier. Each of the nutrient solutions was topped up to 1L and the pH adjusted to 5.0.

3.2.5 Planting material, and seed pre-germination

Six rice genotypes were selected for phosphorus use efficiency traits or length QTLs from previous Bangor University studies (Steele *et al*., 2006 and Virk *et al*., 2003) and MRQ 76 was from the Malaysian Agricultural Research and Development Institute (MARDI). The two NILs selected at the BC_3F_3 generation were derived from the same cross as PY 84 but they each contained only one of the root QTLs (Steele *et al*., 2006) (**Table 3.3**). The seeds are surface sterilized by first soaking in distilled water for 5 minutes and then submerged in 1% sodium hypochlorite (Sauer and Burroghs, 1986) for 10-20 minutes with constant stirring or aeration. Seeds were then rinsed 4 times with distilled and dried using a paper towel. The sterilized seeds were then placed equidistantly in a plastic container lined with filter papers and wetted with sterilized distilled water and germinated at 25°C for 3 d in the dark.

Table 3.1 Preparation of macro and micronutrients hydroponic stock solution.

Elements	Reagents	Preparation (g/100ml solution)
Macronutrient		
N	Ammonium nitrate (NH_4NO_3)	9.14
P	Sodium phosphate, monobasic ($NaH_2PO_4.H_2O$)	3.56
K	Potassium sulfate (K_2SO_4)	7.14
Ca	Calcium chloride, dehydrate ($CaCl_2.2H_2O$)	11.73
Mg	Magnesium sulfate, 7-hydrate ($MgSO_4.7H_2O$)	32.4
Micronutrient	**Dissolve each reagent separately and mix in 1L Scott bottle then add 50ml H_2SO_4 and make up volume to 1L**	**Preparation (g/1L solution)**
Mn	Manganous chloride, 4-hydrate ($MnCl_3.4H_2O$)	1.5
Mo	Ammonium molybdate, 4-hydrate [$(NH_4)6Mo_7O_{24}.4H_2O$]	0.074
Zn	Zinc sulfate, 7-hydrate ($ZnSO_4.7H_2O$)	0.035
B	Boric acid (H_3BO_3)	0.934
Cu	Cupric sulfate, 5-hydrate ($CuSO_4.5H_2O$)	0.031
Fe	Ferric chloride, 6-hydrate ($FeCl_3.6H_2O$)	7.70
	Citric acid, monohydrate ($C_6H_8O_7.H_2O$)	11.90
	1 M Sulphuric Acid (H_2SO_4)	50 ml

Table 3.2 Element composition of nutrient solution.

Elements	Reagents	mL of stock solution/ 1L nutrient solution			Concentration of element in nutrient solution (ppm)
		Full P	½ P	0 P	
Macronutrient					
N	NH_4NO_3	1.25	1.25	1.25	40
P	$NaH_2PO_4.H_2O$	1.25	0.625	0	10/5/0
K	K_2SO_4	1.25	1.25	1.25	40
Ca	$CaCl_2.2H_2O$	1.25	1.25	1.25	40
Mg	$MgSO_4.7H_2O$	1.25	1.25	1.25	40
Micronutrient					
Mn	$MnCl_2.4H_2O$	1.25	1.25	1.25	0.50
Mo	$(NH_4)6Mo_7O_{24}.4H_2O$	1.25	1.25	1.25	0.05
Zn	$ZnSO_4.7H_2O$	1.25	1.25	1.25	0.01
B	H_3BO_3	1.25	1.25	1.25	0.20
Cu	$CuSO_4.5H_2O$	1.25	1.25	1.25	0.01
Fe	$FeCl_3.6H_2O$	1.25	1.25	1.25	2.00

Source: Adapted from Yoshida *et al*. (1976)

Table 3.3 Rice cultivars (*Oryza sativa*) used for gene expressions of phosphorus effects on root phenotypes

Genotype	**Name country of release**	**Target environment**	**Candidate gene or region hypothesized to be acting**
Ashoka 228	India	Upland	LOC_Os05g02310
Ashoka 200F	India	Upland	LOC_Os05g02310
PY 84 (QTL2, QTL7, QTL9, QTL11)	India	Upland	LOC_Os09g31430
Kalinga III	India	Upland	-
MRQ 76	Malaysia	Irrigated (aromatic)	-
NIL (QTL7)	-	Upland	-
NIL (QTL9)	-	Upland	LOC_Os09g31430

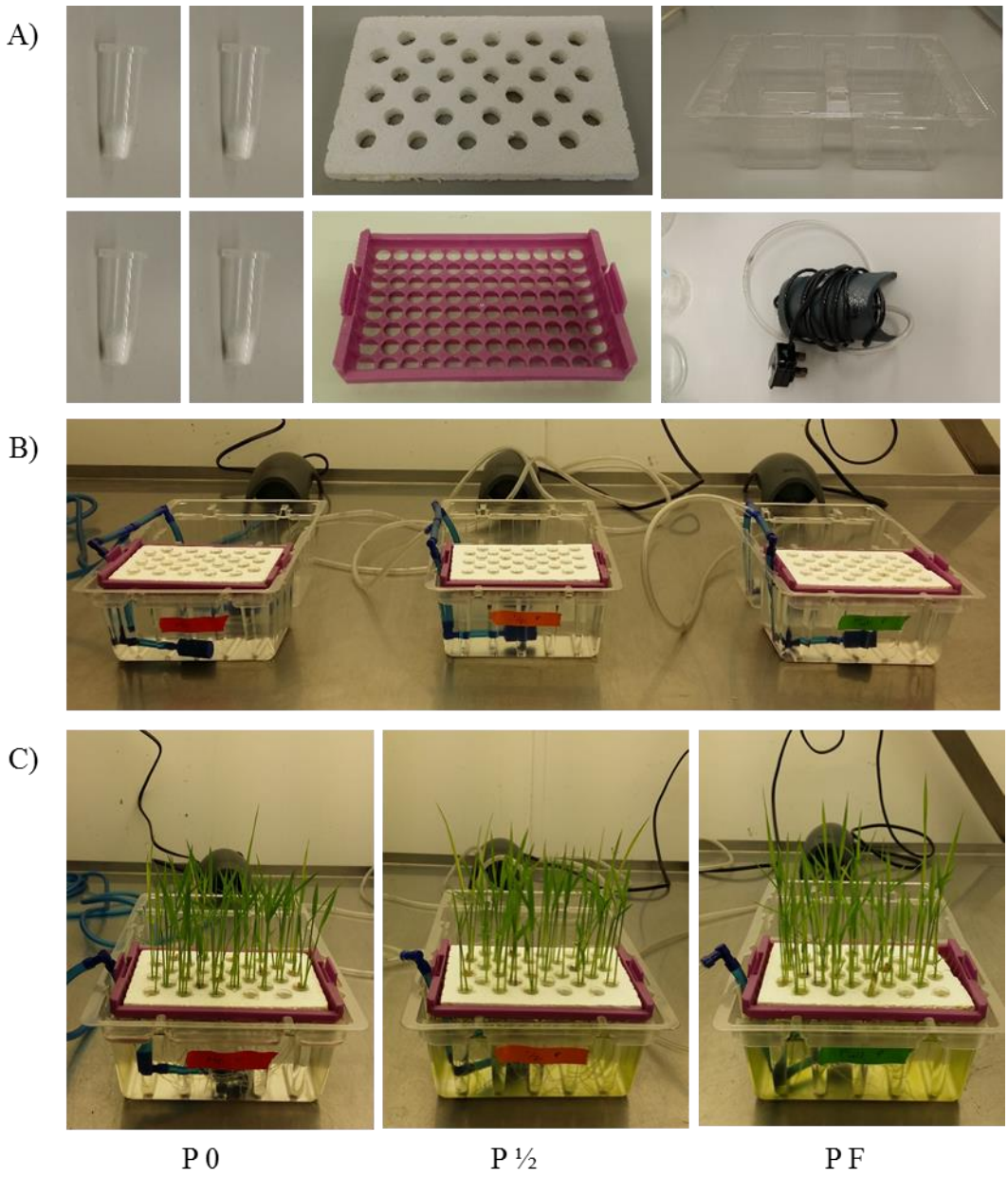


Figure 3.3 Aerated hydroponics system (AHS) set up. (A) 0.5 ml PCR tubes, cotton as support, 1cm thick polystyrene, Gilson pipette tips holder, plastic tank container and aquarium pump (B) AHS set up with seedling at day 0 (C) Seedlings were grown in AHS with Yoshida's nutrient (0 P, ½ P and Full P) conditions at day 7.

3.2.6 Rice hydroponic growth conditions

After pre-germination, the seedlings were transferred into a modified hydroponic modified aerated hydroponic system (AHS) and treatments began. The seven rice genotypes were grown by placing three pre-germinated seeds per tube on a Styrofoam float secured on Gilson pipette tray (**Figure 3.3**). The reason three seeds/genotype/replication/treatment were

used was to make sure the amount of roots and shoots produced were ample enough for the downstream process (total RNA extraction and qRT-PCR). The trays containing seeds were suspended by placing in a container filled with 600 ml of Yoshida's nutrient solution treatments. The experiment was carried out in a controlled environmental growth room situated in Thoday Building, SENRGy, Bangor University.

The growth conditions were set to 26°C ± 2°C; 16-h day/8- h night cycle period for 7 days and a relative humidity was maintained at ~ 60-70%. Due to the temperature and humidity condition of the hydroponics, there was a small loss of nutrient solution volume. Therefore, the container was completely replenished/replaced every two to three days a week to the level of touching the Gilson trays and monitored daily similar to experiments by Yang *et al*. (1994) and Junior *et al*. (2010). At day 7, all of the samples were removed from the AHS system and the roots were washed and cleaned using distilled water and dried using laboratory tissue paper. The samples were laid flat on a dry surface and lengths of the shoot and the longest root were measured using a ruler and recorded (**Appendix 3.1**). Each genotype shoot and root parts were then separated and snap frozen in liquid nitrogen and stored in -70°C until further use.

3.2.7 MIQE guidelines

Important steps to ensure scientific accuracy must be followed when carrying out quantitative analysis of RNA by ($\Delta\Delta C_T$) qRT-PCR. These are specific designs for experimental procedures, the control group to be tested against tested sample, good quality RNA, a suitable number of replicates, suitable reference genes used, controlled experimental conditions and accurate sample handling. During and post qRT-PCR it was important to use housekeeping genes as the internal control to verify the efficiency of the internal control and target gene amplification in subsequent statistical analysis of the data. For this study, except for a few practical limitations, efforts were made to follow closely to the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin *et al*., 2009).

3.2.8 RNA extraction

Total RNA was extracted from frozen samples (see **section 3.2.6**), which were processed in batches to avoid RNA degradation as in **section 3.2.10**.

3.2.9 End point PCR for IPP gene (LOC_Os05g02310) and ß-glucosidase (LOC_Os09g31430)

The three primer pairs were tested using conventional PCR before proceeding to qRT-PCR. The PCR amplification was carried as described in **Table 3.4** by using Kalinga III extracted DNA as the template. One pair of primers for IPP and two primer pairs for ß-glucosidase were designed from the sequences of the genes from the Nipponbare reference genome (**Table 3.5**). Total volume 20 µl PCR reaction was prepared using 1 µl of template DNA (50ng), 10 µl of BioMix PCR reaction mix (Bioline, GmbH, Germany) and 1µl (20pM) of each primer in 0.2ml 8-strip PCR tubes. The PCR was carried out on PTC-100 Programmable Thermo Controller (MJ Research INC., USA) with PCR conditions were followed initial denaturation at 94°C for 2 mins, followed by 35 cycles of 94°C for 30 sec, 57°C for 30 sec and 72 °C for 1 min, with a final extension at 72°C for 5 min. The PCR products were ran on a 3% agarose gel at 90V for 60 minutes alongside Bioline easy ladder I.

Table 3.4 Concentration of the components used for PCR Reaction

Component	Volume/reaction	Final concentration
BioMix PCR reaction mix (2x)	10	1 x
Forward Primer (20pM)	1	1 pM
Reverse Primer (20pM)	1	1 pM
ddH_20	7	
Template DNA	1	50ng/reaction
Total reaction volume	20	

3.2.10 RNA extraction and quantification

A clean bench surface and tools (e.g. spatula, mortar and pestle) were prepared prior to the experiment by spraying with RNaseZap® RNase Decontamination Solution (Thermo Fisher Scientific), and all experiment was rinsed with DEPC treated deionized water. 100 mg samples from the leaves and root tissues (7 days) of the hydroponics experiment were ground into a fine powder in a mortar and pestle using liquid nitrogen. The required amount of ground fine powder were scraped with a spatula into 1.5 ml Eppendorf tubes that had been chilled in liquid nitrogen. The total RNA extraction was performed using ZR Plant RNA MiniPrep™ kit (Zymo Research: Cambridge Bioscience, UK) and treated with DNase to remove trace amount

of DNA, purified per manufacturer's instructions and stored at -70°C. Total RNA yield and quantity were later determined by using Qubit® RNA BR Assay kit (Life Technologies) and Qubit® 2.0 Fluorometer (Invitrogen) according to manufacturer's instruction.

3.2.11 DNase treatment of total RNA

The RNA samples were treated using DNase 1 (Promega) according to manufacturer's instruction. The DNase digestion reaction were set up by adding 1 µl of RQ1 RNase-free DNase 10x reaction buffer, 1 µl of RQ1 RNase-Free DNase (1U/µg RNA), 1 µg of total RNA and topped up with nuclease-free water to a final volume of 10 µl. The digestion reactions were then incubated at 37°C for 30 minutes followed by the addition of 1 µl of RQ1 DNase Stop solution to terminate the reaction. Samples were then incubated at 65°C for 10 minutes to inactivate the DNase 1 enzyme.

3.2.12 Pooled RNA

1 µl of total RNA from four replications of each samples was aliquoted. The RNA was precipitated overnight with 2.5 x volume of 100% ethanol at -20°C. The RNA was pelleted, washed with 70% cold ethanol, dried briefly under vacuum and suspended in DEPC-treated deionized water or TE buffer. The pooled total RNA are then used to check the integrity structure via RNA gel analysis.

3.2.13 Agarose RNA Gel Analysis

Total shoot and root RNA quality and integrity extracted were assessed by running on a formaldehyde-agarose (FA) denaturing gel electrophoresis with 1 µg RNA from each sample along with transcript RNA markers 0.28-6.6 kb (Sigma-Aldrich). All samples and markers mixed with RNA Sample loading buffer (Sigma-Aldrich) were heated at 65°C for 5 mins and immediately cooled on ice before loaded onto gels. Electrophoresis was performed on a horizontal 1.2% (w/v) 1 x MOPS buffer (Fisher Scientific) and gel stained with safe view nucleic acid stain (NBS Biologicals) that was cast (approximately 5mm thick) in a 15 x 15 cm gel tank (Labnet International, Inc.). The electrophoresis ran at 90 V for approximately 2 hours. The gel was later visualized and photographed with a High-Performance 2UV™ Transilluminator (Ultra-Violet Products Ltd., Cambridge, Cambridgeshire) and imaging software.

3.2.14 Reference genes and target genes

In order to have a specific gene expression analysis of quantitative reverse transcription PCR (qRT-PCR), nucleotide sequences that are specific towards the candidate genes (IPP) (LOC_Os05g02310) and QTL 9 (LOC_Os09g31430) (Lakshmanan *et al*., 2013) are used. Housekeeping genes: Actin, ß-Tubulin, Cyclophilin, EF1-α, GADPH and ubiquitin (Omar *et al.*, 2016 and Pabuayon *et al.*, 2016) acts as an endogenous controls to normalize gene expression differences in sample quantity. The housekeeping gene primers have been reported to be reliable due to their robustness in analyzing gene expression analysis across different tissues types, developmental stages, and genotypes in rice for drought tolerance. These targets were amplified with qRT-PCR from treated total RNA extraction using the oligonucleotides primers as described in **Table 3.5**. All primers were synthesized by Eurofins (Eurofins Genomics, Ebensburg, Germany).

Table 3.5 Information regarding six selected reference housekeeping genes (control) and two candidate genes. Soluble inorganic pyrophosphatase 1 is related to Phosphate Use Efficiency (PUE), and two sequences were tested in the ß-glucosidase which is the candidate gene for QTL9 root related.

Type	Gene name	Gene symbol	MSU ID	Forward primer (5'-3')	Reverse Primer (5'-3')	Amplicon size (bp)	Reference
Control	Ubiquitin conjugating enzyme	Ubiq	LOC_Os03g13170	GTATCATCGAGCCGTCGCTTC	CATAGCATTTGCGGCAGATCA	76	Pabuayon *et al.* (2016)
Control	Actin	Act	LOC_Os11g06390	AGTGTCTGGATAGGAGGGTCCA	TGCTACCTCATAAAGTGTAGGCGT		Pabuayon *et al*.(2016)
Control	ß-Tubulin chain	ß-Tub	AK072502	GCTGACCACACCTAGCTTTGG	AGGGAACCTTAGGCAGCATGT	82	Bevitori e*t al.* (2014)
Control	Elongation factor 1-α	EF1- α	MGG_03641 (XM_003716200)	CATCTTAACGTCGTCGTCATC	AGTGGCCGGTAGTCGTGG	62	Omar *et al.* (2016)
Control	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	LOC_Os08g03290	AATGGCAAGCTTACGGGAATGT	TGAGGCAGCCTTCTCGATTCTA		Pabuayon *et al.* (2016)
Control	Cyclophilin	Cyc	LOC_Os02g02890	GTGGTGTTAGTCTTTTTATGAGTTCGT	ACCAAACCATGGGCGATCT		Pabuayon *et al.* (2016)
Candidate[1]	Inorganic pyrophosphatase 1	IPP	LOC_Os05g02310	AAGCCGTGGAGAAGAAGACA	TGCCCCTAGGAATCTCAATG	615	Steele & Price, unpublished microarray screen[1]
Candidate[1]	ß-glucosidase	Os9bglu30	LOC_Os09g31430_10	TTTCCGGTTCTCCATTGCGT	CTCCGCGAAGTCCACGTATT	903	Lakshmanan *et al*. (2013)
Candidate[1]	ß-glucosidase	Os9bglu30	LOC_Os09g31430_41	GGGCTCCTACGACTTCATCG	ATGAGGCCGAACCTGTCAAG	705	Lakshmanan *et al*. (2013)

[1] The primers for all three candidate target were designed by K. Steele from *Japonica* Reference genome (NCBI database) using free online Primer Designer tools.

3.2.15 Establishing efficiency and standard curve

Quantitative Real time PCR based on QuantiFast® SYBR® Green RT-PCR one-step kit (Qiagen) assay was carried out using control gene primers in QuantStudio™ 6 Flex Real-Time PCR System (Thermo Scientific, Europe) system. Even though absolute quantitative qRT-PCR via $\Delta\Delta C_T$ does not require a standard curve, it was carried out for this exercise in order to optimize the assay and make sure the efficiency of the assay was optimum. A known Kalinga III pooled total RNA sample (40 ng/µl) was used as a starting template material. The actin housekeeping gene primer was used as one of the internal control for later qRT-PCR assay, it was diluted 1:2 fold from 20 pM to 2.5 pM. All the reactions were carried out using QuantiFast® SYBR® Green RT-PCR one-step kit (Qiagen) per manufacturer's instructions. The diluted samples were added to the qRTPCR mix and prepared in triplicates and aliquoted into MicroAmp® Fast 96 well reaction plate (Life Technologies Ltd, Netherlands). The assays ran using QuantStudio™ 6 Flex Real-Time PCR System (Thermo Scientific, Europe).

The qRT-PCR cycle number was plotted versus change in ΔRn which represents the difference between normalised reporter signal (Rn) and baseline signal established in the first 23 cycles. Rn is obtained by dividing the fluorescence signal of reporter ROX dye by the SYBR Green I fluorescence signal of the passive dye included in the 2x QuantiFast SYBR Green RT-PCR Master Mix Qiagen buffer. The ΔRn increases during the PCR cycles as actin amplicon increases until the reaction reaches a plateau and negative template control (NTC) sample is indicated (green line) below the threshold line. The standard curve plot is presented as a log of gene copy number versus C_T (threshold cycle). While the C_T represents PCR cycle numbers at which significant fluorescence signal was first detected during the exponential phase of the PCR amplification itself. The amount of copy numbers amplified is based on the amount of the starting material, i.e input template or primers concentration. The efficiency of the qRT-PCR assay can be determined according to the amount of gene copy number produced with the serial dilutions to establish a linear standard curve by calculating the slope curve obtained:

$$\text{qRT-PCR efficiency (E)} = 10^{(-1/\text{slope})} \times 100$$

This also tells the efficiency and consistency of the assays performed across replicates. A good qRT-PCR assay run with a slope between -3.1 and -3.6 and with a reaction efficiency of 90-105% is typically considered high efficiency.

3.2.16 Quantitative Real-Time PCR (qRT-PCR)

Each target gene along with 6 reference genes (**Table 3.5**) and no template control (NTC) was analyzed by qRT-PCR. The qRT-PCR amplification reactions were performed and

run in MicroAmp® Fast 96 well reaction plate (Life Technologies Ltd, Netherlands) using a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Scientific, Europe). The experiment was setup using QuantiFast® SYBR® Green RT-PCR one-step kit (Qiagen) per manufacturer's instructions (**Table 3.6**). Each RT-PCR reaction mix consisted of 12.5 µl of QuantiFast® SYBR® Green RT-PCR Master Mix (2x), 0.25 µl of QuantiFast® RT Mix, 1 µl of forward and reverse primers (20 pM), and 9.25 µl of RNase free water in a total volume of 24 µl. Finally, 1 µl of approximately 50ng total RNA was added, resulting in a total volume of 25 µl per RT-PCR reaction. In addition, negative template controls (NTCs) for each target sample were also included by performing reactions containing no RNA and were replaced with 1 µl of RNase-free water. To reduce pipetting errors, a master mix was prepared before aliquoting into respective MicroAmp® Fast wells.

All ΔΔCT qRT-PCR plate amplifications followed the thermal cycler steps: reverse transcription at 50°C for 10 min followed by PCR initial activation at 95°C for 5 min, followed by 95°C for 10 sec, repeating this step for 45 cycles and a final extension at 60°C for 1 min. The Fluorescence of the SYBR Green I dye was calibrated against ROX. At the end of the reactions, a dissociation curve analysis was performed by the following profile: 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. By performing the melting curve analysis step, the RT-PCR products specificity and identity can be verified. The results are reported as the fold change in gene expression of the target gene in test samples relative to control samples and were determined by $2\text{-}\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Table 3.6 QuantiFast® SYBR® Green qRT-PCR reaction setup

Component	Volume/reaction (96-well block)	Final concentration
2x QuantiFast SYBR Green RT-PCR Master Mix	12.5 µl	1 x
Forward Primer	1 µl	0.8 pM
Reverse Primer	1 µl	0.8 pM
QuantiFast RT Mix	0.25 µl	
Template RNA	1 µl	50ng/reaction
RNase-free water	9.25 µl	
Total reaction volume	25 µl	

3.2.17 Normalisation, validation of reaction and relative quantification

Normalization of the targeted gene C_T to the reference (housekeeping genes) C_T's was assessed for their expression stability by calculating the ΔC_T of both targeted gene needed to the ΔC_T of the reference genes. The expression ratio or the fold difference was determined as follows:

Sample	Gene	
	C_T IPP (Target)	C_T GADPH (Reference)
Control (Calibrator)	26.89	27.13
Kalinga III (Test)	25.41	24.10

Mean ΔC_T (calibrator) = C_T (target, cal.) - C_T (reference, cal.)
= ΔC_T (control) = 26.892 - 27.132 = **- 0.24**

Mean ΔC_T (test) = C_T (target, test) - C_T (reference, test)
= ΔC_T (A228) = 25.407- 24.104= **1.303**

Next, the ΔC_T of the test sample needs to be normalized to the ΔC_T of the calibrator by:

$\Delta\Delta C_T$ = ΔC_T (test) − ΔC_T (calibrator)
= 1.303 – (-0.24) = **1.543**

The value obtained represents the change in the expression of the gene of interest between the targeted gene and the control (housekeeping gene) conditions normalized for any differences in loading between the reference and the test samples. The obtained value of $\Delta\Delta C_T$, the fold difference in gene expression level between the two genes can be calculated:

$$\mathbf{RE = 2^{-\Delta\Delta C\ T}} = \text{Normalised expression ratio}$$
$$2^{-(1.543)} = \mathbf{0.34}$$

This indicated that the IPP gene expressed at a 0.34 fold higher level than the GADPH control gene.

The expression levels of the genes tested were compared in both shoot and root tissues and the fold change calculated is a representation of a log 10 value. Any value lower than 1.00 is considered downregulated relative to the control (i.e. 1.5 =150%, 2 = 200%, 10 = 1,000%) and any value greater than 1.00 is considered upregulated relative to the control. While 1.00 value represents no change in expression.

The samples across all treatments ran in three replications and the C_T mean, ΔC_T mean, ΔC_T SE and $\Delta\Delta C_T$ standard deviations were calculated using the Livak method/$\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) in order to quantitatively determine the gene expressions level relative to the endogenous controls genes. The RQ value showed the change in fold of gene expression in relation to Kalinga III after being normalized to six endogenous controls: Actin, ß-Tubulin, Cyclophillin, GAPDH, EF1-α and Ubiquitin. The analysis were then plotted on a linear graph.

3.2.18 Analysis of Relative Quantification ($\Delta\Delta C_T$) qRT-PCR results

For quantifications of gene expression, the threshold cycle values (C_T) for each gene transcript obtained from qRT-PCR experiments were imported into the QuantStudio™ Real-Time PCR Software v.12 (Applied Biosystems; Thermo Fisher). C_T values are the point whereby the fluorescence signal detected rises appropriately above the background fluorescence. The qPCR machine determined this value along with the baseline and threshold signal of fluorescence. In addition, ΔC_T and $\Delta\Delta C_T$ values are used to calculate the fold or change in gene expression levels normalised against the references genes can be defined as the relative quantity (RQ) of a target gene in a treatment sample relative to a control sample.

3.2.19 Statistical Analysis

Statistical analyses were performed with SPSS statistics software package version 22.0 (IBM Corporation). Gene expression data were analyzed by a univariate generalized linear model (GLM) with a Tukey's HSD post-hoc test to assess for significance differences effect of different levels of Phosphorus nutrient treatments on genotypes shoot and root growth. A probability level of $p < 0.05$ with the confidence interval at 95% was considered statistically significant. All of the values analysed were expressed as means ± the standard error of the mean (SEM).

Relative expression values of candidate genes of tested samples in relation to control sample was calculated using the $2^{-\Delta\Delta CT}$ method normalised to six housekeeping genes (**Table 3.5**) as internal control. The data are presented in as mean ± SEM and fold change in percentage. All statistical calculations were performed by Quantstudio6 Flex software.

Test between subjects effect of univariate analysis of variance (ANOVA) was conducted to test the hypothesis that there would be one or more mean difference between P treatments and genotypes. A post-hoc analyses (Tukey's HSD) were performed to examine individual pairwise mean difference comparisons across all three levels of P concentration treatments for the combined results of seven genotypes for shoot height and root length.

3.3 Results and Analysis

3.3.1 Effect of different P treatments on plant shoot and root growth

Shoot height and root length of seven rice genotypes at 7 days grown in hydroponics condition with different P concentrations are presented in **Appendix 3.1** and analysis of overall shoot height and root length **Table 3.7**. ANOVA for overall treatments revealed that P concentration had no significant effect on overall shoot height [$F(2, 63) = 0.17$, $p = 0.84$, ns] but it had a significant effect on overall root length [$F(2, 63) = 19.52$, $p < 0.001$]. Mean cumulative root length from all genotypes under half P (8.31 ± 0.38 cm) were 2.36 cm longer than those treated under full P (5.95 ± 0.41 cm) (**figure 3.4)**. There were no significant effects for treatment by genotype interactions.

3.3.2 Comparison of genotypes on root length and shoot height

A statistically significant difference was observed when comparing between overall genotypes for shoot height [$F(6, 77) = 2.62$, $p < 0.05$] and overall root length [$F(6, 77) = 4.27$,

$p < 0.01$]. There was no significant interaction between treatment and genotypes for shoot height and root length (**Table 3.8)**.

Shoot height under no P treatment showed that PY 84 (10.13 ± 0.77 cm) was significantly taller compared to MRQ 76 (6.88 ± 0.31 cm) by 3.25 cm ($p < 0.001$) (**figure 3.5**). There were no significant difference between genotypes or genotypes by treatment interaction for height under half P or full P. However, under combined shoot length of genotypes from all treatments showed that NIL (BC3F3) (10.22 ± 0.35 cm) was significantly taller than MRQ 76 (7.83 ± 0.42 cm) by 2.39 cm.

Comparatively, genotypes grown under 0 P treatment shown to have a significant effect on root length between the all of the genotypes tested where MRQ 76 (10.75 ± 0.32 cm) was longer compared with all the others. Ashoka 200F (9.53 ± 0.29 cm) and NIL (QTL7) (7.85 ± 0.43 cm) fairs reasonably in length while Kalinga III (7.70 ± 0.56 cm), NIL (QTL9) (7.30 ± 0.24 cm), PY 84 (7.13 ± 0.39 cm) had the shorter root length but Ashoka 228 (6.88 ± 0.24 cm) had the shortest root length (**figure 3.6**). Even under full P treatment, MRQ 76 (9.0 ± 0.41 cm) shown to have a longer root compared to Kalinga III (4.2 ± 0.49 cm) when treated at full P ($p < 0.05$). A combined mean root length of all genotypes treatments also proved that MRQ (9.83 ± 0.31 cm) was longer than Ashoka 228 (6.31 ± 0.66 cm) by 3.52 cm ($p <0.01$).

Table 3.7 Mean ± standard error of total shoot height and root length of seven rice genotypes at 7 days grown in hydroponics with different P concentrations.

	0 Phosphorus	½ Phosphorus	Full Phosphorus	Mean of shoot treatments
Shoot height (cm)	8.97 ± 0.23^{a}	9.25 ± 0.42^{a}	9.15 ± 0.39^{a}	9.12 ± 0.20
Root length (cm)	8.16 ± 0.28^{b}	8.31 ± 0.38^{b}	5.95 ± 0.41^{a}	7.47 ± 0.24

Means ($n = 28$) within the same row followed by different letters are significantly different between treatments ($p < 0.05$).

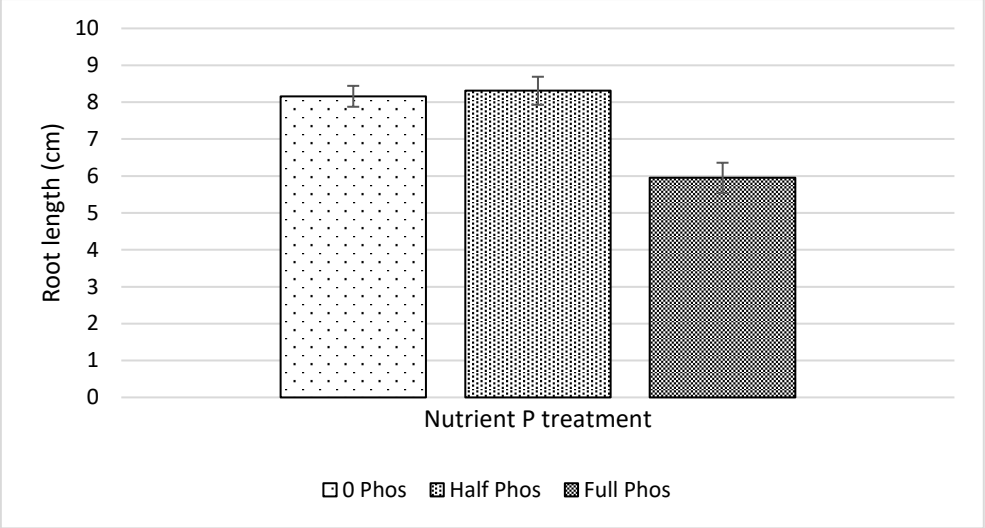


Figure 3.4 Root length of combined rice genotypes grown in hydroponic conditions with three different P treatments. Bars shown are based on observed means for genotypes in each treatment (n = 28). Vertical bars indicate the standard error of the mean (± SEM).

Table 3.8 Mean of shoot height and root length of seven rice genotypes at 7 days grown in hydroponics condition with different P concentrations.

	Shoot height (cm)				**Root length (cm)**			
Genotype	0 Phosphorus	½ Phosphorus	Full Phosphorus	Mean of shoot treatments	0 Phosphorus	½ Phosphorus	Full Phosphorus	Mean of root treatments
Ashoka 200F	8.63 ± 0.17^{b}	8.40 ± 0.45^{a}	8.45 ± 0.60^{a}	8.49 ± 0.23^{ab}	9.53 ± 0.29^{bc}	9.33 ± 1.19^{a}	5.38 ± 1.69^{ab}	8.08 ± 0.85^{ab}
Ashoka 228	9.95 ± 0.21^{b}	8.63 ± 2.88^{a}	10.38 ± 1.23^{a}	9.65 ± 0.97^{ab}	6.88 ± 0.24^{a}	5.98 ± 2.00^{a}	6.08 ± 0.75^{ab}	6.31 ± 0.66^{a}
Kalinga III	8.75 ± 0.25^{b}	9.15 ± 0.53^{a}	8.20 ± 1.36^{a}	8.70 ± 0.46^{ab}	7.70 ± 0.56^{a}	8.75 ± 0.26^{a}	4.18 ± 0.49^{a}	6.88 ± 0.64^{a}
MRQ 76	6.88 ± 0.31^{a}	8.58 ± 0.73^{a}	8.05 ± 0.92^{a}	7.83 ± 0.42^{a}	10.75 ± 0.32^{c}	9.73 ± 0.52^{a}	9.03 ± 0.41^{b}	9.83 ± 0.31^{b}
PY 84	10.13 ± 0.77^{b}	9.28 ± 0.41^{a}	8.43 ± 0.79^{a}	9.28 ± 0.41^{ab}	7.13 ± 0.39^{a}	7.43 ± 0.22^{a}	5.73 ± 1.18^{ab}	6.76 ± 0.44^{a}
NIL (QTL 7)	9.45 ± 0.10^{b}	10.15 ± 0.25^{a}	11.05 ± 0.93^{a}	10.22 ± 0.35^{b}	7.85 ± 0.43^{ab}	8.68 ± 0.18^{a}	5.35 ± 0.47^{ab}	7.29 ± 0.47^{a}
NIL (QTL 9)	9.00 ± 0.20^{b}	10.55 ± 0.42^{a}	9.53 ± 0.84^{a}	9.69 ± 0.35^{ab}	7.30 ± 0.24^{a}	8.33 ± 0.30^{a}	5.90 ± 0.94^{ab}	7.18 ± 0.43^{a}
Genotype (G)	**	ns	ns	*	**	ns	ns	**

*, ** and ns; Significance at the $p < 0.05$, $p < 0.01$ and non-significant. Values represent as means ($n = 4$) and ± standard error of the mean (±SEM). Means with different letters within the same treatment column are significantly different ($p < 0.05$) according to Tukey's HSD.

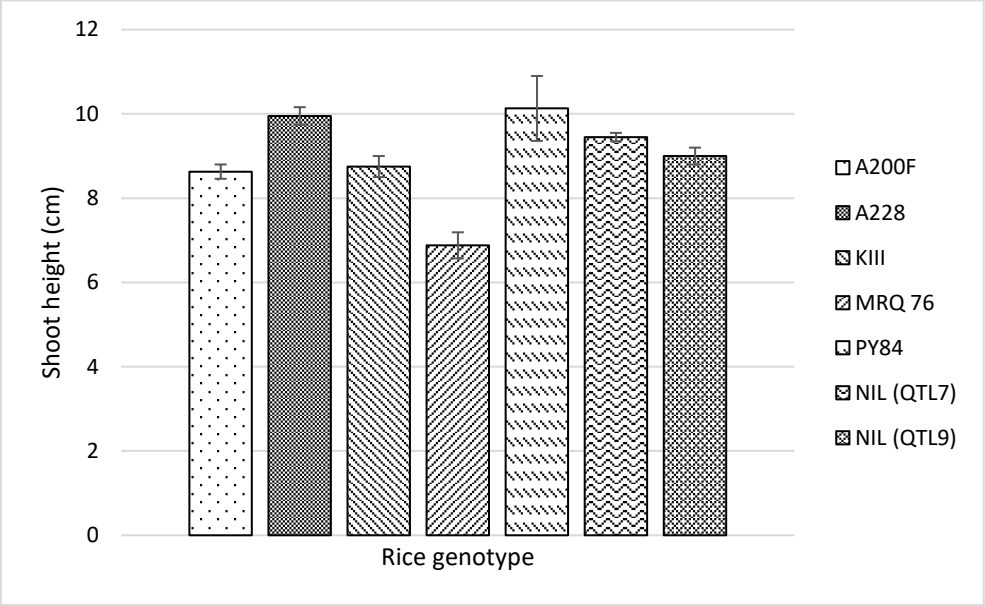


Figure 3.5 Shoot height of seven rice genotypes grown in hydroponic conditions at 0 P treatment. Bars shown are based on observed means for varieties in each treatment (*n* = 4). Vertical bars indicate the standard error of the mean (± SEM).

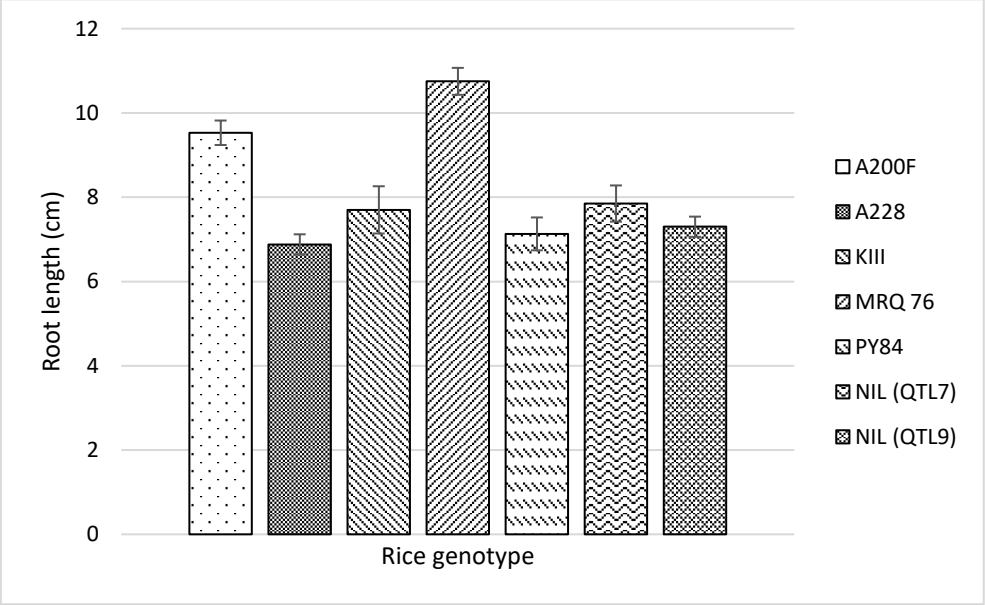


Figure 3.6 Root length of seven rice genotypes grown in hydroponic conditions at 0 P treatment. Bars shown are based on observed means for varieties in each treatment (*n* = 4). Vertical bars indicate the standard error of the mean (± SEM).

3.3.4 RNA integrity

3.3.4.1 Genotype

The pooled total RNA of each of the genotypes that were treated with three different P treatments were ran on 1.2% formaldehyde agarose (FA) gel (**Figure 3.7**)**.** Comparing the gel image to the analysed total RNA quantified with Qubit® RNA BR Assay kit and Qubit® 2.0 Fluorometer, the ANOVA analysis showed that there were a significant difference in the overall genotype total RNA in shoot [F (6, 63) = 2.72, $p < 0.05$] and root [F (6, 63) = 2.47, $p < 0.05$]. There were no significant differences in the interaction between genotype and treatment for RNA yield in the shoot and root tissues.

At full P, there was a slight significance in genotypes for the shoot total RNA of NIL (QTL9) (959 ± 77.03 ng/µl) and Kalinga III (541.5 ± 159.47 ng/µl) $p = 0.05$. No other significance were found for all the genotypes total RNA contents in 0 P and half P treatments. While the analysis for total RNA for root tissues in 0 P showed there are near a significance between genotypes [F (6, 21) = 2.58, $p = 0.049$]. Post hoc analysis between NIL (QTL9) (293.0 ± 5.80 ng/µl) and Kalinga III (141.0 ± 47.22 ng/µl) was only $p = 0.081$) (**Table 3.9**).

RNA extracted from 0 P treatment for MRQ 76 (174.1 ± 42.65 ng/µl) (**Figure 3.7**; gel A; lane 16) and full P treatment for Kalinga III (114 ± 50.61 ng/µl) (**Figure 3.7**; gel C; lane 10) root tissue were particularly low in yield when compared to all other genotypes in the respective treatments (**Table 3.10**). Due to the varying yield of total RNA from each genotypes and replication in all three treatments, adjustments were made for downstream process by diluting all samples to 50ng/µl of total RNA as a template in qRT-PCR reactions as samples with low yield are limited.

3.3.4.2 Treatment

Based on **Figure 3.7**, the mean total RNA extracted from all seven genotypes from shoot (781.9 ± 25.65 ng/µl) versus root (253.0 ± 15.17 ng/µl) tissue are vastly different in the terms of the amount of yield obtained giving a better resolution on the gels. ANOVA analysis in between treatments effect for all seven genotypes in shoot tissues are not significant but found that there was a significant difference in the mean amount of root total RNA between full P (197.2 ± 18.39 ng/µl) and half P (349.9 ± 31.81 ng/µl) treatment ($p < 0.001$) (**Table 3.10**).

Table 3.9 Qubit® RNA BR Assay kit (Life Technologies) and Qubit® 2.0 Fluorometer (Invitrogen) determination of total RNA (ng/µl) extracted from shoot and root tissue parts of seven rice genotypes treated in three different phosphorus nutrient concentrarions.

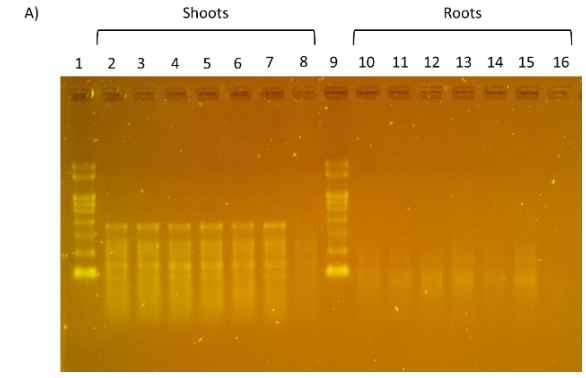
	Shoot [RNA] (ng/µl)			**Root [RNA]] (ng/µl)**		
	0 Phosphorus	**½ Phosphorus**	**Full Phosphorus**	**0 Phosphorus**	**½ Phosphorus**	**Full Phosphorus**
Ashoka 200F	795.5 ± 58.88[a]	597.0 ± 127.38[a]	797.5 ± 31.61[a]	236.1 ± 36.54[a]	202.5 ± 84.77[a]	165.6 ± 66.47[a]
Ashoka 228	684.5 ± 86.48[a]	618.0 ± 228.78[a]	699.5 ± 62.59[a]	157.2 ± 38.14[a]	234.5 ± 109.43[a]	189.4 ± 37.41[a]
Kalinga III	573.5 ± 156.75[a]	981.0 ± 55.02[a]	541.5 ± 159.47[a]	141.0 ± 47.22[a]	454.5 ± 51.14[a]	114.0 ± 50.61[a]
MRQ 76	620.0 ± 162.80[a]	843.5 ± 92.15[a]	940.0 ± 88.23[a]	174.1 ± 42.65[a]	462.5 ± 70.28[a]	254.0 ± 44.25[a]
NIL (QTL 7)	692.5 ± 63.40[a]	752.0 ± 59.98[a]	832.5 ± 68.17[a]	214.7 ± 36.56[a]	347.5 ± 77.26[a]	172.1 ± 43.91[a]
NIL (QTL 9)	833.5 ± 33.33[a]	1092.5 ± 151.84[a]	959.0 ± 77.03[a]	293.0 ±5.80[a]	431.7 ± 83.95[a]	282.8 ± 46.92[a]
PY 84	861.5 ± 65.03[a]	798.5 ± 19.91[a]	905.5 ± 95.67[a]	268.0 ± 26.50[a]	316.0 ± 43.01[a]	202.5 ± 21.22[a]
Genotype (G)	ns	ns	ns	ns	ns	ns

Values represent means ($n = 4$). Means within the same treatment column followed by different letters are significantly different between genotypes within treatments *($p < 0.05$) by Tukey's HSD test

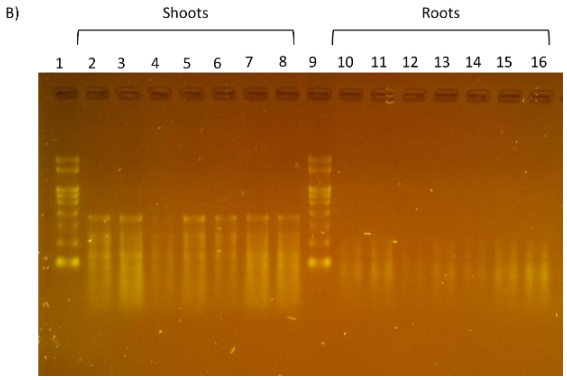
Table 3.10 Mean total RNA (ng/µl) of seven rice genotypes in shoot and root tissue parts treated in three different phosphorus nutrient concentrarions.

	Shoot [RNA] (ng/µl)				Root [RNA]] (ng/µl)			
	0 Phosphorus	**½ Phosphorus**	**Full Phosphorus**	**Mean of shoot treatments**	**0 Phosphorus**	**½ Phosphorus**	**Full Phosphorus**	**Mean of root treatments**
Mean of all 7 genotypes	723.0 ± 38.91[a]	811.8 ± 52.32[a]	810.0 ± 40.29[a]	781.9 ± 25.65	212.0 ± 15.69[a]	349.9 ± 31.81[b]	197.2 ± 18.39[a]	253.0 ± 15.17

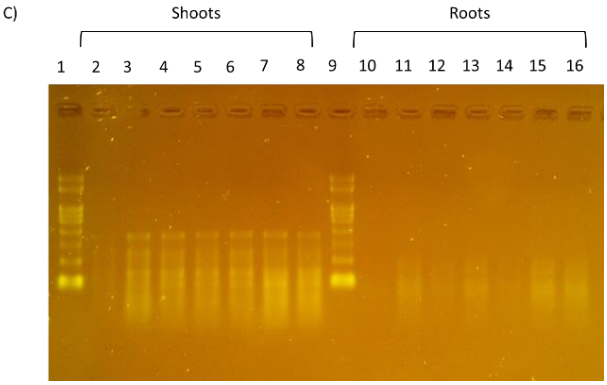
Values represent means (n = 28). Means within the same tissue sample row followed by different letters are significantly different ($p < 0.001$) by Tukey's HSD test



0 Phosphorus



Half Phosphorus



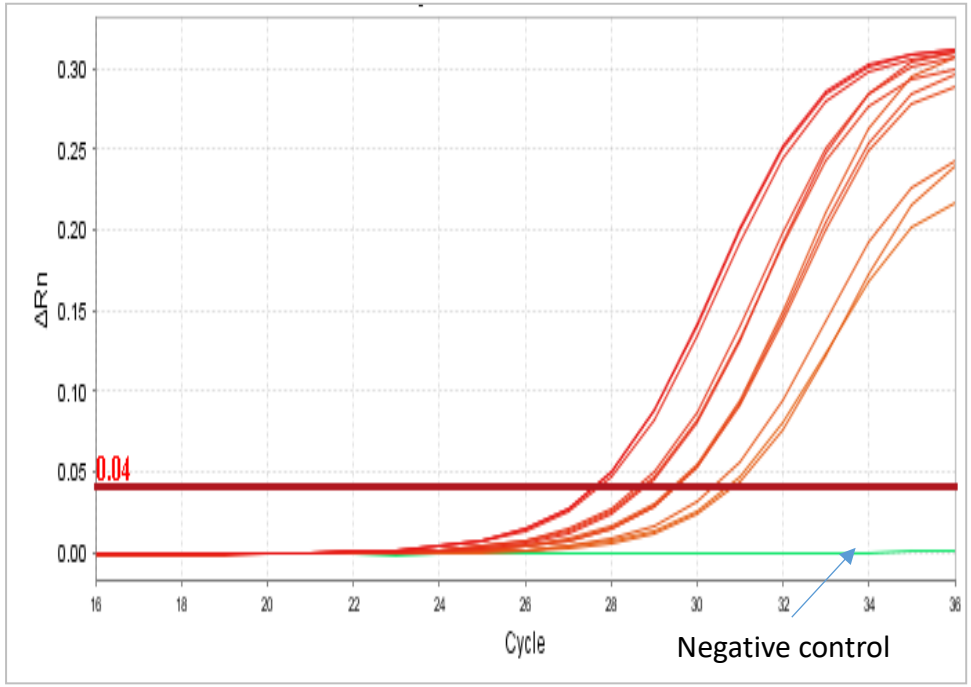
Full Phosphorus

Figure 3.7 Electrophoretic analysis of pooled total RNA extracted from shoot and root of seven rice genotypes treated in Yoshida's nutrient solution A) 0 Phosphorus; B) Half Phosphorus and C) Full Phosphorus, performed on 1.2% Formaldehyde-agarose (FA) denaturing gel electrophoresis (90 V/120 mins). Lane: 1) transcript RNA markers 0.28-6.6 kb (Sigma-Aldrich); 2) Kalinga III; 3) Ashoka 228; 4) Ashoka 200F; 5) PY 84; 6) NIL (QTL 7); 7) NIL (QTL 9); 8) MRQ 76; 9) transcript RNA markers 0.28-6.6 kb (Sigma-Aldrich); 10) Kalinga III; 11) Ashoka 228; 12) Ashoka 200F; 13) PY 84; 14) NIL (QTL7); 15) NIL (QTL 9); 16) MRQ 76.

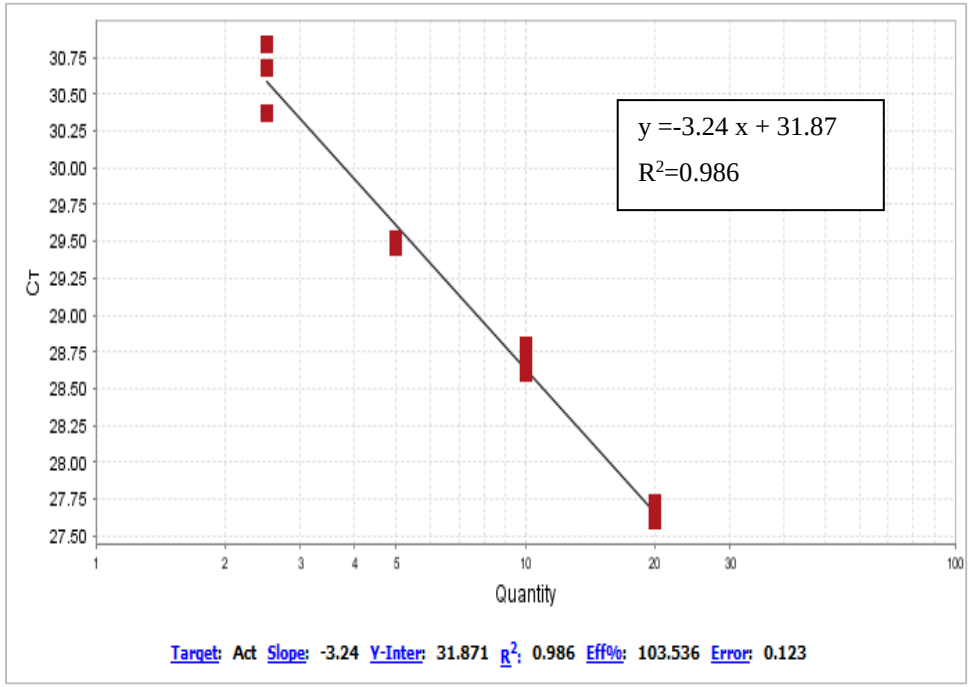
3.3.5 Standard curve and amplification efficiency

To assay the accuracy of the quantification system, a standard curve by C_T value against several serial dilution of primers with the amplification of Kalinga III total RNA (40ng/ul) with serially diluted (½ fold) actin primers were amplified in triplicates. **Figure 3.8 a)** shows a sigmoid shaped amplification graph of Actin gene with ΔRn is plotted against PCR cycle number. The C_T had a threshold line of 0.04 which signifies where the intersection between an amplification curve of actin and a threshold line (red) and a relative measure of the concentration of target in the qRT-PCR assay. The graph plots the relative concentration of actin in PCR cycle with decreasing amount of template. No amplification was detected for the no template control (NTC) that ran simultaneously with the Actin dilution assay (indicated by arrow).

The efficiency of the qRT-PCR reaction was determined by a standard curve (**Figure 3.8 b**). The standard curves for serial dilution Actin, each ranging from $1 \times 10^{2.5}$ to 1×10^{20} copies/µl. The curve were linear in the range tested ($R^2 = 0.986$) by the triplicate reactions. The slopes of the standard curves for actin were -3.24, and Y-intercept of 31.871, which is considered as acceptable. From the slope value, a high amplification efficiency of 103.5% was determined for actin and that the investigated minimal range for a primer concentration of 2.5 pM had no problem of producing a high qRT-PCR amplification efficiency for other relative quantification.



a)



b)

Figure 3.8 qRT-PCR amplification and standard curves for Actin. Amplification curves represents cDNA detected in sample with varying dilutions of Actin. **a)** Kalinga III Actin's ΔRn against PCR cycle with negative control (green line) versus C_T (threshold cycle) **b)** Standard curve of relative Actin dilution assay. Ct plotted against log Actin copy number.

3.3.6 Validation of the $\Delta\Delta C_T$ method

3.3.6.1 Os05g02310 Inorganic Pyrophosphatase (IPP) gene expression in KIII, A228 and A200F

The results from relative quantification and the gene expression fold difference ($2^{-\Delta\Delta Ct}$) assay calculated relative to Kalinga III after being normalised with all six housekeeping genes are shown in **Table 3.11.** The fold change and expression of the Ashoka series relative to Kalinga III IPP gene of each treatment are shown in **Figure 3.9**. This study has demonstrated that there is no statistical significance in expression levels in Ashoka varieties relative to Kalinga III for the IPP gene tested from shoot tissue at 0 P or full P. Even though Ashoka 228 (RQ=0.88) and Ashoka 200F (RQ=0.78) had lower expression than Kalinga III by 12% and 22%, respectively, when treated under 0 P they were not significantly different. There was a statistical significant difference of expression in genotypes tested in half P treatment [$F(2, 6) = 9.67$, $p < 0.05$) between Ashoka 228 (RQ = 0.34) and Ashoka 200F (RQ = 1.06) a difference of 72% ($p < 0.05$).

For root, there was a significant difference in expression for overall treatment [$F(2, 18) = 3.67$, $p < 0.05$) but there were no significant differences between the genotypes or genotypes by treatments interactions. The results did not show any difference in all three treatments or genotype IPP expression levels. However, both Ashoka 228 and Ashoka 200F did have less expression than Kalinga III by 18% (RQ=0.82) and 19% (RQ=0.81) under 0 P treatment but it was not significant. In contrast, Ashoka 228 (RQ=1.12) had a higher expression (but not significant) than Ashoka 200 F (RQ=0.84) when treated with half P. While the reverse was observed for high P treatment where Ashoka 228 (RQ=0.68) expression was lower by 32% but Ashoka 200F (RQ=1.37) experienced non-significant upregulation of expression by 37% relative to Kalinga III (**Table 3.12**). The fold change of the Ashoka series IPP gene expression of each treatment are shown in **Figure 3.10**.

Table 3.11 Expression analysis in Ashoka 228 and Ashoka 200F shoot tissue of candidate gene Os05g02310 after 7 days in 0 P, half P and full P, calculated by $\Delta\Delta C_T$ method relative to Kalinga III. Values represents means and the standard error as mean ± SE (n = 3). Letters down a column of treatment are significantly different between genotypes within treatments (p < 0.05).

Treatment	Tissue	Genotype	C_T Mean ±SD	ΔC_T Mean ± SE	$\Delta\Delta C_T$	RQ ($2^{-\Delta\Delta Ct}$)	RQ min	RQ max
0 P	Shoot	Kalinga III	24.81 ± 0.48	0.28 ± 0.44	0	1.00[a]	0.53	1.89
		Ashoka 228	25.34 ± 0.36	0.46 ± 0.25	0.19	0.88[a]	0.61	1.26
		Ashoka 200F	25.02 ± 0.39	0.64 ± 0.29	0.36	0.78[a]	0.52	1.18
Half P	Shoot	Kalinga III	23.74 ± 0.77	-0.97 ± 0.55	0.00	1.00[a]	0.45	0.22
		Ashoka 228	25.39 ± 0.15	0.57 ± 0.38	1.54	0.34[b]	0.20	0.60
		Ashoka 200F	24.01 ± 0.34	-1.05 ± 0.32	-0.08	1.06[a]	0.66	1.69
Full P	Shoot	Kalinga III	25.81 ± 0.49	0.74 ± 0.53	0.00	1.00[a]	0.47	2.15
		Ashoka 228	26.18 ± 0.28	1.19 ± 0.49	0.45	0.73[a]	0.36	1.49
		Ashoka 200F	26.43 ± 0.08	0.64 ± 0.24	-0.09	1.07[a]	0.75	1.52

Table 3.12 Expression analysis in Ashoka 228 and Ashoka 200F root tissue of candidate gene Os05g02310 after 7 days in 0 P, half P and full P, calculated by $\Delta\Delta C_T$ method relative to Kalinga III. Values represents means and the standard error as mean ± SE (n = 3). Letters down a column of treatment are significantly different between genotypes within treatments (p < 0.05).

Treatment	Tissue	Genotype	C_T Mean ±SD	ΔC_T Mean ± SE	$\Delta\Delta C_T$	RQ ($2^{-\Delta\Delta Ct}$)	RQ min	RQ max
0 P	Root	Kalinga III	26.32 ± 0.23	-0.37 ± 0.21	0.00	1.00[a]	0.73	1.37
		Ashoka 228	26.51 ±0.07	-0.09 ± 0.11	0.28	0.82[a]	0.71	0.96
		Ashoka 200F	26.47 ± 0.53	-0.07 ± 0.38	0.30	0.81[a]	0.47	1.40
Half P	Root	Kalinga III	26.15 ± 0.12	0.23 ± 0.18	0.00	1.00[a]	0.77	1.29
		Ashoka 228	25.87 ± 0.40	0.06 ± 0.36	-0.17	1.12[a]	0.67	1.90
		Ashoka 200F	25.88 ± 0.46	0.49 ± 0.41	0.26	0.84[a]	0.46	1.53
Full P	Root	Kalinga III	25.80 ± 0.51	0.20 ± 0.39	0.00	1.00[a]	0.57	1.76
		Ashoka 228	26.40 ± 0.33	0.75 ± 0.28	0.56	0.68[a]	0.45	1.02
		Ashoka 200F	25.35 ± 1.01	-0.26 ± 0.65	-0.45	1.37[a]	0.53	3.52

RQ = relative quantities of expression of each of the target gene in samples

RQmin/RQmax = the values calculated by the RQ sample tested that represents the statistically variability in the calculations of each sample's RQ value. These values are represented in the gene expression plot as error bars.

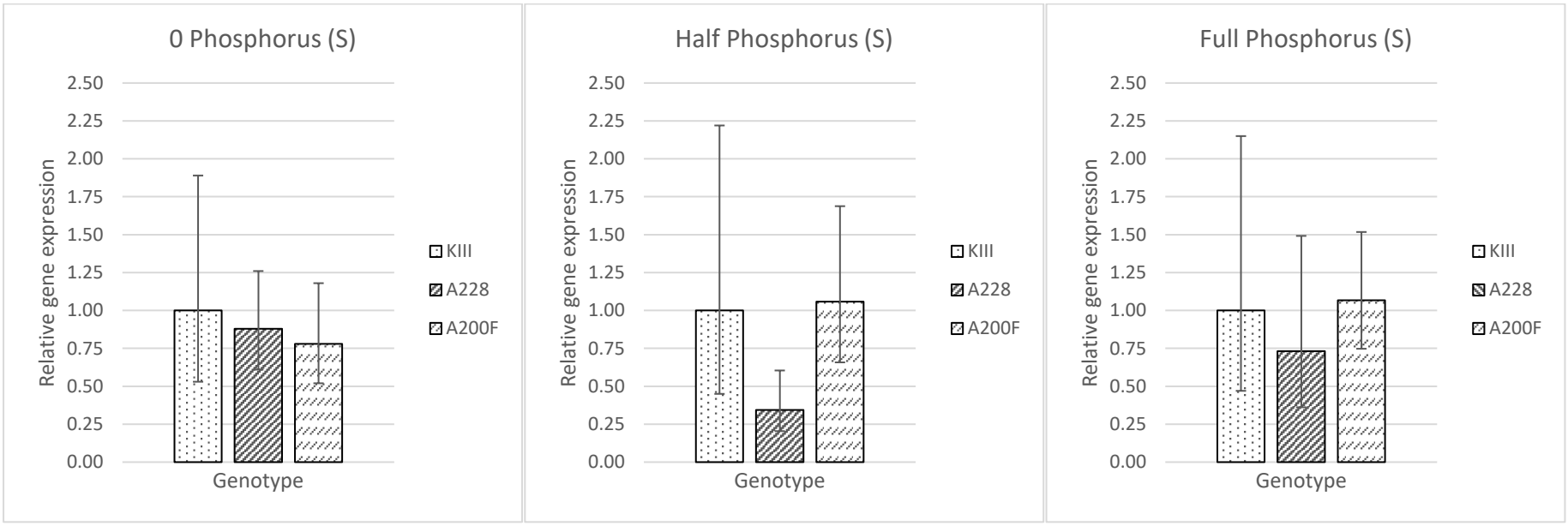


Figure 3.9 Expression analysis of IPP gene in shoot tissues of Ashoka 228 and Ashoka 200F in different P nutrient treatments. All expression levels was determined by the normalization against the 6 housekeeping reference genes. The X-axis shows the different genotype and Y-axis shows the expression level in log2 scale. Data represents mean values and the standard error as mean ± SE (*n*=3) for each data point. The results are relative to LOC_Os05g02310 (IPP) in Kalinga III.

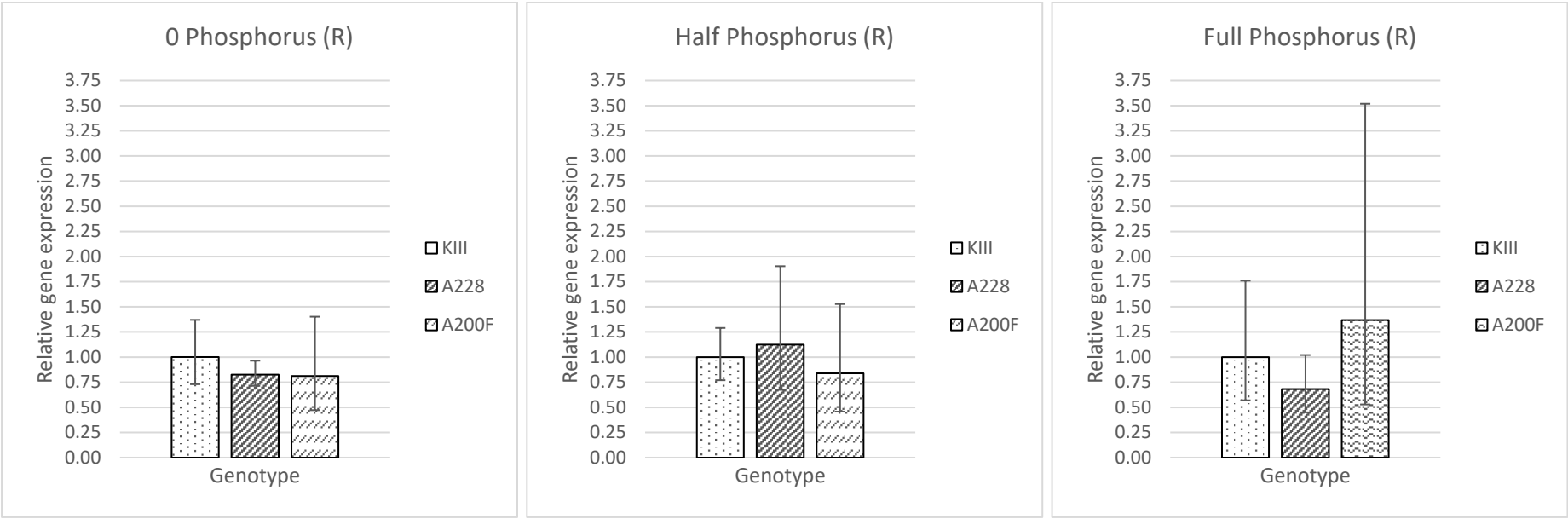


Figure 3.10 Expression analysis of IPP gene in root tissues of Ashoka 228 and Ashoka 200F in different P nutrient treatments. All expression levels was determined by the normalization against the 6 housekeeping reference genes. The X-axis shows the different genotype and Y-axis shows the expression level in log2 scale. Data represents mean values and the standard error as mean ± SE (*n*=3) for each data point. The results are relative to LOC_Os05g02310 (IPP) in Kalinga III.

3.3.6.2 ß-glucosidase (Os09g31430_10) gene expressions in Kalinga III and PY 84

The result from relative quantification of gene expression ($2^{-\Delta\Delta Ct}$) assay in PY 84 was calculated relative to Kalinga III. The RQ value showed the change in fold of gene expression in relation to Kalinga III shoot tissue after being normalized to six endogenous controls (**Table 3.13**). The expression of the PY 84 gene expression for each treatment and tissues are shown in **Figure 3.11** and **Figure 3.12**.

There were no statistical significance found in the expression of Os09g31430_10 in the shoot tissues in all of the three treatments (**Table 3.13)**. PY 84 roots treated in 0 P and full P treatment did not show any significance difference in the expression levels but Os09g31430_10 expression was significantly lower in half P treatment for PY 84 (RQ = 0.37, $p < 0.05$) which was downregulated by 63% relative to Kalinga III (**Table 3.14**).

Table 3.13 Expression in PY 84 shoot tissue of candidate gene Os09g31430_10 after 7 days in 0 P, half P and full P, calculated by $\Delta\Delta C_T$ method relative to Kalinga III. Values represents means and the standard error as mean ± SE ($n = 3$). Letters down a column of treatment are significantly different between genotypes within treatments ($p < 0.05$).

Treatment	Tissue	Genotype	C_T Mean ± SD	ΔC_T Mean ± SE	$\Delta\Delta C_T$	RQ ($2^{-\Delta\Delta Ct}$)	RQ min	RQ max
0 P	Shoot	KalingaIII	25.76 ± 0.08	-0.02 ± 0.22	0.00	1.00[a]	0.73	1.37
		PY84	25.66 ± 0.16	0.24 ± 0.14	0.25	0.84[a]	0.68	1.03
Half P	Shoot	KalingaIII	27.32 ± 0.06	1.53 ± 0.15	0.00	1.00[a]	0.80	1.25
		PY84	27.14 ± 0.18	1.30 ± 0.19	-0.23	1.17[a]	0.89	1.54
Full P	Shoot	KalingaIII	26.76 ± 0.25	1.44 ± 0.26	0.00	1.00[a]	0.68	1.46
		PY84	26.44 ± 0.28	1.39 ± 0.19	-0.05	1.04[a]	0.79	1.36

RQ = relative quantities of expression of each of the target gene in samples

RQmin/RQmax = the values calculated by the RQ sample tested that represents the statistically variability in the calculations of each sample's RQ value. These values are represented in the gene expression plot as error bars.

Table 3.14 Expression in PY 84 root tissue of candidate gene Os09g31430_10 after 7 days in 0 P, half P and full P, calculated by $\Delta\Delta C_T$ method relative to Kalinga III. Values represents means and the standard error as mean ± SE (n = 3). Letters down a column of treatment are significantly different between genotypes within treatments ($p < 0.05$).

Treatment	Tissue	Genotype	C_T Mean ± SD	ΔC_T Mean ± SE	$\Delta\Delta C_T$	RQ ($2^{-\Delta\Delta Ct}$)	RQ min	RQ max
0 P	Root	KalingaIII	26.65 ± 0.41	2.49 ± 0.29	0.00	1.00[a]	0.65	1.53
		PY84	26.12 ± 0.24	1.95 ± 0.17	-0.54	1.45[a]	1.13	1.87
Half P	Root	KalingaIII	25.47 ± 1.02	-0.37 ± 0.69	0.00	1.00[a]	0.37	2.74
		PY84	27.10 ± 0.31	1.04 ± 0.22	1.42	0.37[b]	0.27	0.52
Full P	Root	KalingaIII	28.52 ± 0.46	1.72 ± 0.39	0.00	1.00[a]	0.57	1.76
		PY84	28.30 ± 0.26	1.35 ± 0.21	-0.37	1.29[a]	0.95	1.76

RQ = relative quantities of expression of each of the target gene in samples

RQmin/RQmax = the values calculated by the RQ sample tested that represents the statistically variability in the calculations of each sample's RQ value. These values are represented in the gene expression plot as error bars.

3.3.6.3 ß-glucosidase (Os09g31430_41) gene expressions in Kalinga III and PY 84

The result from relative quantification of gene expression ($2^{-\Delta\Delta Ct}$) assay in PY 84 was calculated relative to Kalinga III and the RQ value showed the change in fold of gene expression after being normalized to six endogenous controls. The expression in shoot and root tissue of PY 84 and Kalinga III are shown in Figure fold change of the Ashoka series IPP gene expression of each treatment are shown in **Figure 3.11** and **Figure 3.12**.

There were no significant difference detected in shoot tissue for all samples in all three treatments (**Table 3.15**). In contrast, there was a significant difference between Os09g31430_41 in PY 84 (RQ = 2.14, $p < 0.05$) compared to Kalinga III, a 114% increase of expression in root when treated in 0 P treatment. Similarly in half P treatment where Os09g31430_41 in PY 84 (RQ = 2.87, $p < 0.05$) was significantly expressed by 187% increase as to that of Kalinga III. While full P also revealed a moderate 91% increase of expression in PY 84 Os09g31430_41 (RQ = 1.91, $p < 0.05$) relative to Kalinga III (**Table 3.16**).

Table 3.15 Expression in PY 84 shoot tissue of candidate gene Os09g31430_41 after 7 days in 0 P, half P and full P, calculated by $\Delta\Delta C_T$ method relative to Kalinga III. Values represents means and the standard error as mean ± SE (n = 3). Letters down a column of treatment are significantly different between genotypes within treatments ($p < 0.05$).

Treatment	Tissue	Genotype	C_T Mean ± SD	Sample ΔC_T Mean ± SE	$\Delta\Delta C_T$	RQ ($2^{-\Delta\Delta Ct}$)	RQ min	RQ max
0 P	Shoot	KalingaIII	27.89 ± 0.14	2.12 ± 0.23	0.00	1.00[a]	0.72	1.39
		PY84	27.34 ± 0.26	1.92 ± 0.18	-0.20	1.15[a]	0.88	1.50
Half P	Shoot	KalingaIII	27.90 ± 0.20	2.11 ± 0.19	0.00	1.00[a]	0.76	1.31
		PY84	27.72 ± 0.14	1.88 ± 0.18	-0.23	1.17[a]	0.90	1.52
Full P	Shoot	KalingaIII	28.22 ± 0.15	2.90 ± 0.23	0.00	1.00[a]	0.71	1.40
		PY84	27.54 ± 0.47	2.49 ± 0.29	-0.41	1.33[a]	0.88	2.01

RQ = relative quantities of expression of each of the target gene in samples

RQmin/RQmax = the values calculated by the RQ sample tested that represents the statistically variability in the calculations of each sample's RQ value. These values are represented in the gene expression plot as error bars.

Table 3.16 Expression in PY 84 root tissue of candidate gene Os09g31430_41 after 7 days in 0 P, half P and full P, calculated by $\Delta\Delta C_T$ method relative to Kalinga III. Values represents means and the standard error as mean ± SE (n = 3). Letters down a column of treatment are significantly different between genotypes within treatments ($p < 0.05$).

Treatment	Tissue	Genotype	C_T Mean ± SD	Sample ΔC_T Mean ± SE	$\Delta\Delta C_T$	RQ ($2^{-\Delta\Delta Ct}$)	RQ min	RQ max
0 P	Root	KalingaIII	27.47 ± 0.27	3.30 ± 0.23	0.00	1.00[a]	0.71	1.40
		PY84	26.38 ± 0.16	2.20 ± 0.14	-1.10	2.14[b]	1.74	2.64
Half P	Root	KalingaIII	28.13 ± 0.17	2.28 ± 0.38	0.00	1.00[a]	0.58	1.73
		PY84	26.82 ± 0.27	0.76 ± 0.20	-1.52	2.87[b]	2.13	3.85
Full P	Root	KalingaIII	28.66 ± 0.17	1.86 ± 0.30	0.00	1.00[a]	0.64	1.55
		PY84	27.89 ± 0.14	0.93 ± 0.17	-0.93	1.91[b]	1.48	2.45

RQ = relative quantities of expression of each of the target gene in samples

RQmin/RQmax = the values calculated by the RQ sample tested that represents the statistically variability in the calculations of each sample's RQ value. These values are represented in the gene expression plot as error bars.

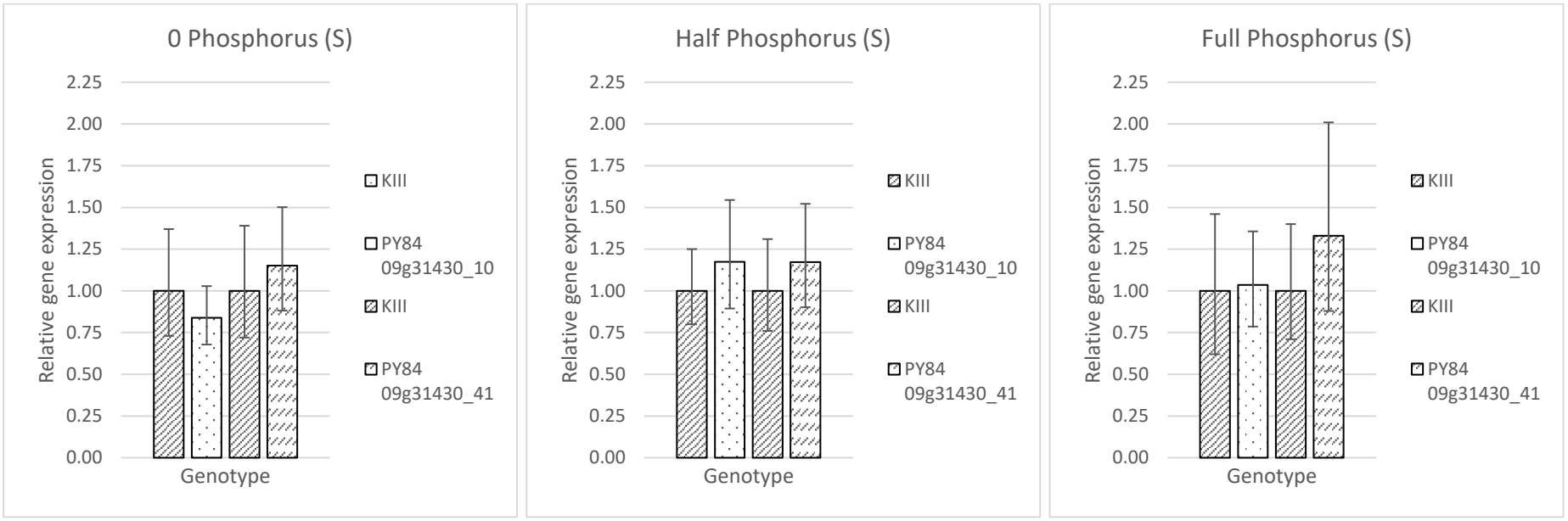


Figure 3.11 Expression analysis of LOC_Os09g31430_10 and of LOC_Os09g31430_41 gene in shoot tissues of PY 84 in different treatments of P nutrient concentrations. All expression level was determined by the normalization against 6 housekeeping reference genes. The x-axis shows the different genotype and treatments and Y-axis shows the expression level in log2 scale. Data represents values and the standard error as mean ± SE (*n*=3) for each data point. The PY 84 results are relative to a value of one for LOC_Os09g31430_10 (left hand bar) and LOC_Os09g31430_41 (third bar from left) tested in Kalinga III.

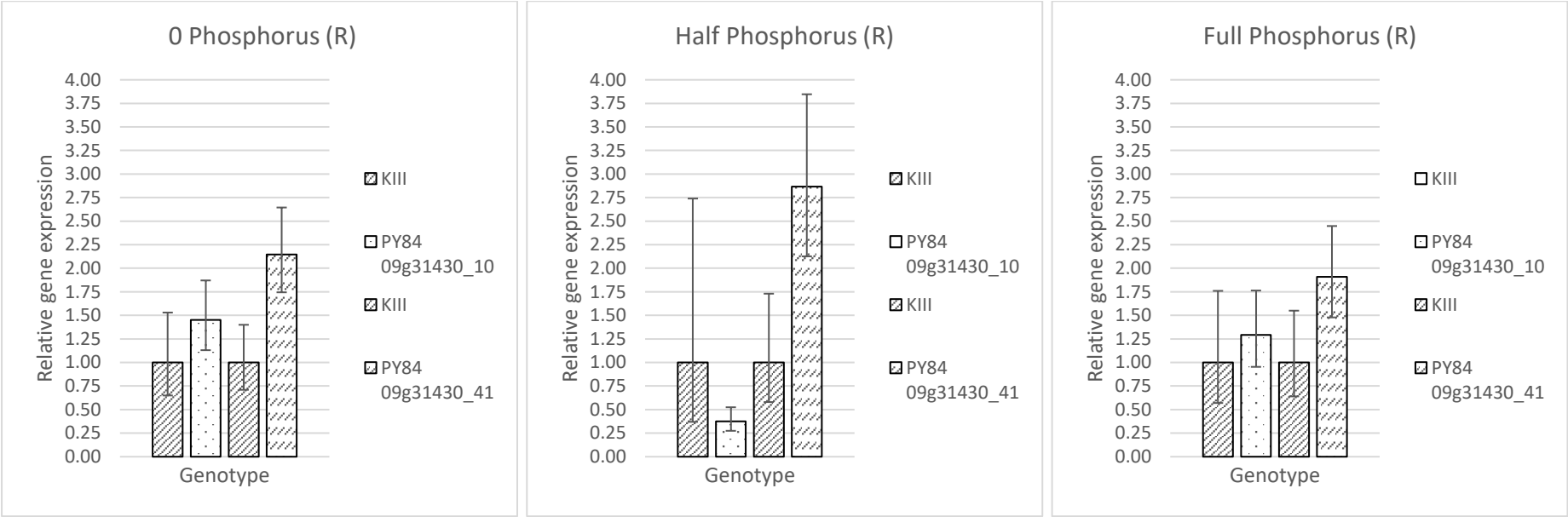


Figure 3.12 Expression analysis of LOC_Os09g31430_10 and of LOC_Os09g31430_41genes in root tissues of PY 84 in different treatments of P nutrient concentrations. All expression level was determined by the normalization against 6 housekeeping reference genes. The x-axis shows the different genotype and treatments and Y-axis shows the expression level in log2 scale. Data represents values and the standard error as mean ± SE (*n*=3) for each data point. The PY 84 results are relative to a value of one for LOC_Os09g31430_10 (left hand bar) and LOC_Os09g31430_41 (third bar from left) tested in Kalinga III.

3.4 Discussion

3.4.1 Effect of different hydroponics P treatments on plant shoot and root growth

The recorded hydroponics measurements showed that all three treatments given did not significantly affect the overall height of seven rice genotypes tested. However, the roots length did have an effect due to the P level given whereby half P had a slightly longer mean cumulative root length (8.31 ± 0.38 cm) followed by no P (8.16 ± 0.28 cm) than in full P (5.95 ± 0.41 cm) after 7 days of treatment. This suggest that all genotypes roots tested grew longer ($p < 0.001$) under low P presence with optimal growth conditions. As it has being noted before, P is required as part of macronutrient source for rice crop for many physiological and biochemical functions that increases tillering in cereals crops in the later part of developmental stage growth especially crucial in improving root growth during rice crop initial growth. (Fageria *et al.*, 2006; Fageria 2007; Fageria and dos Santos, 2008).

Comparing more closely between the seven genotypes, analysis of variance indicated that a significant difference ($p < 0.001$) of 3.25cm in shoot height between PY 84 (10.13 ± 0.77 cm) and MRQ 76 (6.88 ± 0.31 cm) with no P treatment. While Ashoka 228 (9.95 ± 0.21 cm) was second tallest sample and Ashoka 200F (8.63 ± 0.17 cm) had a medium height. The cumulative shoot height of all treatment analysed that NIL (BC3F3) had the tallest shoot height (10.22 ± 0.35 cm) and MRQ 76 was still the shortest (7.83 ± 0.42 cm). Similarly, there were genotypic differences in the responses for root length ($p < 0.05$) tested with no P in the Yoshida's nutrient solution. However, MRQ 76 performed better than shoot height by having the longest root (10.75 ± 0.32 cm) compared to Ashoka 200F (9.53 ± 0.29 cm), Kalinga III (7.70 ± 0.56 cm), PY 84 (7.13 ± 0.39 cm) and Ashoka 228 (6.88 ± 0.24 cm). Half P and full P treatment also gave indication that MRQ 76 outperform all other genotypes. This would indicate that the rice genotypes developed its rooting system better than others to adapt in P deficient environment. Another key point to note that genotypes developing longer roots could have a better P acquisition and a higher phosphate use efficiency as plant species that are adjusting to P deficient environment will increase its root length (Fitter, 1985 and Hill *et al*., 2006). By having more branches roots per unit of root mass, it will also increases the surface area and chances of acquiring more nutrients from the soil/nutrient medium.

The hydroponic experiment concluded that low P supply enhances the adventitious root elongation as well as the lateral root development and elongation in rice (Kirk & van Du, 1997) and in beans (Liao *et al*., 2001). The modified aerated hydroponic system also is a useful method to document P deficiency effect of physiological responses of shoot and root growth at the early stages of seedling growth similar to Negi *et al*. (2016) findings. The hydroponic

results were in agreement with a study by Vejchasarn *et al*. (2016) that there was genetic variation in roots tested under low P. Root QTL genes could have acted as the underlying factor in determining its traits particularly in P deficient environment. This are later explored through qRT-PCR analysis of root and PUE candidate genes.

3.4.2 Total RNA extraction yield

In extracting the RNA, there are several factors considered when quantifying the RNA yield and quality made in this experiment. The extractions were carried out in batches. After carefully removing the plants from the modified aerated hydroponics system (MAHS) then snap freezing the shoot and root tissue parts in liquid nitrogen and stored at -70°C until needed in order to minimize RNA degradation. However, each processed batch took longer than the ZR Plant RNA MiniPrep™ kit (Zymo Research: Cambridge Bioscience, UK) manufacturer's instructions due to limited number of available centrifuge rotor chambers that can be spun each time. Delay in processing the batch could have affected the final RNA yield, especially for root RNA. Although some differences in RNA yield (**section 3.3.4.1**) were detected, these were adjusted for in dilutions for qRT-PCR.

Extraction of shoot and root total RNA yield using ZR Plant RNA MiniPrep™ kit (Zymo Research: Cambridge Bioscience, UK) and quantified by using Qubit® RNA BR Assay kit (Life Technologies) and Qubit® 2.0 Fluorometer (Invitrogen) as presented in **Table 3.9**. There were some minor errors in using the RNA extraction kits' column due to similarities in shape and size of Zymo spin IIIC column that allows for high-capacity DNA elimination and the Zymo spin IIC column that efficiently adsorbs total RNA that affected the final total RNA yield eluted. In hindsight, the final yield could have been optimal if the correct columns had been used in the correct sequence. However, the company representative via e-mail communication reassured that it did not affect the overall extraction process. In retrospect, it is advisable to use colour-coded columns rather than white translucent columns of different sized filters, which are difficult to differentiate whilst working at a pace. Coding text both on the equipment (columns) and in the instruction manual need to be more distinctive and therefore more easily distinguished eliminating the possibility of any errors.

From the experiment, a cumulative average yield of 781 ng/µl from shoot total RNA and 253 ng/µl from root total RNA were eluted from the column of ZR Plant RNA MiniPrep™ kit due to reasons mentioned above. Mean total RNA ratio eluted from shoots and roots tissue part of the seven genotypes are 3:1. There were no significant differences in the shoot total RNA extracted in all seven genotypes for all three P treatments but there were significant difference in genotypes total RNA eluted in full P treatment where NIL (MABC Advance

Lines) yields slightly more than Kalinga III (p = 0.05). On the other hand, ANOVA analysis for root total RNA does not seem to show any significant difference in between genotypes but does show that half P treatment (349.9 ± 31.81 ng/µl) eluted significantly more total RNA than no P treatment (212.0 ± 15.69 ng/µl) compared to full P treatment (197.2 ± 18.39 ng/µl).

There was no purity assessment carried out on the total RNA extracted as the extraction were performed according to manufacturer's instruction kit and the manufacturer claimed a maximum ~50µg RNA binding capacity with high quality total RNA elution recovery (A_{260}/A_{280} > 1.8, A_{260}/A_{230} > 1.8). In addition, according to the MIQE guidelines, the purity and yield assessment was desirable and not necessarily essential to be included for publication of qRT-PCR experiments (Bustin *et al*., 2009). The total RNA extraction for shoot and root tissue parts from each genotype P treatment was more than enough for a downstream molecular analysis as only a minimum of 50 ng/reaction were needed for the qRT-PCR assay testing the candidate genes.

3.4.3 Technical difficulties of using qRT-PCR

In order to validate and rule out any effect of inaccuracies in determining the qRT-PCR product concentration and errors due to dilution and pipetting variations, normalisation against a reference gene is integrated as an essential step the real time PCR. It circumvents the need for accurate quantification of the starting material and can be advantageous whereby the starting material are limited providing amplification efficiencies are near 100% and differ within 5% of each other, whereby most optimized reactions fall within this criteria. The relative quantification (expression values) between targeted gene and control gene can be evaluated by the $\Delta\Delta C_T$ method (Livak and Schmitten, 2001). It requires the determination of the difference in ΔC_T value from at least two samples. This method requires the use of known reference genes that exhibits constant and stable expression levels in all samples investigated in order to quantify accurately.

Earlier pilot run of assays produced multiple melt curves and negative template controls (NTCs) having the same Tm point as tested samples. Therefore, optimization of qRT-PCR assay were carried before the actual run of samples and candidate genes. Due to the limitations in time and resources qRT-PCR was not able to be carried out on all of the original seven rice genotypes listed (**section 3.2.5**) and it was only carried out on Ashoka 200F, Ashoka 228 (for IPP), PY 84 (for ß-glucosidase) and Kalinga III for both candidates due to time and resource constrains. A total four replications were required for each genotype per assay but only the three best replications results were used for statistical analysis because some of the replications did not give a good resolution of amplification or melting curve. A minimum of three

replication is considered acceptable for qRT-PCR results under MIQE guidelines (Bustin *et al*., 2009).

3.4.4 Os05g02310 Inorganic Pyrophosphatase (IPP) gene expression in Kalinga III, Ashoka 228 and Ashoka 200F

The roots are the main organs that is involved during initial sensing and response to plant nutrient level particularly in P deficient environment (Richardson, 2009). In this chapter, the aim of a hydroponics based experiment was carried out to study if LOC_ Os05g02310 IPP gene might be responsible for different levels of phosphate use or uptake with different levels of expression in Ashoka 200F and Ashoka 228 compared to Kalinga III.

From the qRT-PCR gene expression data (**section 3.3.6.1**) it was observed that the gene expression fold difference or relative quantity (RQ) of the Ashoka varieties were not significantly expressed in shoot tissue compared to Kalinga III in both 0 P and full P treatment. Treatment in 0 P caused Ashoka 200F and Ashoka 228 to have a lower IPP gene expression to Kalinga III by 12% and 22%, respectively. While full P gave Ashoka 200F only a slight 7% upregulation compared to Ashoka 228 which was downregulated by 27% when compared to Kalinga III but otherwise were not significantly different. However, at half P treatment, Ashoka 228 was significantly downregulated by 72% when compared to Ashoka 200F. Similarly in root tissues, there were no significant IPP gene expression seen between both the Ashoka varieties across all P treatments. Eventhough, Ashoka 228 were slightly upregulated by 12% in half P and Ashoka 200F upregulated by 37% in full P, both varieties were similarly downregulated by nearly 20% compared to Kalinga III when treated in 0 P treatment.

This findings is similar to Huang *et al*. (2008) report, where the gene expression of inorganic PPiase was either unchanged or only slightly upregulated in anoxic *Arabidopsis* (Loreti *et al.*, 2005). However, in anoxic rice coleoptiles, Os05g02310 were significantly downregulated in anoxic rice coleoptile (Lashanti-Kudahettige *et al*., 2007) which results in the reduction of PPi degradation by inorganic PPiases and hence allowing available inorganic phosphorus could be directed to other essential metabolic processes.

Ashoka 228 shoot expression were highly downregulated (66%) in half P compared to Kalinga III, this differs from the hydroponics experiment where the shoot height of Ashoka 228 (8.63 ± 2.88 cm) was not significantly different when compared to Ashoka 200F (8.40 ± 0.45 cm) and Kalinga III (9.15 ± 0.53 cm). Our findings suggested that the IPP Os05g02310 gene was not upregulated during the early germination period of rice germination. This could be due to Ashoka 228 shoot growth might be utilizing its slightly higher phosphorus content

from the grains (4.9 ± 0.77 mg/g) as reported in Chapter 2 (Appendix 2.7) under 0 P treatment during the early germination period.

3.4.5 ß-glucosidase (Os09g31430_10) and (Os09g31430_41) gene expressions in Kalinga III and PY 84

Phosphate (Pi) is needed as an essential macronutrient for plant growth and development but limiting in soils due to its slow rate of diffusion (Raghothama, 1999). Rice plants have developed a complex root system architecture (RSA) to acquire P nutrient optimally under various environments (Gruber *et al,* 2013). Previous studies were carried out on Arabidopsis (Alatorre-Cobos, 2014) and rice (Negi *et al*., 2016) using modified hydroponics systems to document the effects of root growth under Pi deficiency. It is well reported that PY84 carry all four introgressed QTLs (QTL 2, QTL 7, QTL 9, QTL 11) associated with root related phenotypes that results in increase of yield (Steele *et al.,* 2013). Our study looked into possible validation of introgressed LOC_Os09g31430 in PY 84 that could differ from Kalinga III under P deficient conditions.

The expression of two candidate genes of ß-Glucosidase (LOC_Os09g31430) did not show any significant expression level in PY84 shoot tissues when compared to Kalinga III. Os09g31430_10 saw a slight upregulation of 4 and 17% in PY 84 treated in half P and full P but was down regulated by 16% in 0 P. While Os09g31430_41 saw a much higher upregulation of 15%, 17% and 33% going as the P concentration increases in 0 P, half P and full P, respectively. Similarly, there were no significant gene expression of Os09g31430_10 in the root tissues of PY 84 treated in 0 P and full P, but were highly downregulated by 63% under half P treatment in comparison to Kalinga III. On the contrary, candidate gene LOC_Os09g31430_41was significantly expressed in PY 84 root tissues of all of the three treatments showed a marked 114%, 187% and 91% upregulation in 0 P, half P and full P, respectively.

Our findings also concurs with previous unpublished study by Bangor University MSc. student, Prabhu Manickam (2016) where he found that LOC_Os09g31430 was upregulated in Kalinga III compared to Nipponbare. This proves the hypothesis that this gene might be potentially responsible for deeper rooting phenotyping with different expression in PY 84 compared to Kalinga III.

3.5 Conclusion

Effects of P deprivation can be seen for both shoots and roots tissue parts when morphological data analysis (**Table 3.8**) on the selected upland rice genotypes. Although the ANOVA analysis of shoot height of combined genotypes were not significant across all three treatments, root length saw a significant effect of half P (8.31 ± 0.38 cm) and low P (8.16 ± 0.28 cm) where combined genotype root length grew longer when compared to full P treatment (5.95 ± 0.41 cm). Ashoka 228 (9.95 ± 0.21 cm) was closer to PY 84 shoot height (10.13 ± 0.77 cm) than Ashoka 200F (8.63 ± 0.17) or Kalinga III (8.75 ± 0.25 cm) after 7 days under 0 P treatment. However, there was a significant difference of genotypic in the root growth response to low P condition between the varieties tested. Ashoka 200F (9.53 ± 0.29 cm) grew longer roots than PY84 (7.13 ± 0.39 cm) and Kalinga III (7.70 ± 0.56 cm) while Ashoka 228 (6.88 ± 0.24 cm) was the shortest under 0 P.

Gene expression study showed no significant difference towards IPP (LOC_Os05g02310) expression in shoot tissue from both varieties although it was downregulated by nearly 20% in comparison to Kalinga III under 0 P treatment. However, Ashoka 228 saw a significant down regulation of expression by 66% when compared to Kalinga III in half P. Meanwhile Ashoka 200F was slightly upregulated by 6% and 7% in half and full P treatment, respectively. There were also no significant expression observed in the root tissues across all treatments. The lack of expression of the IPP gene which confers to P use efficiency may be due to the Ashoka varieties probably starts to utilise external P source once the stored P from grain has depleted after its initial 7 days of germination.

The expression of ß-Glucosidase (LOC_Os09g31430) in PY84 compared to Kalinga III did not saw any significance from both candidate gene in shoot tissues across different P treatments. On the contrary, candidate gene LOC_Os09g31430_41was significantly expressed in all of the three treatments with PY 84 showed a marked 114%, 187% and 91% upregulation in 0 P, half P and full P, respectively.

CHAPTER 4

4 General discussion

4.1 Introduction

This study has aimed to identify the morphological and genetic response of upland rice plants growth performance, particularly root traits, associated with phosphate (P) use efficiency (PUE) in upland rice grown under different P levels. In addition, trying to answer if rice root length and PUE candidate genes show variation (expression) with morphological traits associated with adaptation to low P growth medium. A glasshouse (soil) and laboratory (nutrient solution) based experiment was designed to mimic P nutrient stress conditions as experienced by the rice plants growing in the field. This study used qRT-PCR gene expression analysis to see if the plants' responses relate to any upregulation of the QTLs genes associated with PUE and root length under the P deficient environment.

4.2 Relevance of upland rice plants responses in P deficient environment

Presently, China, India and South Eastern regions of Asia account for approximately 91% of the world total rice production (Khush, 2004; Zeigler and Barclay, 2008; FAOSTAT database). Cultivated of rice varieties vary for adaptation to the irrigated lowlands (approx. 60%), rain-fed lowland (approx. 19%) rain-fed upland (approx. 15%) and other ecosystems including flooded lands (GRiSP, 2013). From this, nearly 24% of all rice grown in Asia are on marginal land that often has poor and low nutrient soils. These problematic lands are prone to a multitude of conditions of biotic and abiotic stresses. In addition to this, the lack of resources and limited access to input such as nitrogen, phosphorus and potassium (N:P:K) fertilizers perpetuates a vicious circle of low crop yields, making subsistence agriculture difficult in areas of South Asia particularly in the Eastern India and Nepal (Garrity and O'Toole, 1994; Babu *et al*., 2004).

The current global climate environment increases constraints on crop growers and stress on crops and presents challenges in order to sustain a level of food security and meeting the demands of world population and its consumption. Researchers, breeders and rice growers' understanding of how to respond to the stresses is the basis for improving existing varieties and landraces will lead to improvement in breeding for yield under environmental stress (drought/water stress) and increasing productivity with lower levels of fertilizers (including phosphorus) would be more crucial.

Rice productivity is limited by the phosphorus (P) availability as this finite resource is have relatively poor mobility in soil resulting only in small fraction of available P absorbed by plant crops (Castro *et al*., 2013; Arredondo *et al*., 2014). Inefficient acquisition and efficient use of P limits potential crop growth and yield (Withers *et al*., 2014), particularly in poor soil deficient areas. Therefore, the logical approach is to identify optimal rice genotypes with high productivity and to identify root QTL as well as PUE related genes that are expressed in plants that responds positively when grown under low P environment to further improve rice yield.

4.3 Upland rice growth performance associated with PUE grown under different P levels

The specific objectives under this section were to study the relationship of upland rice genotypes' agro-morphological responses, for both Ashoka varieties, throughout the vegetative and reproductive stages under different P nutrient concentrations administered to a low P soil comparing to Kalinga III.

The hypothesis was that the Ashoka varieties would perform better under low P nutrient condition. Different response to 0 P, half P and high P for some traits and similar response to certain other traits were observed. In the pilot experiment (2015) Ashoka 200F grains weighed 1.8 g more than Ashoka 228 as well as producing more grains compared to both Ashoka 228 and Kalinga III. However, looking at individual grain weight, it seems Ashoka 200F weighed less by almost 4.4 mg than both Ashoka 228 and Kalinga III. It was also noted that both Ashoka varieties have 1.5 mg/g less shoot P content than Kalinga III. Therefore, in this aspect, the Ashoka 200F does seem to utilize low available P in the soil to produce more grain numbers compared to the other two entries.

While the main experiment (2016), the initial booting stage leading up to flowering stage showed that Ashoka 228 grew taller and tiller production than Ashoka 200F and Kalinga III. It was later outperformed by Ashoka 200F which ultimately had more tillers, longer root length, height, number of grains produced and biomass weight at maturity compared to Ashoka 228 and Kalinga III. It is also worth mentioning that each variety does have a different length of growing periods at which it goes through the vegetative, reproductive, spikelet filling and finally harvest dates. The glasshouse experiment were carried out in batches by germinating and transplanting the seedlings according to each variety's growing periods. Meaning that the variety with the longest harvesting dates were transplanted first followed by variety with the shortest harvesting dates so that sampling time and data can be collected can be synchronized during vegetative, booting, flowering and harvesting days.

This agrees with the findings of J.R. Witcombe (Pers. Comm.) who has observed Ashoka 200F to be more productive than Ashoka 228 in many trials across India and Nepal. Comparing between the Ashoka varieties, it showed that shoots grow slower in Ashoka 200F before booting but the growth rate increases at booting so it grows taller as well as producing more tillers, longer roots after booting stage under low P. The Ashoka 200F also produced more grain numbers per plant than other two tested plants but were lower in grain weight. In terms of PER and PUE, the Ashoka varieties had less P uptake efficiency than Kalinga III at full P.

The chapter 2 findings are in agreement with Fageria *et al*. (1988) where upland rice varieties tested under greenhouse conditions were found to be performing well under low P. This was also demonstrated in various annual crops such as maize, soybean, dry bean and upland rice. Upland rice P uptake significantly increased in an exponential quadratic fashion with advancement of plant age where towards its harvesting stage, P uptake was seen to be higher in shoot compared to grain (**Table 2.8**; **Table 2.12**). The PUE for grain production in upland rice was also associated with higher yield compared to legumes species (Fageria *et al*., 2013).

4.4 The expression of candidate genes in rice roots for PUE grown in hydroponics

The combination of morphological and quantitative molecular approaches using qRT-PCR allows gene expression studies that can inform future selection of genotypes for increased use of phosphorus and root length traits in upland rice genotypes. A main hypothesis was formulated, tested and confirmed that Ashoka 200F had longer root growth response than PY84 under low P nutrient condition than Kalinga III (**Table 3.8; Figure 3.6**).

The following objective was set to study candidate genes expressions:

- Ashoka 228 and Ashoka 200F expression of IPP compared to Kalinga III under different P concentrations.
- PY 84 expression of ß-Glucosidase compared to Kalinga III under different P concentrations.

The chosen method of studying gene expression was through qRT-PCR approach which is widely used in gene expression studies. The MIQE recommendations were followed carefully in order to generate robust data. A candidate gene associated with Phosphorus use efficiency in Ashoka varieties and a candidate gene associated with a root length QTL from in PY 84 were further investigated. The candidate genes identified were from chromosome 5 (LOC_Os05g02310) and chromosome 9 (LOC_Os09g31430) based on evidence from rice

from previous studies (see Chapter 3). The IPP (LOC_Os05g02310) gene was hypothesized to be responsible for different levels of phosphate use or uptake. However, we found that there were no significant upregulation when tested in both Ashoka varieties tissue parts across all treatments except there were a significant downregulation in Ashoka 228 shoot under half P. This did not agree with previous expression analyses on Ashoka varieties, Kalinga III and IR64, but those experiments were less well designed and replicated than the current study. Therefore the hypothesis has been rejected that the Ashoka varieties up-regulate IPP compared to Kalinga III and further investigation is recommended. It is plausible that the lack of IPP gene expression in both shoot and root of the Ashoka upland rice varieties tested to that of control (Kalinga III) could be due to epigenetic effects, so future experiments should be designed to test for epigenetic effects.

Epigenetic variation could have caused the expression study to be non-significant by adaptation to varying macronutrient supply in previous generations of the lines used and it may have conditioned traits that are valued in crop improvement similarly observed in barley (Raboy, V. unpublished, Pers. Comm). Among other epigenetic inheritance, one is called "trans-generational adaptation", where throughout the plant growth stages the plants "intra-generational" have adaptive responses towards environmental variations of biotic or abiotic stresses (Suzuki *et al*., 2014; Zhu, 2016).

The other candidate gene tested; LOC_OS09g31430 is a gene encoding for ß-Glucosidase associated with physiologically important processes in plant especially response towards abiotic stresses and lignification and hydrolysis of cell wall oligosaccharides (Opassiri *et al*., 2006). Previous qRT-PCR unpublished study by Bangor University MSc. student, Prabhu Manickam (2016) also found that LOC_Os09g31430 was upregulated in Kalinga III (*indica*) compared to Nipponbare (*japonica*). The hypothesis that this gene is potentially responsible for deeper rooting phenotyping were proven with one pair of primers for of the candidate gene LOC_Os09g31430_41 showing a significant expression in PY 84 compared to Kalinga III. However, LOC_Os09g31430_10 did not have much difference in expression in root tissues except for a down regulation in PY 84 under Half P.

4.5 Final conclusion

The demand for rice production are on the rise due to increasing world's population consumption living mainly on rice-based diets as their daily nutrient intake. The figures are set to increase as the prediction by FAO suggests that rice consumption demand will increase from 395.4 metric million tonnes in the year 2000 to 533 million metric tonnes by 2030 (Abdullah *et al*., 2008; Alexandratos and Bruinsma, 2012). However, the present challenges of cultivating

rice are met with adverse global climate trends and scarcity of agricultural land area. This is exacerbated by biotic, abiotic stresses and other factors (Lobell *et al*., 2011; Weller *et al*., 2016) have caused large grain yield to decline by 10% for each 1°C increase associated with global warming (Peng *et al*., 2004).

The data presented in this thesis demonstrate that the effect of rice growth in P deficient environment caused a genotypic effect on Ashoka 200F genotype where it responded respond by growing deeper root compared to Ashoka 228 and Kalinga III. As the plants approaches harvesting stage, Ashoka 200F grew taller and producing extra tillers with higher grain count per plant, higher dry shoot weight, root weight and SPAD value, higher plant P uptake and more efficient at absorbing phosphorus (PUE) under low P conditions compared to Ashoka 228 and Kalinga III. On the contrary, both Ashoka varieties produced a lighter grain weight than its control Kalinga III and there were no significant differences found on the root length between all three genotypes irrespective of treatments given.

Additional analysis on the morphological traits of upland rice varieties from aerated hydroponic system (AHS) experiment found that the roots grew longer under P deficient environment after 7 days of sowing. Low P supply enhances the adventitious root elongation as well as the lateral root development and elongation in rice (Kirk & van Du, 1997). PY 84 and Ashoka 228 shoot grew taller than Ashoka 200F and Kalinga III under 0 P treatment but a significant genotypic effect were seen in root length where Ashoka 200F had longer roots compared to PY 84, Kalinga III and Ashoka 228 under similar conditions. The P deficiency effect of physiological responses of shoot and root growth at the early stages of seedling growth were similar to Negi *et al*. (2016) findings and also in agreement with a study that there was genetic variation in roots tested under low P (Vejchasarn *et al*., 2016).

The qRT-PCR gene expression analysis on IPP (LOC_Os05g02310) gene showed no significant difference on genotypes across all treatment for root tissues, except for Ashoka 228 gene expression of shoot tissue was highly down regulated under half P treatment. It could be worth to invest future investigation looking into the epigenetic variation of "trans generational adaptation" to better understand as to why this occurs. On the contrary, ß-Glucosidase candidate gene LOC_Os09g31430_41was significantly upregulated in the root tissues of PY84 across all P treatments. This supports the hypothesis that this gene underlies the effect of QTL9 on roots, but further investigation, such as reverse and forward genetic screens are required. These findings are a step towards providing great potential strategies in breeding drought tolerant/resistance traits of upland rice varieties for future breeding programmes in drought prone areas including Malaysia.

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4.6 Knowledge gaps and suggestions for further exploration and recommendation

The present study depicts the complexity of P uptake and P use efficiency and traits associated with low P environment. Morphological traits were identified in three levels of P concentration and QTL gene associated for these traits were also studied under the same P concentration gradient. Although the aims of this investigation have been met, there are several list of further work has been noted throughout the experiments. The main findings are summarised as follows:

- The current study demonstrates the usefulness of phenotypic traits screening in glasshouse set up. The upland rice varieties alongside Malaysian genotype(s) need to be further tested on either small, medium or large scale via rhizotron to study the phenotypic root growth under different levels of P conditions.
- The current study also validated the fidelity of the aerated hydroponic system (AHS) in recording phosphorus related effects on physiological and responses. It also demonstrates the potential for comparable molecular effects of phosphorus deficiency on root traits and PUE in upland rice varieties.
- Further test need to be conducted on the Malaysian rice genotype MRQ 76 (irrigated rice) for phosphorus efficiency and root QTL as MRQ 76 has shown to have the longest roots (10.75 ± 0.32 cm) compared to the all six genotypes tested under 0 P hydroponic conditions.
- The potential of candidate gene LOC_Os09g31430 has shown to be potentially useful in detecting introgressed root QTL in *indica* upland rice genotypes. This could perhaps be further explored with Malaysian irrigated rice genotypes as well. It is possible that a functional marker for its selection could be designed based on the sequences used for the primers for LOC_Os09g31430_41.
- The plant phosphorus requirements and root growth in rice plants are well now understood but the underlying knowledge of how its regulation and gene expressions and epigenetic effects on genotypes associated with response to specific environment is still limited.

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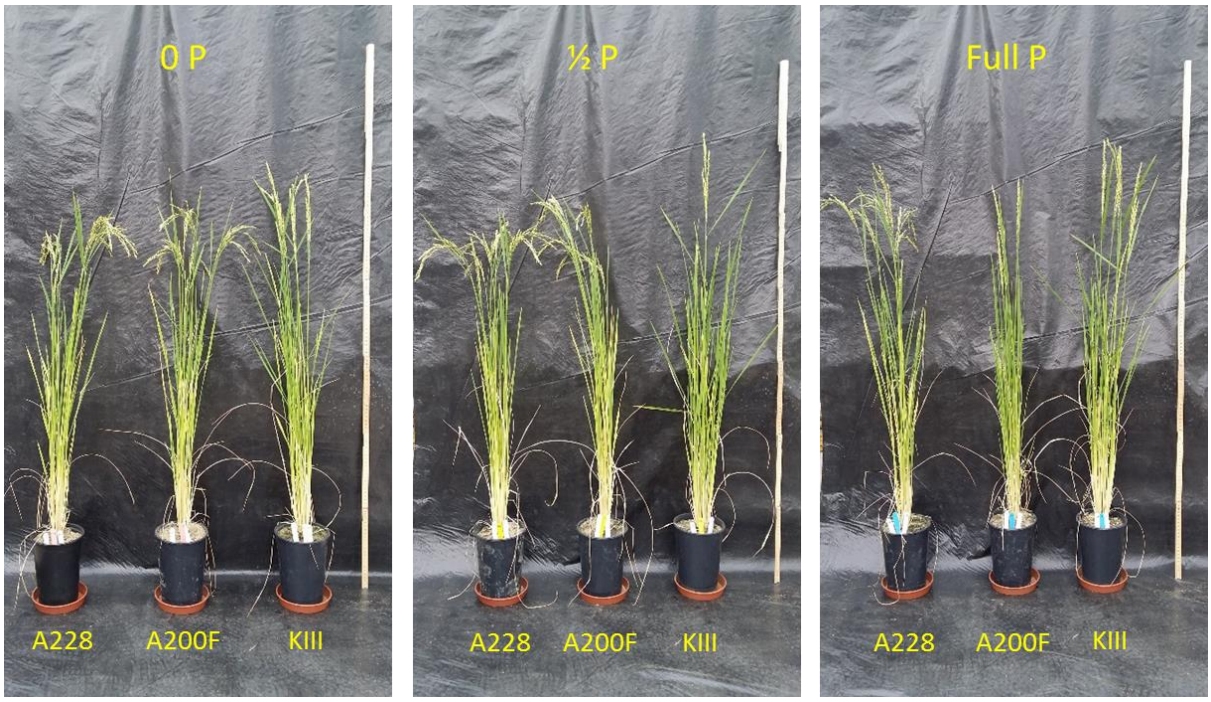
Appendices



Appendix 2.1 Plant height comparisons on Ashoka 228, Ashoka 200F and Kalinga III treated with 0 P, half P and full P at day 95 after sowing.

Appendix 2.2 Measurements of shoot and root length on day 75 of rice varieties treated with 0 P, medium and full P nutrient solutions respectively.

Appendix 2.3 Phosphorus concentration (mg/g) in shoot, root and grain of Ashoka 228, Ashoka 200F and Kalinga III at day 45 (tillering), day 75 (flowering) and day 120 (harvesting) treated in Yoshida's nutrient solution of varying phosphorus concentrations; 0 P, half P and Full P.

Treatment	Genotype	Replication	P conc. (mg/g)						
			day 45		day 75		day 120		
			Shoot	Root	Shoot	Root	Shoot	Root	Grain
0P	Ashoka 228	1	3.48	1.86	2.37	1.35	0.54	0.59	7.13
0P	Ashoka 228	2	2.61	0.96	2.42	1.23	0.85	0.43	4.33
0P	Ashoka 228	3	3.01	2.41	2.47	1.47	0.47	0.88	3.72
0P	Ashoka 228	4	3.71	2.41	2.59	1.26	0.49	0.37	4.31
0P	Ashoka 200F	1	3.83	1.07	2.90	0.59	0.23	0.42	5.84
0P	Ashoka 200F	2	2.98	1.73	2.15	1.09	0.08	0.49	3.97
0P	Ashoka 200F	3	3.66	1.69	2.54	1.44	0.55	0.20	3.89
0P	Ashoka 200F	4	2.78	1.70	2.45	1.26	0.28	0.46	4.34
0P	Kalinga III	1	2.94	1.51	2.70	1.23	0.20	0.75	3.81
0P	Kalinga III	2	3.59	2.97	2.74	0.75	0.34	0.65	5.33
0P	Kalinga III	3	3.93	2.04	3.04	1.40	0.74	0.59	4.26
0P	Kalinga III	4	3.26	1.64	2.51	0.79	0.50	0.57	4.25
Half P	Ashoka 228	1	4.16	1.12	2.25	1.75	1.76	0.43	4.80
Half P	Ashoka 228	2	4.36	1.61	2.68	1.28	1.39	0.71	4.21
Half P	Ashoka 228	3	4.14	1.42	3.47	1.33	1.48	0.57	4.29
Half P	Ashoka 228	4	3.61	1.25	2.63	1.54	1.40	0.62	4.91
Half P	Ashoka 200F	1	3.85	1.68	3.82	1.52	0.68	0.57	4.42
Half P	Ashoka 200F	2	4.78	2.18	3.11	1.71	1.17	0.75	4.75
Half P	Ashoka 200F	3	3.73	2.53	2.84	1.88	1.20	0.57	4.54
Half P	Ashoka 200F	4	3.63	1.91	2.57	1.33	0.96	0.67	3.62
Half P	Kalinga III	1	4.71	1.51	3.67	1.31	1.60	0.78	3.81
Half P	Kalinga III	2	4.07	1.73	3.08	1.42	1.38	0.77	4.19
Half P	Kalinga III	3	4.41	1.04	2.74	1.69	1.07	0.76	4.08
Half P	Kalinga III	4	3.95	1.88	2.95	1.17	1.19	0.84	3.71
Full P	Ashoka 228	1	4.92	1.41	4.35	1.05	1.53	1.06	4.22
Full P	Ashoka 228	2	4.41	2.02	3.25	2.37	1.63	0.53	4.02
Full P	Ashoka 228	3	4.83	1.29	2.58	1.77	1.91	0.78	5.67
Full P	Ashoka 228	4	5.46	1.46	3.78	0.78	1.53	0.97	4.43
Full P	Ashoka 200F	1	3.97	1.00	2.89	1.53	1.38	0.46	4.46
Full P	Ashoka 200F	2	4.33	2.20	3.38	0.83	1.63	0.49	5.21
Full P	Ashoka 200F	3	4.69	1.16	3.54	0.80	2.37	0.26	4.59
Full P	Ashoka 200F	4	4.54	1.36	3.68	1.07	2.01	0.29	5.54
Full P	Kalinga III	1	4.34	1.64	3.14	1.11	1.36	1.07	3.56
Full P	Kalinga III	2	4.85	1.49	2.96	1.40	1.02	0.79	3.73
Full P	Kalinga III	3	5.24	2.32	3.87	1.33	2.57	0.45	4.05
Full P	Kalinga III	4	4.75	1.36	4.07	0.43	1.44	1.04	3.96

Appendix 2.4 A summary table showing the significance of genotype, treatment and their interaction effect towards phosphorus treatments at day 45, day 75 and day 120.

Variables	Number of growth days								
	45			75			120		
	Genotype	Treatment	GxT	Genotype	Treatment	GxT	Genotype	Treatment	GxT
Plant height (cm)	ns	ns	ns	**	ns	ns	**	ns	ns
Root length (cm)	*	ns	ns	ns	ns	ns	ns	*	ns
Tillers per plant	ns	ns	ns	*	ns	ns	*	ns	ns
SPAD Reading	**	ns	ns	**	ns	ns	**	ns	ns
Dry shoot wt. (g/plant)	ns	ns	ns	ns	ns	ns	**	**	ns
Dry root wt. (g/plant)	ns	ns	ns	ns	ns	ns	*	ns	ns
Counted grain/pot	n/a	n/a	n/a	n/a	n/a	n/a	*	ns	ns
Total grain weight (g)	n/a	n/a	n/a	n/a	n/a	n/a	*	ns	ns
Single grain weight (mg)	n/a	n/a	n/a	n/a	n/a	n/a	**	ns	ns
P conc. in shoot ($mg.g^{-1}$)	ns	**	ns	ns	**	ns	ns	**	ns
P uptake in shoot (mg/g)	ns	**	ns	ns	**	ns	ns	**	ns
P conc. in root ($mg.g^{-1}$)	ns	ns	ns	ns	ns	ns	**	ns	ns
P uptake in root (mg/g)	ns	ns	ns	ns	ns	ns	ns	*	ns
P conc. in grain ($mg.g^{-1}$)	n/a	n/a	n/a	n/a	n/a	n/a	ns	ns	ns
P uptake in grain (mg/g)	n/a	n/a	n/a	n/a	n/a	n/a	ns	ns	ns
Total plant P uptake (mg/g)	ns	**	ns	ns	*	ns	ns	*	ns
P utilization efficiency (PUE) (mg/mg)									

*, **,ns, n/a; Significance at the $p < 0.05$, $p < 0.01$, non-significant, not applicable.

Appendix 2.5 Effect of different P concentrations on plant growth variables of Ashoka 200F, Ashoka 228 and Kalinga III at 45 days after sowing.

45 day	**Treatments**								
	0 P (0 mg.kg^{-1})			**Half P (49 mg.kg^{-1})**			**Full P (98 mg.kg^{-1})**		
Variables	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**
Plant height (cm)	59.6 ± 3.58a	56.9 ± 1.04a	59.3 ± 0.79a	61.5 ± 3.36a	55.1 ± 1.89a	61.5 ± 1.86a	58.7 ± 2.16a	59.1 ± 1.47a	61.6 ± 1.61a
Root length (cm)	31.1 ± 1.36b	24.0 ± 0.54a	24.9 ± 1.20a	30.0 ± 1.90b	25.4 ± 0.83ab	23.9 ± 0.75a	26.9 ± 2.68a	28.1 ± 1.95a	29.1 ± 2.75a
Tillers per plant	7.8 ± 0.63a	7.5 ± 0.29a	7.5 ± 0.96a	7.5 ± 0.50a	8.5 ± 0.50a	7.3 ± 0.48a	8.8 ± 0.48a	8.3 ± 0.75a	7.8 ± 0.75a
SPAD Reading	29.8 ± 1.31a	34.2 ± 1.19b	29.8 ± 0.60a	27.5 ± 0.87a	32.7 ± 1.41b	30.6 ± 0.85ab	29.2 ± 1.28a	31.2 ± 1.12a	30.3 ± 1.29a
Dry shoot wt. (g/plant)	2.8 ± 0.03a	2.6 ± 0.11a	2.6 ± 0.21a	2.9 ± 0.16a	2.9 ± 0.09a	2.7 ± 0.15a	3.0 ± 0.17a	2.8 ± 0.10a	2.8 ± 0.17a
Dry root wt. (g/plant)	4.0 ± 0.49a	3.1 ± 1.27a	2.0 ± 0.32a	2.1 ± 0.41a	3.7 ± 0.74a	4.0 ± 1.03a	4.9 ± 1.18a	4.1 ± 0.69a	3.3 ± 0.55a
P conc. in shoot (mg.g^{-1})	3.3 ± 0.26a	3.2 ± 0.25a	3.4 ± 0.21a	4.0 ± 0.26a	4.1 ± 0.16a	4.3 ± 0.17a	4.4 ± 0.16a	4.9 ± 0.22a	4.8 ± 0.18a
P uptake in shoot (mg/g)	9.1 ± 0.26a	8.2 ± 0.25a	9.0 ± 0.21a	11.3 ± 0.26a	11.9 ± 0.16a	11.7 ± 0.17a	13.0 ± 0.16a	13.6 ± 0.22a	13.4 ± 0.18a
P conc. in root (mg.g^{-1})	1.5 ± 0.49a	1.9 ± 1.27a	2.0 ± 0.32a	2.1 ± 0.41b	1.4 ± 0.74a	1.5 ± 1.03ab	1.4 ± 1.18a	1.5 ± 0.69a	1.7 ± 0.55a
P uptake in root (mg/g)	6.0 ± 0.83a	4.8 ± 0.68a	3.8 ± 0.41a	4.2 ± 0.46a	4.9 ± 0.75a	5.6 ± 0.88a	6.1 ± 0.66a	6.2 ± 0.76a	5.2 ± 0.44a
Total Plant P uptake (mg/g)	32.6 ± 2.67a	27.0 ± 2.79a	25.0 ± 2.37a	29.9 ± 2.20a	35.9 ± 4.38a	39.3 ± 5.53a	44.3 ± 5.07a	44.5 ± 4.72a	38.9 ± 3.17a

Different P treatments and variables for Ashoka 200F, Ashoka 228 and Kalinga III at 45 days. Values represent means (n=4) and ± standard error of the mean (± SEM). Means with different letters within same row are significantly different between genotypes within treatments ($p < 0.05$).

Appendix 2.6 Effect of different P concentrations on plant growth variables of Ashoka 200F, Ashoka 228 and Kalinga III at 75 days after sowing.

75 day	Treatments								
	0 P (0 mg.kg^{-1})			Half P (49 mg.kg^{-1})			Full P (98 mg.kg^{-1})		
Variables	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**
Plant height (cm)	69.4 ± 3.60a	84.6 ± 2.00b	70.6 ± 0.75a	73.1 ± 1.51a	84.6 ± 1.39b	76.1 ± 3.35ab	69.5 ± 2.60a	86.6 ± 1.30c	77.2 ± 14.61b
Root length (cm)	31.5 ± 0.98b	23.4 ± 0.94a	30.3 ± 2.54b	31.4 ± 2.44a	31.0 ± 2.80a	29.6 ± 2.56a	32.8 ± 3.06a	30.4 ± 2.15a	33.5 ± 4.93a
Tillers per plant	13.0 ± 0.41b	10.0 ± 0.91a	11.3 ± 0.25ab	13.3 ± 0.95a	12.5 ± 0.65a	10.8 ± 0.48a	12.8 ± 1.75a	11.8 ± 0.25a	11.8 ± 0.75a
SPAD Reading	36.3 ± 0.55a	37.8 ± 0.25a	39.7 ± 0.24b	37.3 ± 0.46a	36.2 ± 1.09a	38.7 ± 0.88a	35.4 ± 0.56a	37.3 ± 0.85ab	39.1 ± 0.94b
Dry shoot wt. (g/plant)	8.4 ± 0.13a	9.1 ± 0.24a	8.6 ± 0.41a	8.9 ± 0.20a	9.1 ± 0.27a	9.0 ± 0.63a	9.5 ± 0.21a	9.6 ± 0.32a	9.0 ± 0.22a
Dry root wt. (g/plant)	11.4 ± 3.99a	5.2 ± 0.43a	7.0 ± 1.83a	7.2 ± 2.05a	4.7 ± 0.86a	6.2 ± 1.14a	11.5 ± 2.46a	7.7 ± 1.98a	13.6 ± 4.59a
P conc. in shoot (mg.g^{-1})	2.5 ± 0.15a	2.5 ± 0.05a	2.8 ± 0.11a	3.1 ± 0.27a	2.8 ± 0.21a	3.1 ± 0.20a	3.4 ± 0.17a	3.5 ± 0.38a	3.5 ± 0.27a
P uptake in shoot (mg/g)	21.1 ± 1.15a	22.4 ± 0.95a	23.5 ± 0.44a	27.2 ± 2.06a	25.9 ± 2.05a	27.6 ± 1.59a	31.9 ± 1.73a	33.4 ± 3.03a	31.4 ± 2.10a
P conc. in root (mg.g^{-1})	1.1 ± 0.18a	1.3 ± 0.05a	1.0 ± 0.16a	1.6 ± 0.12a	1.5 ± 0.11a	1.4 ± 0.11a	1.1 ± 0.17a	1.5 ± 0.36a	1.1 ± 0.22a
P uptake in root (mg/g)	10.5 ± 3.02a	6.9 ± 0.83a	6.6 ± 1.09a	11.3 ± 2.95a	6.9 ± 1.37a	8.5 ± 1.45a	11.4 ± 2.31a	9.8 ± 1.71a	11.5 ± 1.72a
Total plant P uptake (mg/g)	69.7 ± 11.80a	54.1 ± 2.54a	57.5 ± 4.57a	76.4 ± 13.25a	59.5 ± 2.69a	68.5 ± 6.80a	93.3 ± 13.07a	86.0 ± 9.72a	102.4 ± 19.84a

Different P treatments and variables for Ashoka 200F, Ashoka 228 and Kalinga III at 75 days. Values represent means (n=4) and ± standard error of the mean (± SEM). Means with different letters within same row are significantly different between genotypes within treatments ($p < 0.05$).

Appendix 2.7 Effect of different P concentrations on plant growth variables of Ashoka 200F, Ashoka 228 and Kalinga III at 120 days after sowing.

120 day	**Treatments**								
	0 P (0 mg.kg^{-1})			**Half P (49 mg.kg^{-1})**			**Full P (98 mg.kg^{-1})**		
Variables	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**	**Ashoka 200F**	**Ashoka 228**	**Kalinga III**
Plant height (cm)	101.1 ± 3.14a	95.5 ± 0.98a	99.2 ± 1.23a	108.2 ± 2.14b	91.7 ± 3.14a	102.2 ± 2.75ab	99.2 ± 5.63a	98.2 ± 2.40a	102.2 ± 0.76a
Root length (cm)	24.8 ± 0.43a	26.6 ± 2.46a	27.9 ± 3.54a	28.0 ± 1.21a	27.0 ± 2.19a	27.5 ± 4.51a	36.3 ± 3.47a	29.1 ± 3.64a	32.9 ± 0.77a
Tillers per plant	13.0 ± 0.41b	10.0 ± 0.91a	11.3 ± 0.25ab	13.3 ± 0.95a	12.5 ± 0.65a	10.8 ± 0.48a	12.8 ± 1.75a	11.8 ± 0.25a	11.8 ± 0.75a
SPAD Reading	32.9 ± 1.12b	20.1 ± 1.27a	30.2 ± 2.66b	35.9 ± 1.32b	22.1 ± 0.50a	29.9 ± 2.81b	32.5 ± 1.18b	25.1 ± 2.32a	29.4 ± 1.79ab
Dry shoot wt. (g/plant)	11.3 ± 0.21b	10.0 ± 0.21a	10.7 ± 0.18ab	12.5 ± 0.34b	10.8 ± 0.23a	11.5 ± 0.43ab	12.9 ± 0.48a	11.9 ± 0.31a	13.1 ± 0.34b
Dry root wt. (g/plant)	13.0 ± 4.93b	6.0 ± 0.98a	6.1 ± 0.75a	8.0 ± 1.12a	7.0 ± 1.58a	5.5 ± 0.53a	13.9 ± 1.56b	5.4 ± 1.00a	8.6 ± 3.39a
Dry grain wt. (g/plant)	11.1 ± 0.36a	10.4 ± 0.40a	11.2 ± 0.23a	10.6 ± 0.35ab	10.1 ± 0.43a	12.2 ± 0.64b	11.1 ± 0.23a	10.3 ± 0.31a	12.8 ± 0.21b
Counted grain/pot	609.3 ± 26.25a	596.0 ± 81.82a	525.0 ± 14.23a	600.8 ± 10.24b	508.5 ± 20.07a	549.3 ± 9.86ab	641.3 ± 14.46b	494.5 ± 17.13a	598.0 ± 21.87b
P conc. in shoot (mg.g^{-1})	0.3 ± 0.10a	0.6 ± 0.09a	0.5 ± 0.12a	1.0 ± 0.12a	1.5 ± 0.09a	1.3 ± 0.12a	1.9 ± 0.22a	1.7 ± 0.09a	1.6 ± 0.34a
P uptake in shoot (mg/g)	3.2 ± 1.09a	5.9 ± 0.95a	4.8 ± 1.22a	12.6 ± 1.78b	16.4 ± 1.30b	15.0 ±1.30b	23.7 ± 2.16a	19.7 ± 1.53a	20.8 ± 4.30a
P conc. in root (mg.g^{-1})	0.4 ± 0.07a	0.6 ± 0.11a	0.6 ± 0.04a	0.6 ± 0.04ab	0.6 ± 0.06a	0.8 ± 0.02b	0.4 ± 0.06a	0.8 ± 0.12b	0.8 ± 0.14b
P uptake in root (mg/g)	4.2 ± 0.67a	3.1 ± 0.20a	3.8 ± 0.31a	5.2 ± 0.92a	3.8 ± 0.45a	4.4 ± 0.50a	4.9 ± 0.27a	4.4 ± 0.90a	5.9 ± 0.87a
P conc. in grain (mg.g^{-1})	4.5 ± 0.45a	4.9 ± 0.77a	4.4 ± 0.32a	4.3 ± 0.25a	4.6 ± 0.18a	3.9 ± 0.11a	5.0 ± 0.23b	4.6 ± 0.37ab	3.8 ± 0.11a
P uptake in grain (mg/g)	49.6 ± 4.25a	51.0 ± 9.04a	49.1 ± 2.90a	45.8 ± 2.73a	45.9 ± 3.25a	48.0 ± 2.13a	55.3 ± 3.55a	47.3 ± 4.50a	48.8 ± 1.57a
Total plant P uptake (mg/g)	181.3 ± 24.01a	159.8 ± 21.45a	153.7 ± 8.58a	185.0 ± 10.17a	186.1 ± 18.18a	176.4 ± 6.13a	273.9 ± 16.80a	194.3 ± 10.91a	217.9 ± 31.73a

Different P treatments and variables for Ashoka 200F, Ashoka 228 and Kalinga III at 120 days. Values represent means (n=4) and ± standard error of the mean (± SEM). Means with different letters within same row are significantly different between genotypes within treatments ($p < 0.05$).

Appendix 3.1. Shoot height and root length of seven rice genotypes (Kalinga III, Ashoka 228, Ashoka 200F, PY 84, QTL7, QTL9 and MRQ 76) treated in Yoshida's nutrient solution of varying Phosphorus concentrations; 0 P, Half P and Full P at day 7.

0 P	Rep	MRQ 76	Ashoka 228	Ashoka 200F	Kalinga III	PY 84	QTL7	QTL9
Shoot height (cm)	1	7.0	9.8	8.4	9.0	12.3	9.4	9.5
	2	7.0	9.5	9.0	8.5	10.0	9.7	8.5
	3	6.0	10.0	8.8	8.2	8.8	9.5	9.0
	4	7.5	10.5	8.3	9.3	9.4	9.2	9.0
Root length (cm)	1	10.5	7.0	8.7	7.0	7.5	6.8	8.0
	2	11.5	6.5	9.6	6.5	8.0	8.5	7.2
	3	11.0	6.5	9.8	8.5	6.2	8.6	7.0
	4	10.0	7.5	10.0	8.8	6.8	7.5	7.0

Half P	Rep	MRQ 76	Ashoka 228	Ashoka 200F	Kalinga III	PY 84	QTL7	QTL9
Shoot height (cm)	1	9.5	12	9.4	7.8	9.3	10.6	11.5
	2	10.0	0.0	8.5	10.0	10.3	10.5	10.0
	3	8.0	11.5	7.2	10.0	9.2	9.5	9.7
	4	6.8	11	8.5	8.8	8.3	10.0	11.0
Root length (cm)	1	11.2	8.0	11.0	8.3	7.0	9.2	8.5
	2	9.4	0.0	10.5	9.5	8.0	8.5	9.1
	3	9.5	7.4	5.8	8.7	7.5	8.5	7.9
	4	8.8	8.5	10.0	8.5	7.2	8.5	7.8

Full P	Rep	MRQ 76	Ashoka 228	Ashoka 200F	Kalinga III	PY 84	QTL7	QTL9
Shoot height (cm)	1	9.8	9.0	10.0	8.9	10.5	9.4	9.5
	2	6.0	11.4	7.5	11.7	8.5	12.3	9.3
	3	9.4	13.3	7.5	6.7	6.7	9.5	7.6
	4	7.0	7.8	8.8	5.5	8.0	13.0	11.7
Shoot height (cm)	1	10.2	4.5	9.0	5.4	8.4	4.0	8.5
	2	8.5	5.4	2.5	4.5	4.0	6.0	4.2
	3	9.0	6.4	2.5	3.5	3.5	5.4	4.9
	4	8.4	8.0	7.5	3.3	7.0	6.0	6.0