*Journal of Biotechnology* **16**(4): 621-631, 2018

# INVESTIGATING THE GENETIC VARIABILITY OF 12 VIETNAMESE RICE ACCESSIONS (*ORYZA SATIVA* L.) IN RESPONSE TO PHOSPHORUS DEFICIENCY

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Received: 10.5.2018
Accepted: 01.10.2018

SUMMARY

Phosphate is the second major factor limiting on crop productivity and leads to various physiological disorders that would consequently affect plant development. In response to phosphate starvation, plants have to improve their root systems for efficiently acquired phosphate. In Vietnam, rice is one of the most important agricultural crops in which 60% of the rain-fed lowland rice is cultured on soil types, which are low in phosphorus or phosphate fixing. This fact along with the scenario in which phosphate resources are running out in fast pace, highly provoked an indispensible need for developing new rice varieties with high productivity under low phosphate condition. An emerging approach in order to accomplish this goal is through genetic improvement of local rice resources. Taking advantage of that, in this study, out of 182 sequenced Vietnamese rice accessions, 12 representatives from 3 distinguishing rice groups: *Indica, Japonica* and admix *Indica* subgroup were chosen. The variation in number of crown root, root length, shoot length, root mass, shoot mass, chlorophyll content and root anatomy were examined as parameters to analyze the effect of phosphorus deficiency (1 µM) compare to full phosphate medium (300 µM). The minimum number of plantlets used for each condition was 15. Each genotype was grown in Yoshida hydroponic medium (changed every 4 days, harvesting was made after 14 days). The experimental results showed a remarkable variation of different rice accessions in response to phosphate deficiency. The starvation of phosphate significantly affected almost all examined traits in which several accessions such as G38, G93, G165, G223, G62 accessions were potential hypersensitive and G11, G177 accessions were potential tolerant to P deficiency. These results are interesting and encouraging to further research for screening our Vietnamese rice collection in order to identify the set of potential genes responsible for tolerance in phosphorus starvation condition.

**Keywords:** *Modern agriculture, Oryza sativa, Phosphorus starvation, Phosphorus tolerant, Root traits*

## INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food crop for more than half of the world's population (Ismail *et al*., 2007). The rapid increase of world population, which is estimated to be 9 billion people in 2050 according to United Nations, puts more pressure on agriculture in order to meet the high demands for crop yields. Rice is currently grown in over a hundred countries, and produces approximately 480 million metric tons of milled rice annually (Muthayya *et al*., 2014). Phosphorus (P) is the second most essential element after Nitrogen for plant to grow and develop, and rice requires P to survive and thrive. Particularly, P involves in nucleic acid synthesis in which P is one of components of nucleotide. P also involves in membrane synthesis and stability in which P together with lipid to make phospholipid layer in membrane. Moreover, it also supplies energy for photosynthesis and respiration process (Niu *et al*., 2013). In soil, P presents in the form of either inorganic which strongly interacts with divalent or trivalent cations or organophosphates which then be hydrolyzed to release P for root uptake (Péret *et al*., 2014).

In rice, P deficiency, which is considered as an abiotic stress for plants, leads to various physiological disorders such as stunted growth, reduced tillering, thin and spindle-like stems, reduced number of grains per panicle. However, most normal soils do not contain sufficient readily available P to meet the high demands of crops. In fact, P concentration in the soil solution is usually much less than 0.3 mg/L P while plant tissues need 300 mg/kg P to develop normally (P uptake in West Africa) (Kauwenbergh, 2010). Most of soil phosphorus is tightly held to the surface of soil particles or is fixed as organic phosphorus compounds, and is therefore relatively unavailable for plant uptake. Thus, modern agriculture relies heavily on P fertilizers. Farmers across the world use 170 million tons of P fertilizers annually. Nevertheless, P rock is a finite, non-renewable resource, and has been predicted variably to be deleted in approximately 300–400 years (Kauwenbergh *et al.*, 2010). Furthermore, a part of fertilizer-derived phosphorus is lost into ground and surface water, which would give the possibility of damaging the surrounding environment, or giving suitable condition for algae to be well developed in river, which will subsequently inhibit aqueous useful living organisms to develop. In Asia including Bangladesh, India, Indonesia, Nepal, Pakistan, South China and Viet Nam, 60% of the rain-fed lowland rice is produced on poor and problematic soils that are naturally low in phosphorus or P fixing (Gamuyao *et al*., 2012) (Figure 1).

In response to P deficiency, plants have developed different adaptive strategies to acquire more P from soil. These strategies include inhibition of primary root length (Svistoonoff *et al*., 2007), proliferation of lateral roots (Pérez-Torres *et al*., 2008), increase in the density and length of root hairs (Pérez-Torres *et al*., 2008), enhanced root cortical aerenchyma formation (Vejchasarn *et al*., 2016), and formation of cluster roots. Beside the natural strategies of the plant itself, in order to create a long-term solution, human also need to develop advanced strategies to cope with P deficiency problem, and the development of rice varieties with high productivity under low P and other stress conditions is a valid and necessary approach to improve yield and enhance food security in rice-dependent countries, especially in Vietnam where rice is considered as one of the staple food crop.

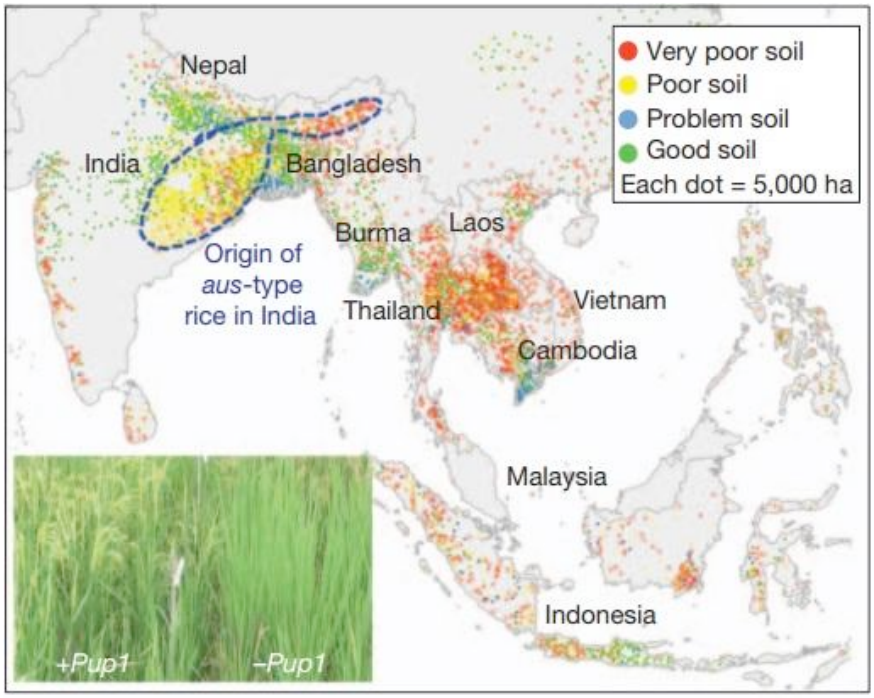



**Figure 1.** Problem soils in Asia and breeding lines with and without the tolerant *Pup1* locus under P-deficient field conditions (Source (Gamuyao *et al*., 2012)).

The search for P deficiency tolerance responsible genes has begun long time ago. In India, the *aus*-type variety Kasalath was found to be tolerant to P deficiency. Further genetic analysis found that there were several regions of the rice genome that are associated with improved phosphorus efficiency, and the first discovered region namely *pup1* was located on chromosome 12 (Gamuyao *et al*., 2012). Phosphorus starvation gene (*PsTOL1*) which belongs to this region acts as an enhancer of early root growth and promotes more P uptake. This discovery was considered as a breakthrough on the way to deal with P starvation condition in the world. To continue this approach, our research focuses on finding potential rice accessions which are able to give material for the next step of quantitative trait loci (QTL). This will help reveal the gene set of P deficiency resistant specifically in the collection of Vietnamese rice. In this study, 12 representative rice cultivars were chosen from Vietnamese rice collection to investigate their genetic variation and plasticity in response to low P.

## MATERIALS AND METHODS

### Plant material and growth condition

*Oryza sativa* accession G11, G38, G53, G62, G93, G119, G125, G138, G150, G165, G177, G223 were provided by Plant Resource Center, Hanoi (Table 1). At first, the seeds were kept in the oven at 50°C for 3 days. Then, seeds were sterile for 2 min with ethanol 70°C and for 25 min with NaClO, followed by several washes by sterile water. After that, seeds were left in the dark at 28°C overnight to absorb water. Then after, seedlings were germinated in MS/4 agar. After 5 days, one set of rice was transferred to Yoshida hydroponic culture with full phosphate (P0) (300 µM) (Yoshida *et al*., 1976). One set of rice was grown in phosphorus deficiency condition (P*) (1 µM). The hydroponic experiments were carried out in a programmable growth chamber (DAIHAN Scientific, ThermoStable GC-450) with light intensity of 12000 Lux followed a 16-h-light (28°C)/8-h-dark (26°C) photoperiod and the humidity was controlled at approximate 80%. The solution was refreshed every 4 days.

**Table 1.** Phosphate deficiency evaluation.

| Accession number | ID | Name | Sub-group | Varietal group | Ecosystem |
|---|---|---|---|---|---|
| 1 | G53 | Lua Can Do | I6 | *Indica* | Unknown |
| 2 | G62 | QuangTrang | I5 | *Indica* | Irrigated |
| 3 | G119 | L26 | Im | Admix | Irrigated |
| 4 | G138 | Nang Quat | Im | Admix | Rainfed lowland |
| 5 | G150 | Nep Dia Phuong | I2 | *Indica* | Irrigated |
| 6 | G223 | BleBlau Tan | J1 | *Japonica* | Upland |
| 7 | G11 | Tam TronHai Duong | I4 | *Indica* | Unknown |
| 8 | G38 | Nep Nuong | J1 | *Japonica* | Upland |
| 9 | G93 | Po Le Po Lau Xa | I5 | *Indica* | Upland |
| 10 | G125 | Nep Nuong | Im | Admix | Unknown |
| 11 | G165 | Giong 90 Ngay | I1 | *Indica* | Rainfed lowland |
| 12 | G177 | Cham Hom | J1 | *Japonica* | Unknown |

### Analyzing plant traits

After 15 days, rice plants were harvested to analyze traits (number of crown root, root length, shoot length). After measuring these trait, rice plants were dried in desiccator at 70°C for at least 5 days to be completely dry. Parts of the plant were separated as shoot and root. Shoot is the upper part from the basal and root is the lower part from the basal. The shoot weight and plant weight were measured, the root weight was calculated as the subtraction of total plant weight and the shoot weight. Three fresh rice plants were used to extract chlorophyll. All experiments were performed in triplicate.

### Chlorophyll extraction

Adapted from Rajalakshmi and Banu (2015) with some modifications, about 20 mg of fresh leaf

was cut and weighed precisely. Samples were stored in 2 mL Eppendorf tube in ice to avoid oxidation. Balls were added in each Eppendorf tubes and the whole tube was frozen in liquid nitrogen. Samples were grinded in 2 times of 2 min, 20 rpm by tissue lyser (Qiagen). Subsequently, 1.5 mL of extraction solvent containing 85% acetone, 15% Tris buffer, pH 8.0 was added into the tube, then mixtures were centrifuged at 4°C in 15 min, 12000 g. One mL of supernatant was added into 1.5 mL Eppendorf tubes, stored at 4°C. Samples were diluted with distilled water to obtain the absorbance about 0.1 to 0.3 and then measured at 645 and 663 nm. The chlorophyll content was determined as the equation:

Total chlorophyll (µg/mL) = 20.2 (A645) + 8.02 (A663)

Chlorophyll *a* (µg/mL) = 12.7 (A663) – 2.69 (A645)

Chlorophyll *b* (µg/mL) = 22.9 (A645) – 4.68 (A663)

**Vibratome sectioning**

The samples (5 cm from root tip of crown root) were stored in PBS 1X at 4°C right after harvesting. Root samples were fixed straightly in 6% agar until the agar is solidified. Sections were cut at 7 µm thick by Microm HM 605V (Thermo Scientific). Pictures were taken with Infinity Lumenera 3 microscopy.

**Statistical analysis**

Statistical analysis (two way ANOVA or Student t-test) was conducted using Graphpad Prism 6 (https://www.graphpad.com/).

## RESULTS AND DISCUSSION

In order to achieve a wide range of rice diversity, out of 182 Vietnamese rice accessions, 12 representative rice accessions have been chosen. Based on the genetic nature, these 12 accessions belong to 3 different rice groups: *Indica*, *Japonica* and admix *Indica* subgroup (Table 1) (Phung *et al.*, 2014).

**Change of pH during culture time**

Before entering the main steps of our research, a screening test for gradient of P concentration in Yoshida medium was carried out. The gradient of P concentration had been tested at many different levels, eventually two concentrations which are P (300 µM) and P (1 µM) had been chosen since this range show the optimum impact in term of observing P starvation effect (data not shown). Yoshida medium at 1 µM P and 300 µM P were set as standard low P and standard high P treated conditions, respectively.

Since pH and the onset of P are strongly related, pH changed by time is one of the essential indexes that needed to be examined (Figure 2). Analysis of pH will help determine the range between two times of culture media refreshment in order to achieve the optimal pH condition for rice to uptake P. Generally, P availability to rice is optimum when pH is between 6.0 and 6.5. In acidic condition (pH < 6.0), P is associated with iron and aluminum compounds whereas when pH is greater than 6.5, P is primarily associated with calcium and magnesium that are slowly available to most plants (Cerozi, Fitzsimmons, 2016). Results showed that pH values increased gradually from day 1 to day 3 to reach 6.2 and 6.0 in P0 and P* media respectively. Because in our Yoshida hydroponic media, there was an addition of 0.3 mg/L MES which help to stabilize pH media, we obtained a slow increase instead of decreasing of pH value at the beginning of culture. After 3 days, pH increased rapidly and reached 6.61 and 6.20 at day 4, 6.93 and 6.54 at day 9 which were no longer optimized for root to uptake P. Therefore, day 4 was chosen as the standard pace for changing media in both P0 and P*. The difference in term of pH between P0 and P*(where P0 medium always has a more upward trend compared to P*) can be explained by the dissociation of $NaH_2PO_4$ which is considered as the phosphorus supplier in our hydroponic medium. $NaH_2PO_4$ dissociated and gives rise to the production of Na+ ion, hence increase the pH of the medium. P0 apparently has a higher concentration of $NaH_2PO_4$ that will consequently lead to a more significant increase of pH. The 4-day changing schedule has been tailored for our experiment, yet medium change can be varied in different groups depends on the length of culture and medium composition, i.e., every 3 days in previous article (Gamuyao *et al*., 2012; Vigueira *et al.*, 2016; Neelam *et al*., 2017) or every day in (Mehra *et al*., 2015).

**Genetic variation of 12 representative rice accessions grown under high P condition**

Different traits which normally be affected strongly by P deficiency were chosen including: number of crown root, root length, shoot length, root weight, shoot weight and plant weight to evaluate the

genetic variation of 12 rice accessions grown under normal condition in Viet Nam. Results showed the large phenotypic variations in these traits (Figure 3) which are very interesting and could be useful for genetic mapping studies and identifying potential sources of trait variation. The most significant variation between the 12 traits was observed in root weight trait. They could be divided into 2 distinctive groups: the first group which had much greater root weight included rice accession G38, G93, G125, G165, G177, and the second group which had much lower root weight included rice accession G119, G150, G223. Root development has been shown to play an important role for plant P acquisition (Lynch, 1995). A number of studies have demonstrated the genotypic variations determining the changes in root that will subsequently allow adaptations of plants to P deficiency (Chiou, Lin, 2011; Rouached *et al*., 2010). Root weight is one of the most basic trait to determine root evolution. Despite the fact that this data could not reflect orientation of development in root, the fluctuation in term of root weight between the 12 chosen accessions revealed generally which genetic strategies that accession would utilize to collect P from the environment and how vulnerable the root system of that accession is in term of P adaption. The development of leaf length displays with very low variation between the 12 tested accessions. One notice for this result is that the insignificant differences between accessions do not indicate for a paradox to our hypothesis. Indeed, this is somehow complementary since uptaking P is just the starting point in P metabolism in rice. Regardless of the alteration of root system, the final result, which expressed in shoot length has enhanced the role of P in the general development our subject.

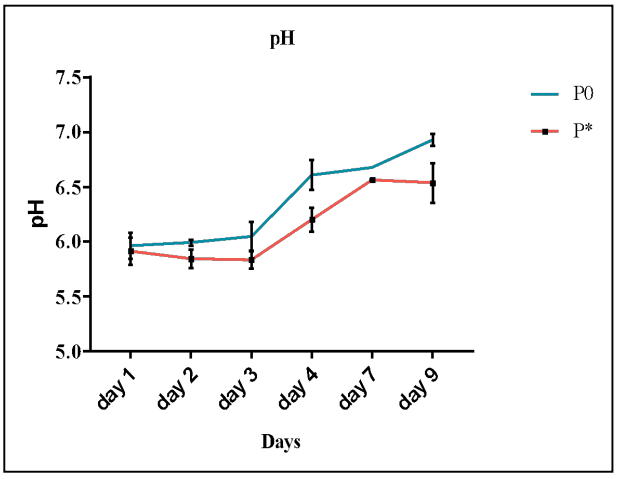



**Figure 2.** pH change by time. P0: medium with high phosphate (300 µM), P*: medium with phosphorus starvation (1 µM). Relative values are represented as mean values ± standard deviation calculated from three replicates.

## Correlation between traits

The correlation between traits was also evaluated (Figure 4). The coefficient of correlation (r) between root weight and shoot weight was 0.87 in P0 medium and was 0.93 in P* medium. R between shoot weight and plant weight was 0.98 in P0 medium and was 0.99 in P* medium. These r-values illustrate the correlation between roots, shoot, and plant weight in term of development pattern, which indicate a strong connection between these three traits regardless the concentration of P within the media. Root weight was also strongly correlated with shoot weight in Pearl millet [*Pennisetum glaucum* (L.) R. Br] in P* medium (r = 0.95) (Gemenet *et al*., 2015). Number of crown root was averagely correlated to root weight and plant weight (r was around 0.4 to 0.5) since root weight and plant weight were calculated including crown root weight. Number of crown root did not associate with root length because crown root was developed from stem nodes and its number often highly correlated with culture condition while root length was measured from seminal roots or lateral roots.

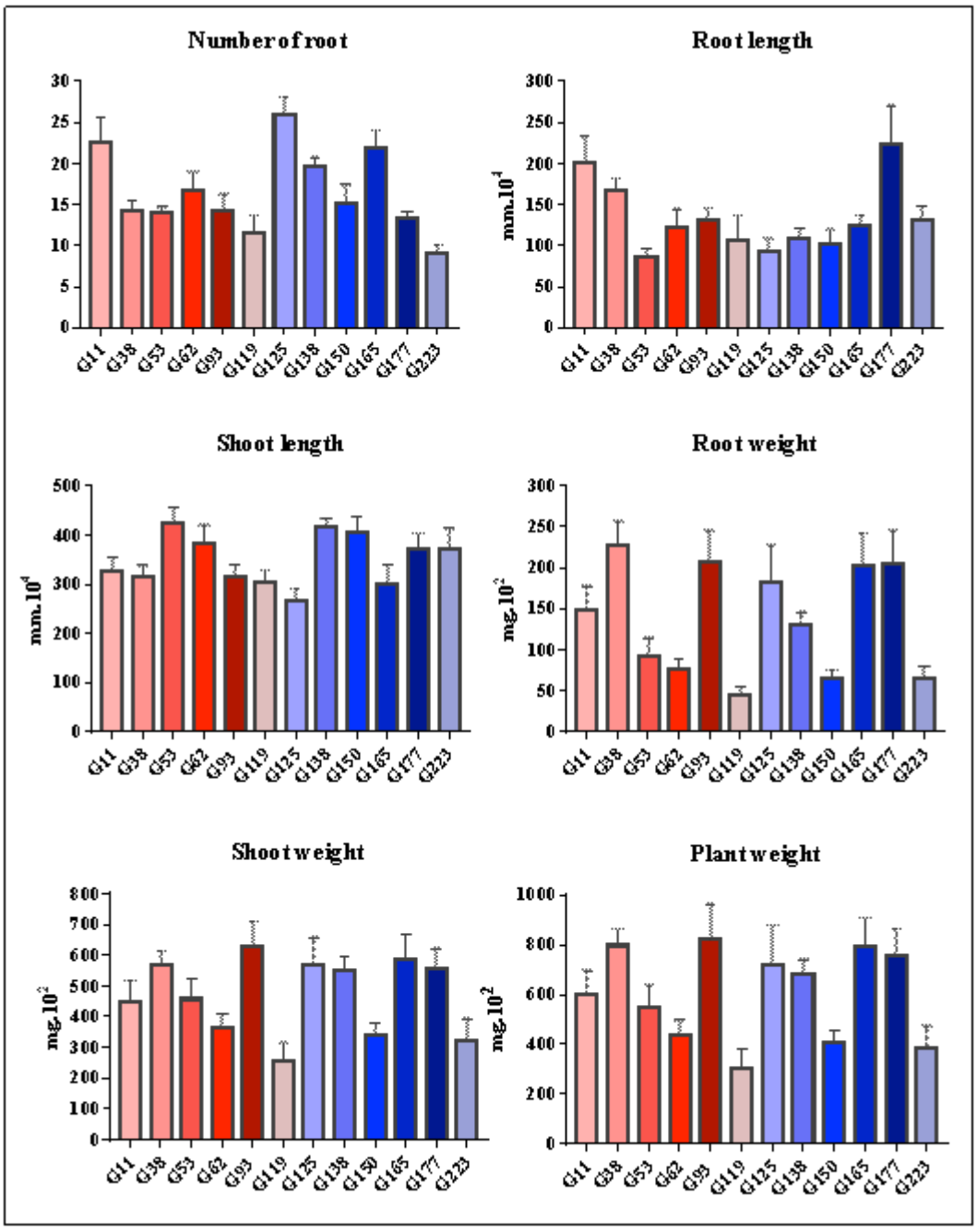
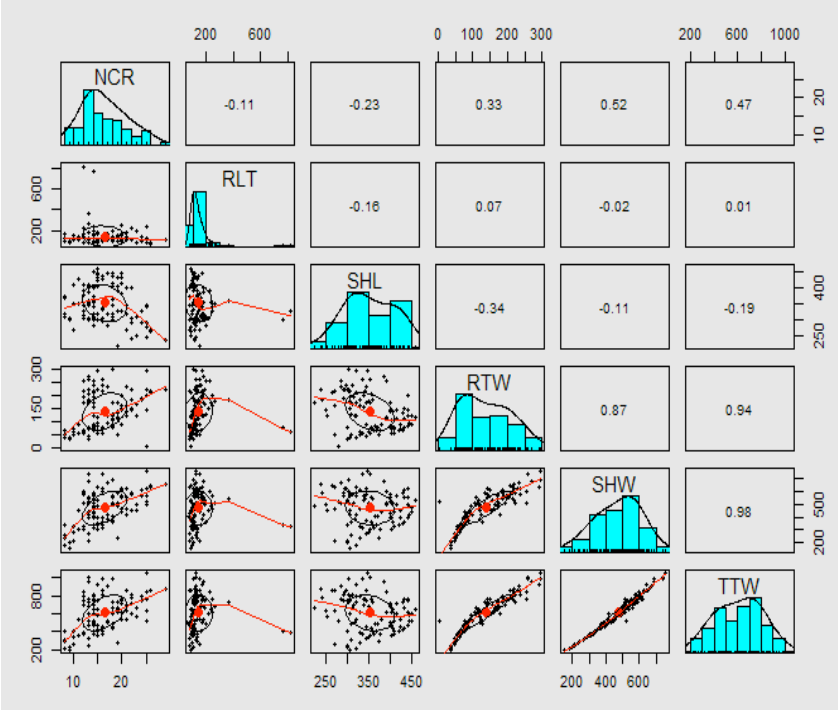
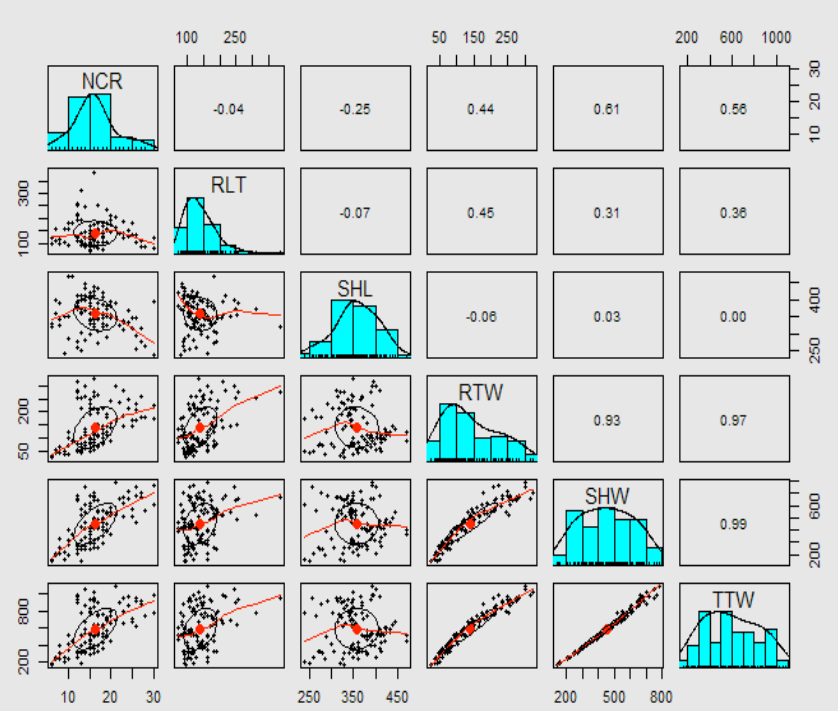



**Figure 3.** The variation of number of crown root, root length, shoot length, root weight, shoot weight and plant weight of 12 rice accessions grown under high phosphate. Relative values are represented as mean values ± standard deviation calculated from 12-15 replicates.




**A**




**B**

**Figure 4.** Correlation between number of crown root (NCR), root length (RTL), shoot length (SHL), root weight (RTM), shoot weight (SHM) and plant weight (TTW) of 12 rice cultivars grown under high phosphate (B) and P starvation (A).

**Root anatomy**

Root system of rice grown under P starvation was highly different compared to the one grown under high P in some accessions (Figure 5). Phenotypic plasticity in root system structure plays a key role in the stress tolerance of crop plants. Results showed that there was genetic variation in 12 tested rice accessions towards numberof root hairs, length of root hairs. Root hairs were much longer, denser in sensitive accessions in response to P starvation while there was nearly no change in these traits in tolerant accessions. The importance of root hair traits for P uptake is well described (Lynch, 2011; Brown *et al*., 2013), and it has been suggested that the more root hairs plants have, the more efficiency of P uptake they acquire (Vejchasarn *et al*., 2016). P starvation also promoted lateral root growth and enhanced root hair formation in *Arabidopsis thaliana* (Nacry *et al*., 2005; Bates, Lynch, 1996). The response to P deprivation was also very strong in cortical aerenchyma formation in sensitive accessions in which living cortical cell (Figure 5B) was replaced by air-filled lacunae (Figure 5C). This could lead to reduce root respiration and the metabolic cost of soil exploration and permit longer root development in maize (Zhu *et al*., 2010; Saengwilai *et al*., 2014). The same phenomenon was also obtained to help maize against drought or can live in low nitrogen condition (Zhu *et al*., 2010; Saengwilai *et al*., 2014).

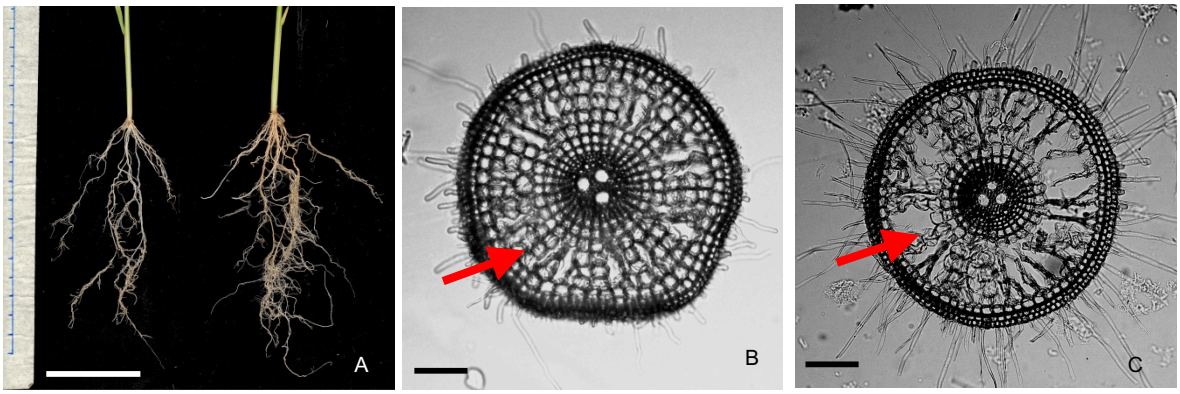



**Figure 5**. Morphological differences in root system of rice grown under high and low P condition. (A) Root system of rice genotype G62 grown under high (left) and low P (right). *Bar 5 cm. (*B, C) Cross section of crown root grown under high and low P, respectively. Red arrow indicates cortical aerenchyma formation. *Bar in B vs C: 10 µm.*

**Effect of P starvation on tested traits**

The difference in P concentration between P* and P0 media affects number of crown root, root length, shoot length, root weight, shoot weight, plant weight and chlorophyll of 12 rice accessions was presented in Figure 6 and Figure 7, respectively. The tolerant accessions to P starvation were defined as the ones having very small absolute Δ which means that P starvation did not affect them strongly, therefore they kept develop normally whereas the sensitive accessions was defined by the ones having very high absolute. Out of the obtained data, G38, G93, G165, G223, G62 were determined as hypersensitive accessions to P deficiency because they have very high absolute Δ and G11, G177 as tolerant accessions to P deficiency because they have very small absolute Δ. These results showed that, there was a large genetic variation in our rice collection in which they have different response to P starvation and can be useful for genetic mapping studies. The substantial genetic variation for all root traits investigated to response to the lack of P was also obtained in 15 rice accessions from Philippines, India, Thailand, Japan, Brazil, Pakistan, Iran, Guinea, United States, and Sri Lanka (Vejchasarn *et al*., 2016).

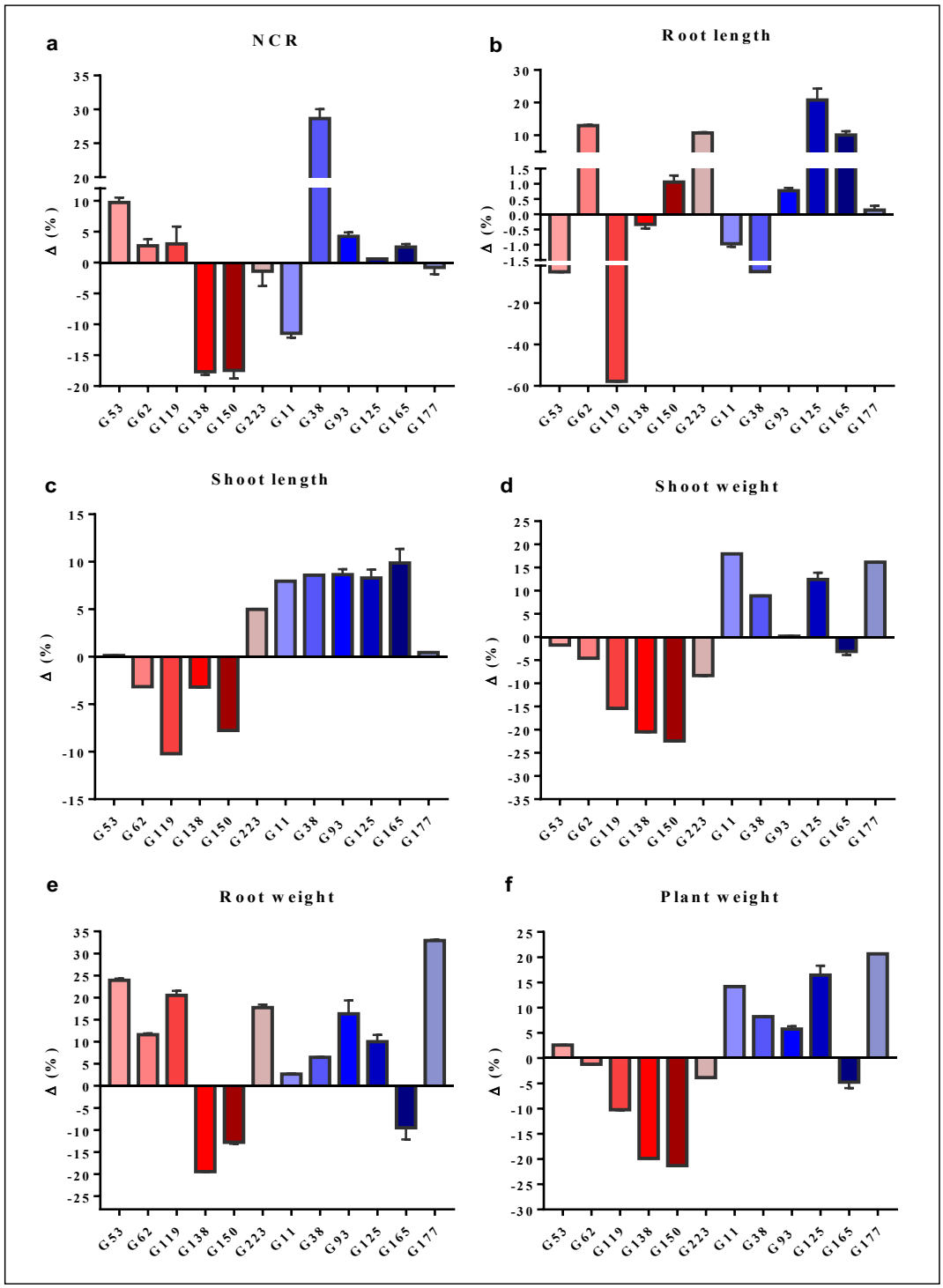



**Figure 6.** The percentage of difference in terms of number of crown root (NCR), root length, shoot length, root weight, shoot weight, plant weigh grown under high P (P0) and P starvation (P*) between 12 rice accessions. Δ = (P*-P0)/P0 x 100. Relative values are represented as mean values ± standard deviation calculated from 12-15 replicates.

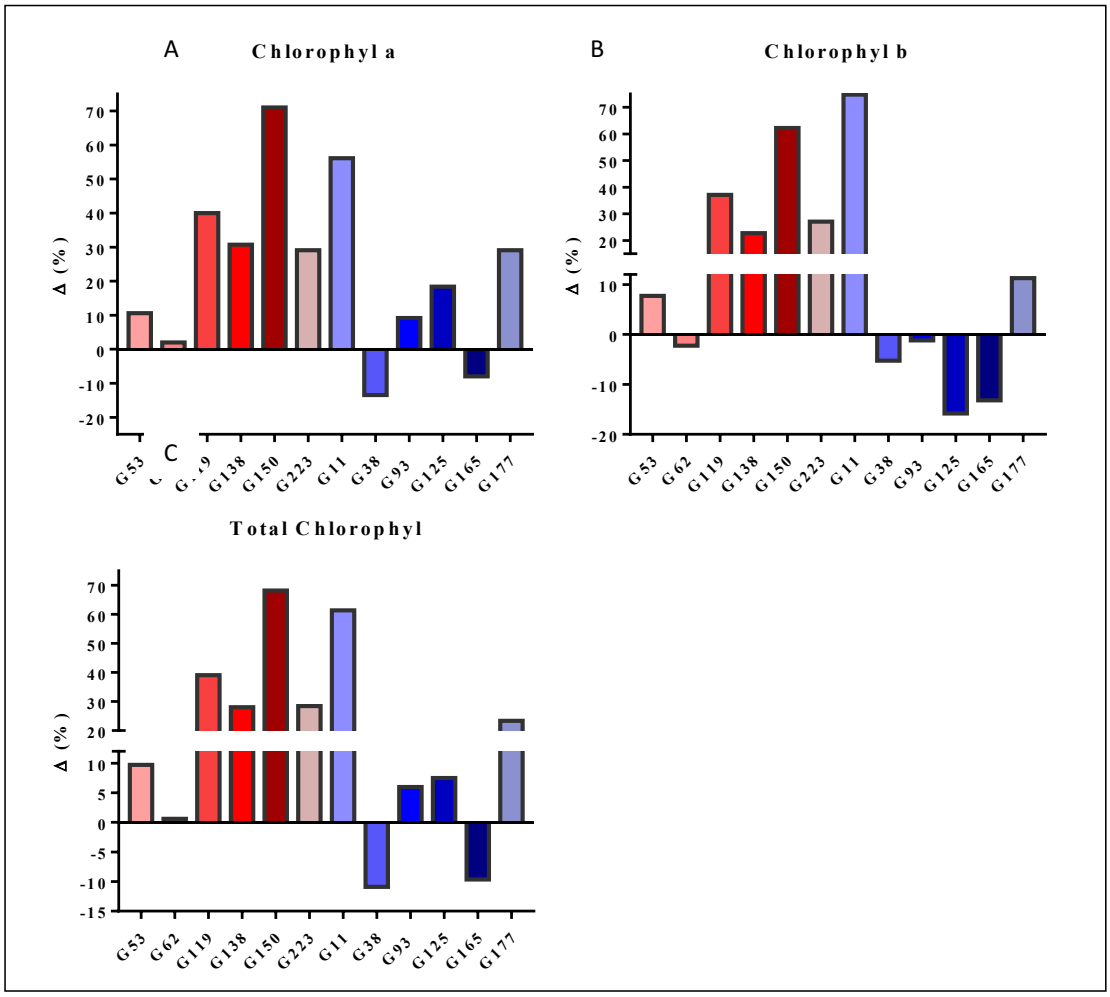



**Figure 7.** The percentage of difference in terms of chlorophyll of 12 rice cultivars grown under high P (P0) and P starvation (P*) between 12 rice accessions. Δ = (P*-P0)/P0 x 100. Relative values are represented as mean values ± standard deviation calculated from 12-15 replicates.

## CONCLUSIONS

Finding rice accessions, which can develop well and have high productivity under P starvation, is a priority in modern agriculture (Rose *et al*., 2013). Our study showed a wide genetic diversity of Vietnamese rice accessions response to low P. Some of them are potential high sensitive to P starvation (G38, G93, G165, G223, G62) while some of them are potential resistant to P starvation (G11, G177). This could pave the way for us to find potential cultivars for breeding program and potential genes responsible for P use efficiency.

**Funding**: *This research is partially funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-NN.03-2016.15.*

**Acknowledgements:** *12 accessions were chosen based on the phenotyping results of the NAFOSTED project (grant number 106-NN.03-2016.15) to Huong TM To. We thank for University of Science and Technology of Hanoi (USTH), Vietnam Academy of Science and Technology (VAST), Direction of Research, Innovation, and Technology Transfer (DRITT- USTH), International Joint Laboratory of Functional Genomics and Biotechnology for Plants*

*and Associated Microorganisms (LMI-RICE 2) for financial support. We thank students of University of Science and Technology of Hanoi (Nguyen Lan Anh, Pham Anh Duc, Huynh Thi Anh Tuyet and Le Vinh Hoa) and high school students from The Olympia School (Do Minh Chau and Nguyen Thao Trang) for helping us in phenotyping experiments*.

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