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Comparing hydroponics, sand and soil medium to evaluate contrasting rice N22 mutants for tolerance to phosphorus deficiency

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Abbreviations: EMS, Ethane methane sulfonate; GS, Glasses with soil; H, Hydroponics; HS, Hydroponics with sand; N22, Nagina22; P, Phosphorus; PS, Pots with soil. ; PUE, Phosphorus use efficiency

Abstract

Soils deficient in P are widespread in major rice ecosystems. In view of declining reserves of rock phosphate and rising costs of P-fertilizers, breeding rice varieties tolerant to low P becomes important for future food security. Four different methods 1. Hydroponics without sand (H), 2. Hydroponics with sand (HS), 3. Large pots with soil (PS) and 4. Glasses with soil (GS) were evaluated using rice aus variety Nagina 22 (N22) and its known gain/loss of function (gof/lof) mutants to screen for low P-tolerance in field. In –P shoot dry weight was significantly more in gof mutant NH787 than in N22 in HS, PS and GS but not in H with fold increase of 1.8 in HS, 5.2 in GS and 9.4 in PS. In HS, in -P, out of 6 traits only shoot dry weight was significantly higher in gof and lower in lof mutants. However, in GS both root and shoot dry weight could confirm gof and lof mutants. It took 40d in GS and 70d in PS to differentiate between growth in –P/ low P and +P and also between gof and lof mutants. Thus shoot dry weight at 30d in HS and both root and shoot dry weight at 40d in GS are best to differentiate between genotypes grown in –P/lowP and +P and also between gof and lof mutants for low P tolerance. The HS method can be carried out in ambient conditions and needs 70% lesser medium compared to H. If germplasm is to be screened for low P tolerance on a large scale, and there is no access to low P soil, then screening using HS is best.

Keywords: Rice, Hydroponics, Low P tolerance, Mutants, -P

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Introduction:

Phosphorus (P) is one of the most important primary macronutrients which promotes plant growth and plays a vital role in improving crop productivity. Nearly 80% of applied inorganic P is wasted in processes such as fixation with iron/aluminum in acidic soils, calcium/magnesium in alkaline soils and slow diffusion leaving only 20% of it to be utilized by the plant. Thus P fertilizer use must be optimised (Yi et al., 2005, Plaxton and Tran, 2011, Vinod and Heuer, 2012, Herrera-Estrella and Lopez-Arredondo, 2016) particularly in India where P fertility of soils is extremely poor (Sanyal et al 2015). Rice is one of the most important cultivated cereal crops in the world feeding more than half of the world's population. In India rice occupies the maximum area under cultivation. Phosphorus is an essential and vital nutrient next only to nitrogen as fertilizer required for rice growth and productivity. There is a threat of exhaustion of rock phosphate in future and combined with the rise in the P fertilizer cost, profit to farmers is drastically reduced. Hence, it is necessary to develop rice genotypes that are tolerant to low P and yet give high yield. Previously, rice variety Rasi and advanced breeding lines IET5854, IET14554, PRH122, IET15328 and IET17467 have been reported to be tolerant to low P condition based on grain yield in field experiments at Directorate of Rice Research, Hyderabad, India (Gopalkrishna et al. 1984; Krishnamurthy et al., 2010) and CAN 5164, CAN 4097, CAN 5170, IR3646-8-1-2, CAN 4137, A8-391 and IAC-47 at National Rice and Bean Research Center of Embrapa (Fageria et al., 1988). Mudgo and DJ123 were identified as tolerant to P deficiency based on above-ground biomass at two locations (Saito et al 2015). In order to identify such genotypes from a large numbers of rice germplasm a simple, reliable, reproducible and cost effective mass screening method is required at laboratory level to reduce the need for large scale field screening.

Screening experiments have been conducted previously for identification of low P tolerant and susceptible rice genotypes using various methods that included screening in field (Fageria et al., 1988, 2014, Krishnamurthy et al., 2010), hydroponics and field (Panigrahy et al. 2014), hydroponics or pots but with large containers and controlled environmental conditions (Wissuwa and Ae 2001b, Rose et al., 2016) or pots with low P soil and field (Saito et al., 2015) or sand and vermiculite (Vejchasarn et al 2016) . Rose et al. (2016) screened six rice genotypes differing in PUE in different concentrations of P in hydroponic and soil based experiments. Yoshida medium and 11 P concentrations were used and 52d old plants were analysed for root shoot traits and P concentration. PUE was significantly influenced by genotype and P supply but there was no significant genotype × P supply interaction. It was recommended that hydroponic cultures supplemented with one or two different P concentrations could help in large scale screening for PUE i.e. shoot biomass produced per unit of shoot P.

Panigrahy et al. (2014) screened 300 EMS induced (N22) mutants under low P field and then shortlisted 4 tolerant and 4 susceptible mutants based on grain yield in low P. These were tested in hydroponics using half strength Hoagland's medium with and without P. Root and shoot traits at 38 d after germination in –P in hydroponics could be indicative of low P tolerance in field but no specific trait was suggested. Vejchasarn et al. 2016 studied effect of P deficiency on root architectural, morphological and anatomical traits in 15 rice genotypes in pots with a mixture of 40 % medium size (0.3–0.5 mm) sand and 60 % vermiculite by volume using low P (2 μM) or sufficient (100 μM) P as 1 % solidphase buffered P. Plants were irrigated once per day with P-free Yoshida nutrient solution via drip irrigation. Phosphorus deficiency caused a 20 % increase in the percent cortical area converted to aerenchyma. Though the direction of low-P response

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was uniform among genotypes, and in every case the genotype by phosphorus interactions were small, the three aus varieties maintained high growth in low P.

For field-based screening the major problem is the non availability of low P soil. Apart from that it is time consuming, requires large space and there are chances of possible intervention of another stress (such as heat, drought, flood) or ambient conditions which cannot be controlled and could mask the performance of a low P tolerant genotype. Maintenance of a growth chamber or controlled glass house also makes it a costly technique. In order to overcome the above problems simple and reliable methods need to be developed that can be carried out in both ambient and laboratory conditions, within a reasonably short time and can serve as a surrogate for field screening. Nagina22 (N22) is a well known *aus* variety used routinely as a check for heat tolerance in rice (Jagadish et al., 2010). In a network project, EMS induced stabilized N22 mutants were evaluated for different traits such as tolerance to herbicide, heat, low P, water deficiency, salinity and resistance to bacterial leaf blight and blast (Panigrahy et al., 2009, 2011, 2014, Poli et al., 2013, Kulkarni et al., 2013, Mohapatra et al., 2014, Kulkarni et al., 2014, Lima et al., 2015, Tella et al., 2016, Mithra et al., 2016). As a part of the network project on functional genomics, a total of 2500 N22 mutants were screened at IIRR under field conditions maintained with low P (<1.8 Olsen P) to identify low P tolerant and susceptible mutants. Some of the best and worst mutants selected in low P under field conditions based on tiller number and grain yield repeatedly for 3 years (6 seasons) were used in the present study to develop a simple screening method.

An experiment was designed using selected N22 mutants whose tolerance/susceptibility to low P in field was previously known. These mutants were different from the mutants used in Panigrahy et al (2014). The objective was to compare four different screening methods Hydroponics

111 without sand (H), Hydroponics with sand (HS), Large pots with soil (PS) and Glasses with soil
112 (GS) and to determine the simplest method and trait to screen large numbers of rice germplasm
113 for low P tolerance. The underlying goal was to be able to differentiate between response of
114 tolerant and susceptible mutants at the earliest stage of growth in the shortest possible time using
115 minimal space and resources.

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Materials and Methods:

Four individual experiments were conducted in wet season 2012. N22 and its mutants induced by ethane methane sulfonate (EMS) were screened in the field continuously from M2 generation to M6 generation in normal field (P 60kg/ha, Olsen P 24) and low P field conditions (no P added since 38 years Olsen P 1.8) (Gopalkrishna et al., 1984), for their tolerance to low phosphorus in field. The M2 seeds were provided by the Coordinator, Network Project. In M7 generation the 8 mutants used in this study were designated as gain of function (gof) and loss of function (lof) based on grain yield in low P field conditions compared to wild type N22 (Supplementary Table1). These 8 mutants showed mean grain yield of 6.77 to 8.99 g/plant in normal P (60Kg P/ha) field conditions. In low P (1.8Kg/ha) field conditions, the per plant yield was 0.8 g in N22, 2.43 to 4.21 g in gof mutants and 0 to 0.47 g in lof mutants. The variety Rasi, well known for its adaptability and tolerance to low P was also used. The mutants are named NH for **N**agina22-**H**yderabad followed by the mutant number. N22 and some gof and lof mutants were then used to see which method and which trait is easiest and best to identify the growth differences between –P/low P and +P or between gof and lof in the shortest time and which confirm the observations in low P field conditions.

<u>Experiment I.</u> Hydroponics without sand (H):

N22 and NH787 a gof mutant were selected. The seeds were surface sterilized with 0.1% sodium hypochlorite and thoroughly washed 4 times with copious amounts of sterile water. Seeds were initially sown on wet filter papers at 25^{o}C in growth chamber for maintaining synchrony in growth. After 14 days of germination, 15 uniform seedlings in three replicates of 5 seedlings each were transferred into full strength Yoshida medium pH 5.7 (Yoshida et al., 1976) with two treatments, -P (no addition of NaH_2PO_4) and +P (0.32mM NaH_2PO_4) in plastic trays

with thermocol on top with three replications. Medium was replaced every four days. Then the trays with seedlings were kept in growth chambers with 16 h light and 8 h darkness and temperature of 25- 28 ^{o}C was maintained for another 40 days. Shifting to growth chamber was necessary as they did not survive in ambient conditions of Hyderabad.

On 40 d the plant samples were separated into shoot and root and kept in a hot air oven at 70^{o}C for 72 hours. Dry weight of each sample was determined separately. P concentration of these samples was then estimated according to the method of Saheki et al. (1985).

Experiment II. Hydroponics with sand (HS):

The seeds of a well known low P tolerant variety Rasi, N22 and 3 gof mutants, NH 767, 776, 787 and one lof mutant NH 210 were surface sterilized as mentioned in experiment I. After 14 days, 4 seedlings were taken and transferred to each plastic pot (4 inches) having 200 g of acid washed white smooth sand in three replicates. At the bottom of the plastic pot a hole was punched in the center and absorbent cotton was placed in the hole. One set of all the lines was kept in small plastic trays containing Yoshida medium pH 5.7 with –P and another set was kept in similar trays having +P medium. Through capillary action medium was absorbed from tray to the pots through absorbent cotton. Media were replaced every 4 days as in Experiment 1. These trays were kept in ambient conditions. All the observations root length, shoot length, root and shoot dry weight, root and shoot P concentration mentioned in experiment I were taken on 30th day after sowing.

Both H and HS were first conducted in ambient conditions, but in H experiment all seedlings died. So H was carried out in controlled growth chamber. The seedlings were grown in growth

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chamber maintained at 28/25^{o}C (day/night) with 16 h light and 8 h dark. The relative humidity was maintained at 70%.

Experiment III: **Pots with soil (PS):**

N22 and gof mutant NH787 were initially sown in normal soil for 25 days and later transferred into respective experimental pots. The plants do not survive if they are sown directly in low P soil. Each pot (12" diameter) was filled with 8 kg soil collected from ICAR-IIRR experimental farm from the low P plot (Olsen P 1.8 = 1.8 mg P/Kg soil). Recommended N, K, Zn (100:40: 12.5 kg / ha) was applied in all the pots. N was given as split doses at basal, tillering and flowering stages. K was applied as basal dose only. Two levels of P were maintained, low P soil obtained from low P field (without any external addition of P) and normal P (60 kg/ha P added to low P soil in pots) maintained in 3 replications and the pots were maintained under natural light conditions till maturity. At harvest, shoot and root dry weight were taken and P concentration determined in root and shoot.

P concentration in root, shoot: Samples of root and shoot after harvest were collected, dried at 70^{o}C and were used for P estimation. Digestion was carried out with tri acid mixture followed by P determination using Phosphovanadate method (Hanson 1950).

Experiment IV: G**lasses with soil (GS):** Small disposable plastic glasses (100 ml capacity) were taken and 200 g of low P soil was added in each glass. Soil was taken from low P plot from ICAR-IIRR farm, powdered and passed through 2 mm sieve. Then the soil was mixed and N, K and Zn fertilizers (100:60:12.5 kg / ha) were added calculated according to soil weight. After this 200 g of soil was weighed and transferred into single disposable glass. P was added in the form

of single super Phosphate (SSP) directly into each glass and mixed. There were six P treatments namely low P, 20P, 30P, 40P, 50P and 60P (P requirement was calculated according to soil weight). Data on low P and 60P is presented in Table3 and data on other levels of P are presented in Supplementary Table 3. N22 and gof mutants NH686 and NH787 and lof mutant NH359 were sown in normal soil for 25 days, then one seedling was transferred into each glass in different treatments. 6 replications were maintained for each treatment. Root and shoot dry weights were taken on 40^{th} day after transfer.

For ease of writing the four experiments are referred to as experiment I Hydroponics (H), experiment II Hydroponics with sand (HS), experiment III Pot with soil (PS), experiment IV Glass with soil (GS). First, root and shoot dry weight of N22 and gof mutant NH787 were compared in +P and -P using all the four methods. Next, dry weight and P concentration in root and shoot in N22 and NH787 were compared in three methods H, HS, PS. Next, in HS, length, dry weight, and P concentration in root and shoot in –P and +P were compared in N22, 3 gof mutants and one lof mutant and the known P tolerant variety Rasi. Finally, in GS, root and shoot dry weight were compared in +P and low P in N22, two gof mutants and one lof mutant. Since this lof mutant was known to show significantly increased grain yield in increasing levels of P in field (data not shown), an additional experiment was set up with four more levels of P (20, 30, 40, 50P) in between low P and 60 P to see the response of mutants to increasing levels of P in GS method only.

There were three replicates for experiment I, II and III (H, HS and PS) and 6 replicates in experiment IV (GS).

Statistical analysis:

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The data from the experiments was analysed by performing analysis of variance (ANOVA) using a statistical computer package Statistix Ver. 8.1. The differences between treatments (+P and-P/ lowP) and genotypes (N22, lof mutants, gof mutants and Rasi) were estimated using least significant differences (LSD) test.

Results

Two hydroponics based methods H and HS and two soil based methods PS and GS were compared using N22 and its gof mutant NH787 for differences in shoot and root dry weight in +P and –P (Table 1). Significant differences were observed between methods. Root dry weight differed significantly between +P and –P only in PS. Shoot dry weight was significantly less in –P than in +P in HS, PS and GS. NH787 showed significantly higher shoot dry weight than N22 in HS, PS and GS with fold increase of 1.8 in HS, 5.2 in GS and 9.4 in PS in -P (Table 1). In HS the differences between growth in –P and +P were significant at 30d, whereas in the other three methods it took 40d to 60d. In HS shoot dry weight was a better trait than root dry weight to distinguish growth in –P from that in +P. Root dry weight of NH787 did not differ in –P and +P in HS. HS method was used next to determine if this method could confirm in –P, the known gain of function (gof) and loss of function (lof) mutants for tolerance to low P in field.

In HS, in general root length was significantly more in all the mutants in –P than in +P at 30 d. It was maximum in NH787 and minimum in NH210 at 30 d (Table 2). On the other hand, shoot length was significantly lower in –P than in +P in all lines. NH776 and 767 showed maximum and NH210 minimum shoot length (Table 2). Root dry weight was significantly less in –P than in +P. In –P, on 30 d maximum root dry weight was in NH787 and least in NH776, both gof mutants (Table 2). Shoot dry weight was also significantly less in –P compared to +P. On 30d highest shoot dry weight was observed in gof mutants NH787 and NH776 and lowest in lof mutant NH210 (Table 2) thus confirming the classification of mutants.

Root and shoot P concentration was measured in three methods H, HS and PS. The differences were significant between the treatments and mutants. In all the methods NH787 had highest root and shoot P concentration compared to N22 (Supplementary Table 1). In H, NH686 and 787 had

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highest root and shoot P concentration and NH359 had lowest root and shoot P concentration in –P (data not shown). In HS, highest P concentration was observed in +P compared to –P. In HS the differences between +P and –P were not significant for root length but were significant for shoot length. Both shoot length and shoot dry weight could identify NH767 and 776, as gof and NH210 as lof compared to N22 but NH787 was not gof based on shoot length. Apart from these mutants Rasi a well known low P tolerant variety was better than N22 based on the shoot dry weight but not on the basis of shoot length. Thus, shoot dry weight is preferred over shoot length. Thus only shoot dry weight in –P could reliably distinguish gof and lof in HS at 30d.

The GS method was conducted to find out if data at 40d in GS could be used instead of 60d in PS to distinguish between lof and gof mutants in low P soil. Hence, two gof and one lof mutant was taken for further studies (Table 3). At 40d in GS the differences were significant between low P and 60P and also between gof and lof. At 40d root dry weight was significantly higher in 60P compared to low P in all lines. In low P, both NH686 and 787 had significantly higher root dry weight than N22 and NH359 had lowest root dry weight. N22 had lowest root dry weight in 60P. At 40d shoot dry weight was significantly more in 60P than in low P in all the lines. Both NH686 and 787 showed significantly more shoot dry weight than all other lines in both low P and 60P. On the other hand NH359 had lowest shoot dry weight in low P and N22 had lowest shoot dry weight in 60P (Table 3). Thus based on GS in low P, NH686 and 787 were confirmed as gof mutants for tolerance to low P. Also NH359 was confirmed as a loss of function mutant for tolerance to low P as it was worse than N22 in low P for both root and shoot dry weight. In 40d itself in soil low P and normal condition gof and lof can be distinguished in GS method compared to 60d in PS. Thus among soil-based methods GS is better than PS.

To further investigate if the shoot dry weight of the same 4 lines changes when 20P, 30P, 40P or 50P is used, these four P concentrations were added to low P soil in different glasses in GS method. The results were significantly different for the lof mutant NH359. At 20P, N22 and lof mutant NH359 could not be distinguished though they were clearly distinguished in low P (Table 3). NH359 would instead be classified as gof instead of lof if tested at 20P or more (Supplementary Table 3). This reveals that there is an optimum below which lof mutant NH359 can be distinguished from N22 and it lies between 1.8P and 20P.

Thus, among the four methods, HS was best to distinguish between both –P and +P and also between gof and lof when observations are taken at 30d itself using shoot dry weight as a criterion. The next best was GS, a soil-based method where P is low (not absent as in HS) and observations are taken at 40d. Differences between –P and +P and between gof and lof were not significant at 20d or 30d (data not shown). H does not distinguish gof from lof even at 40d. It takes 60d in PS to distinguish between –P and +P or between gof and lof mutants but PS is required if plants need to be grown to maturity for seed set analysis.

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Discussion

Among all four methods, HS using sand as a support for roots and GS where low P soil was used in glasses are the two convenient and robust methods that helped screen the mutants for low P tolerance reliably. In HS the differences between gof and lof mutants and also between –P and +P were significant at 30d but took 40 days in GS. Compared to hydroponics, using sand is more close to soil conditions as it provides for aeration and a solid rooting medium. Rice shows different responses to aerobic and flooding conditions and P uptake in particular was reported to be more in aerobic conditions than in flooded condition (Wissuwa and Ae, 2001b). Aeration is required in water culture. We did not use any aeration and this may be one of the reasons H did not work in ambient conditions even though the medium was replaced every 4 days. HS method can be carried out in ambient conditions and saved 70% nutrient medium compared to hydroponics in experiment H. The advantage with HS is that differences are clear and significant at 30 d . In H maintenance of a controlled growth chamber with light, humidity, temperature were essential in our conditions, however, these were not necessary in HS which could be carried out under ambient conditions also. Sand provides a soil–less, cost effective, reusable matrix for screening. Solid phase buffered Al- P method using 40% sand and 60% vermiculite medium was used in a recent study on genetic variation in 15 rice varieties for root traits at 8 leaf stage in the greenhouse (Vejchasarn et al 2016). Sand culture rather than water culture was also recommended to estimate nickel toxicity in barley roots (Lin et al 2016).

Between the two soil-based methods GS was better than PS and 40 d was the earliest time point for easy selection of mutants with gof or lof for tolerance to low P when low P soil was used. The differences between the gof and lof mutants and between low P and 60P were significant and visible only at 40 d. The differences between gof and lof mutants were not very clear at 20 d.

GS method is cheap, requires less labour, roots are easily separated without damage, aerobic conditions are easily maintained and water/nutrient requirement is low. Both glasses and pots were kept in ambient conditions and a growth chamber was not necessary. PS requires more soil, water, more labour and differences between gof and lof mutants or between low and normal P are clear only around 60 d when flowering begins. In GS flowering in general began in 55 days in normal P and 59 days in low P.

In H experiment some of the lof mutants and N22 showed highest shoot length, shoot dry weight at 20 d, but at 40 d some other mutants showed higher root length, shoot length and dry weight. Thus the observations at 20 d can be misleading in H (data not shown). For example there was no difference between the gof mutant NH686 and the lof mutant NH359 in –P at 20d. In H instead of 20 d, observations at 40 d are better for identification of gof and lof mutants for tolerance to –P. The differences within the mutants in –P were not very clear in H. In HS the differences were not clear at 15 d, but at 30 d the differences between gof and lof mutants in –P condition were very significant (data not shown) and in accordance with response in low P field. Compared to H, screening in HS is more reliable for identification of gof and lof mutants. It may be noted that an extreme condition of no P is being used in H and HS which is different from the soil based experiments PS and GS where a minimum amount of P is present in the soil. This may be a cause for the differences in water and soil-based experiments. Secondly, the soil also has biota which were absent in water-based experiments. The soil used in PS and GS was from the same source i.e. low P field and was mixed well before transferring to pots or glasses. So it is assumed that the differences in growth due to soil biota would be minimized. But in any case the purpose of screening for low P tolerance is served in both sand- and soil-based experiments thus reducing field-based screening.

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NH686 showed higher root and shoot biomass in H, HS and PS, and it is also the mutant with highest grain yield in low P in field and in pots experiments. We propose that NH686 can be used as a check in screening experiments for low P tolerance in rice and also as a donor for low P tolerance. Another mutant NH787 also showed equal biomass as NH686. But in PS it was next to NH686 in terms of biomass and grain yield in low P (data not shown).

If germplasm is to be screened for low P tolerance on a large scale, and there is no access to low P soil then screening using HS is best. All the genotypes can be grown in –P in sand keeping some lines as best (eg NH686) and worst (eg NH359) checks. If dry weight of the mutants was lesser than wild type they were confirmed as lof–for tolerance to low P, on the other hand mutants with higher dry weight than wild type were confirmed as gof for tolerance to low P. The mutants we used were previously field tested for tolerance to low P. One gof and one lof genotype is usually used as a check for ranking the genotypes in –P condition. Observations are taken in –P at a time point when the susceptible check genotype dies and the tolerant check genotype is almost as in normal P. It may be noted that most previous experiments also used shoot dry weight as a criterion to classify as tolerant or susceptible (Panigrahy et al., 2014, Saito et al., 2015, Rose et al., 2016, Vejchasarn et al 2016) and phenotyping for shoot root traits or P concentration is usually done at about40 days (Panigrahy et al 2014, Yamaji et al 2016,) which is about the 8 leaf stage used by Vejchasarn et al. (2016).

Rose et al. (2016) screened for phosphorus use efficiency (PUE) rather than tolerance to low P and equal shoot P content of shoot was considered rather than equal P supply thus avoiding bias by P uptake. We considered equal P supply so that the large variation in uptake in low P is also accounted for. They took 6 varieties as checks where Mudgo, Santhi Sufaid, Dawebyan and DJ123 are high PUE lines, TN1 as lowest PUE line and IR64 as moderate PUE line. Out of

these, only IR64 is a popular high yielding variety grown In India. We emphasize yield as a criterion rather than PUE so that high economic yield is obtained irrespective of whether PUE is high or not. We recognize there may be genotypes which take up high P even though their PUE is not high and these would be economically important rather than those with high PUE but ultimately low output of grain or fodder yield. Our experiments were designed to help large scale screening of rice germplasm for tolerance to low P in soil. In the two water-based experiments we compared –P and +P but in soil-based experiments we compared not zero P but low P (Olsen P 1.8 Kg/ha) on one side with higher levels of P on the other. If such a low level of P containing soil is not available, soil with less than 14 KgP/ha can also be taken as low P for screening experiments as the optimum P for rice in India was reported as 14 to 28 Kg/ha(Sanyal et al 2015)

Saito et al. (2015) reported low P soil based experiments in pots and in field. Yoshida medium was used as in our study and above-ground biomass dry weight at 33-35d in pot experiment and 42 days in field experiment was considered for low P tolerance. In the pot experiment higher above-ground biomass was strongly associated with higher root biomass particularly in –P. Based on these studies it was concluded that Mudgo is a P deficiency tolerant variety as it has higher relative biomass in both field and pot experiment and Santhi a susceptible variety as it had least biomass in both field and pot experiments. N22, an aus variety was not among the 12 upland varieties used in their experiments. Three other aus genotypes Kasalath, Jhona309 and Dular showed the highest ability to maintain shoot growth under low P. They also had greater shoot P than other 12 genotypes (Vejchasarn et al 2016). Thus N22 and its gain of function mutants appear to be among the most tolerant lines to low P. Shoot dry weight was the best criterion in both water- and soil-based experiments. In our experiments high grain yield in low P was considered as low P tolerance ignoring PUE when screening large germplasm in field. Our

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results also illustrate that screening in low P (1.8 mg soil extractable P/Kg soil) should be carried out first and selected lines can then be screened under high P conditions as also shown in the double pot technique using low P (2.4-2.6mg soil extractable P/Kg soil) previously (Saito et al 2015). This would help in breeding rice varieties that have least reduction in yield when soil P is low and yet have high absolute yield. It would also help in reducing P removal from fields so that lesser P fertilizer is applied thus increasing both profit to farmer and sustainability.

368 **Conclusion:**

369 We conclude that hydroponics with sand method is better than water when low P soil is not
370 available for screening of genotypes for low P tolerance. When low P soil is available, screening
371 for shoot dry weight in small disposable glasses with low P soil and one seedling per glass is a
372 quicker and reliable method for identifying genotypes for tolerance to low P and also large
373 numbers of genotypes can be screened in 40 days. Overall NH686 can be used as a donor for low
374 P tolerance and NH359 as a line that shows significantly increased growth in response to
375 increasing levels of P. These two are also important gain of function and loss of function mutants
376 respectively for functional genomic studies on P uptake, response, use efficiency and
377 homeostasis in different organs of the plant. Further molecular studies are ongoing to map causal
378 genes for low P tolerance in NH686 and other mutants using MutMap method of Abe et al. 2012.

379 **Conflict of Interest:** The authors state that they have no conflict of interest.

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References:

Abe, A., S. Kosugi, K. Yoshida, S. Natsume, H. Takagi, H. Kanzaki, H. Matsumura, K. Yoshida, C. Mitsuoka, M. Tamiru, H. Innan, L. Cano, S. Kamoun and R. Terauchi. 2012. Genome sequencing reveals agronomically important loci in rice using MutMap. Nat. Biotechnol. 30: 174-178

Fageria, N.K,., Morais, O.P. Baligar, V.C. and Wright, R.J. 1988. Response of rice cultivars to phosphorus supply on an oxisol. Fert Res. 16:195–206

Fageria, N.K., Morais, O.P. Santos, and Vasconcelos, M.J. 2014. Phosphorus use efficiency in upland rice genotypes under field conditions. J Plant Nutr. 37:633–642

Gopalkrishna, K., Krishnamurthy, K. and Ramprasad, A.S. 1984. Studies on varietal tolerance of rice varieties to phosphorus deficiency. Fertilizers and Agriculture. No. 87

Hanson, W.C. 1950. The photometric determination of phosphorus in fertilizers using the phosphovanado- molybdate complex. J Sci Food Agric. 1:172–173

Hewitt, E.J. 1966. Sand and water culture methods used in the study of Plant Nutrition. Experimental Agriculture. 3(02):104–104

Jagadish, S., Muthurajan, R. Oane, R. Wheeler, T. Heuer, S. Bennett, J. and Craufurd. 2010b. Physiological and proteomic approaches to address heat tolerance during anthesis in rice. J Exp Bot. 61:143–156

Krishnamurthy, P., Sreedevi, B. Ram, T. Padmavathi, G. Mahendra Kumar, R. Raghuveer Rao, P. Shobha Rani, N. Latha, P.C. and Singh, S.P. 2010. Evaluation of rice genotypes for phosphorus use efficiency under soil mineral stress conditions. Oryza. 47(1): 29-33

Kulkarni, K.P., Vishwakarma, S. Sahoo, P. Lima, J.M. Manoj, N. Prasad, D. Rajesh, N.G. Mohapatra, T. Robin, S. Sarla, N. Seshashayee, M. Ashok, K.S. Kuldeep, S. Nagendra, K.S. and Sharma, R.P. 2014. A substitution mutation in OsCCD7 cosegregates with dwarf and increased tillering phenotype in rice. Journal of Genetics. 93(2): 389-401

Kulkarni, K.P., Vishwakarma, C. Sahoo, S.P. Lima, J.M. Nath, M. Dokku, P. Gacche, R.N. Mohapatra, T. Robin, S. Sarla, N. Seshashayee, M. Singh, A.K. Singh, K. Singh, N.K. and Sharma, R.P. 2013. Phenotypic characterization and genetic analysis of dwarf and early flowering mutants of rice variety Nagina22. Oryza. 50:18-25

Lima, J.M., Nath, M. Dokku, P. Raman, K.V. Kulkarni, K.P. Vishwakarma, C. and Neelamraju, S. et al. (2015) Physiological, anatomical and transcriptional alterations in a rice mutant leading to enhanced water stress tolerance. AoB Plants. 7, plv02

Lin, Y., Allen, H. E. Di Toro, D. M. 2016. Barley root hair growth and morphology in soil, sand, and water solution media and relationship with nickel toxicity. Environmental toxicology and chemistry. 35(8):2125-2133

Herrera-Estrella, L., and López-Arredondo, D. 2016. Phosphorus: The underrated element for feeding the world. Trends in Plant Science. Volume 21, Issue 6, p461–463, June 2016

Mithra, A.S.V., Kar, M.K. Mohapatra, T. Robin, S. Sarla, N. Seshashayee, M. Singh, K. Singh, A.K. Singh, N.K. and Sharma, R.P. 2016. DBT propelled national effort in creating mutant resource for functional genomics in rice. Current Science. 110(4):543-548

Crop Sci. Accepted Paper, posted 03/03/2017. doi:10.2135/cropsci2016.07.0594

421 Mohapatra, T., Robin, S. Sarla, N. Sheshashayee, M. Singh, A.K. Singh, K.. Singh, N.K. Amitha
422 Mithra, S.V. and Sharma, R.P. 2014. EMS induced mutants of upland rice variety nagina22:
423 generation and characterization. Proc Indian Natn Sci Acad. 80(1):163-172

424 Panigrahy, M., Nageswara Rao, D. and Sarla, N. 2009. Molecular mechanisms in response to
425 phosphate starvation in rice. Biotechnology Advances 27:389–397

426 Panigrahy, M., Neelamraju, S. Nageswara Rao, D. Ramanan, R. 2011. Heat tolerance in rice
427 mutants is associated with reduced accumulation of reactive oxygen species. Biologia Plantarum.
428 55(4): 721-724

429 Panigrahy, M., Rao, D.N. Yugandhar, P. Raju, S.N. Krishnamurthy, P. Voleti, S.R. Reddy,
430 G.A. Mohapatra, T. Robin, S. Singh, A.K. Singh, K. Sheshshayee, M. Sharma, R.P. and Sarla,
431 N. 2014. Hydroponic experiment for identification of tolerance traits developed by rice Nagina
432 22 mutants to low-phosphorus in field condition. Arch Agron Soil Sci. 60:565–576

433 Plaxton, W.C., and Tran, H.T. 2011. Metabolic adaptations of phosphate-starved plants. Plant
434 Physiol. 156:1006–1015

435 Poli, Y., Basava, R.K. Panigrahy, M. Vinukonda, V.P. Dokula, N.R. Voleti, S.R. Desiraju, S. and
436 Neelamraju, S. 2013. Characterization of a Nagina22 rice mutant for heat tolerance and mapping
437 of yield traits. Rice. 6-36

438 Rose, T.J., Mori, A. Cecile, C. Julia. And Wissuwa, M. 2016. Screening for internal phosphorus
439 utilisation efficiency: comparison of genotypes at equal shoot P content is critical. Plant Soil.
440 401:79–91

441 Saheki, S., Takeda, A. and Shimizu, T. 1985. Assay of inorganic phosphate in the mild pH
442 range, suitable for measurement of glycogen phosphorylase activity. Analytical Biochemistry
443 13:3605–3615

444 Saito, K., Vandamme, E. Segda, Z. Fofana, M. and Ahouanton, K. 2015. A screening protocol
445 for vegetative-stage tolerance to phosphorus deficiency in upland rice. Crop Sci. 55:1223–1229

446 Sanyal, S. K., Dwivedi, B. S. Singh, V. K. Majumdar, K. Datta, S. C. Pattanayak, S. K. and
447 Annapurna, K. 2015. Phosphorus in relation to dominant cropping sequences in India: chemistry,
448 fertility relations and management options. Current Science 108 (7): 1262-1270

449 Talla, S.K. Panigrahy, M. Kappara, S. Nirosha, P. Neelamraju, S. and Ramanan, R. 2016.
450 Cytokinin delays dark-induced senescence in rice by maintaining the chlorophyll cycle and
451 photosynthetic complexes. J Exp Bot. 67(6):1839-1851

452 Vandamme, E., Wissuwa, M. Rose, T. Dieng, I. Drame, K.N. Fofana, M. Senthil kumar, K.
453 Venu Prasad, R. Jallow, D. Segda, Z. Suriyagoda, L. Sirisena, D. et al. 2016. Genotypic variation
454 in grain P loading across diverse rice growing environments and implications for field P
455 balances. Frontiers in Plant Science 7, 1435

456 Vejchasarn, P., Jonathan, P. Lynch, and Brown, K.M. 2016. Genetic variability in phosphorus
457 responses of rice root phenotypes. Rice. 9:29

458 Vinod, K.K., and Heuer, S. 2012. Approaches towards nitrogen and phosphorus efficient rice. .
459 AoB PLANTS: pls028;doi:10.1093/aobpla/pls028

Crop Sci. Accepted Paper, posted 03/03/2017. doi:10.2135/cropsci2016.07.0594

460 Wissuwa, M., and Ae, N. 2001b. Further characterization of two QTLs that increase
461 phosphorus uptake of rice (*Oryza sativa* L.) under phosphorus deficiency. Plant and Soil.
462 237:275–286

463 Yamaji, N., Takemoto, Y. Miyaji, T. Mitani-Ueno, N. Yoshida, K. T and Ma, J. F. 2016.
464 Reducing phosphorus accumulation in rice grains with an impaired transporter in the node.
465 doi:10.1038/nature20610

466 Yi, K., Wu, Z. Zhou, J. Du, L. Guo, L. Wu, Y. and Wu, P. 2005. OsPTF1, a novel transcription
467 factor involved in tolerance to phosphate starvation in rice. Plant Physiol. 138:2087–2096

468 Yoshida, S., Forno, D.A. Cock, J.H. Gomez, K.A. 1976. Laboratory manual for physiological
469 studies of rice. 3rd edn. IRRI, Manila

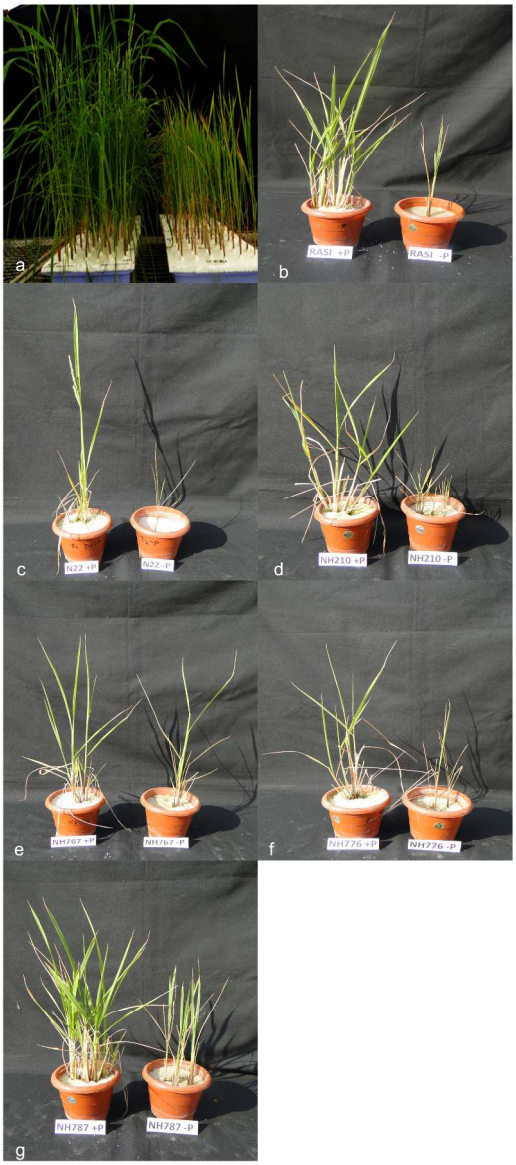

Fig 1: a) Overall view of N22 and mutants in (H) hydroponics Yoshida medium in +P (left) and –P (right). HS Hydroponics with sand culture b) Rasi c) N22 d) NH210 e) NH767 f) NH776 and g) NH787.

152x330mm (300 x 300 DPI)

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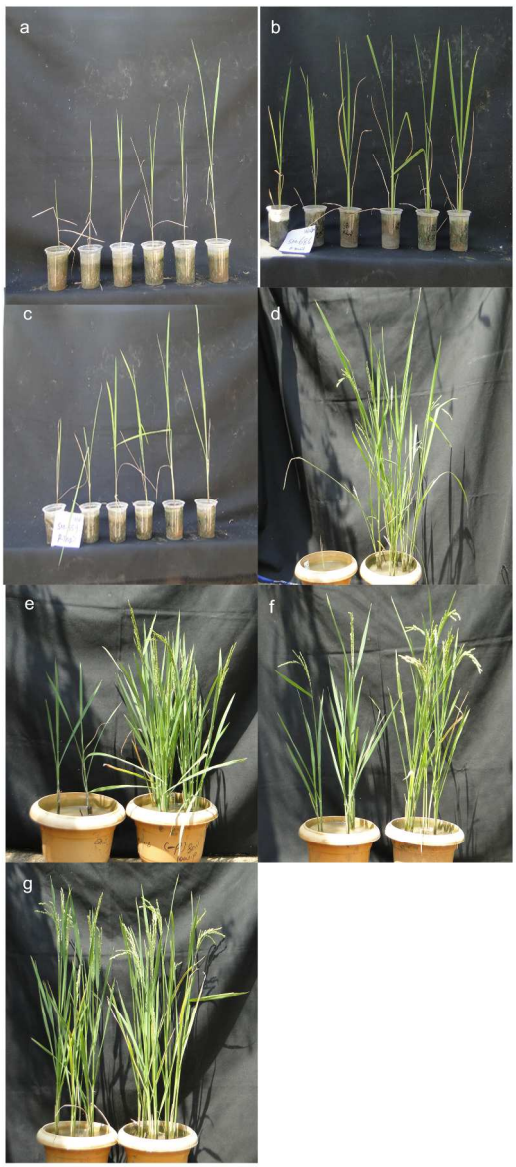

Fig 2. a-c. GS Glass with soil containing left to right low P soil with 20, 30, 40, 50, 60Kg/haP. a. N22, b. NH686, c. NH359. d-g. PS Pot with soil d. N22, e. NH162, f. NH787, g. NH686. low P soil (left) and +P soil (right) in each.

152x330mm (300 x 300 DPI)

Table 1: Root and shoot dry weight in N22 (wild type) and a gain of function mutant NH787 in +P and –P/low P in (Hydroponics) H, (Hydroponics with sand) HS, (Pots with soil) PS and (Glass with soil) GS methods.

S.No	Method	Mutant	Root dry weight (g/plant)		Shoot dry weight (g/plant)	
			+P	-P	+P	-P
1	H	N22	0.009 g‡	0.01 g	0.19 jk	0.08 k
		NH787	0.02 g	0.03 g	0.29 j	0.11 jk
	P x G LSD<0.05		0.26		0.189	
2	HS	N22	0.94 e	0.34 f	9.80 e	4.21 g
		NH787	0.71 de	0.63 e	10.92 d	7.70 f
	P x G LSD<0.05		0.26		0.189	
3	PS	N22	8.62 b	0.68 de	15.85 b	1.34 h
		NH787	9.22 a	4.81 c	17.04 a	12.64 c
	P x G LSD<0.05		0.26		0.189	
4	GS	N22	0.04 g	0.03 g	0.753 i	0.12 jk
		NH787	0.07 fg	0.06 g	1.35 h	0.62 i
	P x G LSD<0.05		0.267		0.189	

‡Means within columns followed by the same letter are not significantly different at $P \leq 0.05$.

Only in PS and GS, very low P (Olsen P 1.8) was considered as –P for comparing the 4 methods

Table 2: Length, dry weight and P concentration in root and shoot of N22 (wild type), gain of function mutants, loss of function mutants (in italics) and a low P tolerant variety Rasi as check in –P and +P on 30th day in HS (hydroponics with sand) method (Experiment 2)

S.No	Entry	Root length (cm)		Shoot length (cm)		Root dry weight (g/plant)		Shoot dry weight (g/plant)		Root P concentration (µg/g DW)		Shoot P concentration (µg/g DW)	
		+P	-P	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P
1	N22	8.95 d‡	11.30 bcd	22.80 cd	18.80 g	0.95 ab	0.35 c	9.80 cd	4.21 g	24.87 e	6.49 i	39.71d	9.65 i
2	NH787	9.70 cd	14.90 a	22.00 cde	19.60 fg	0.71 bc	0.64 bc	10.92 ab	7.70 e	69.16 a	25.89 e	48.73 a	19.92 f
3	NH767	10.35 cd	13.85 ab	28.30 a	20.70 ef	1.08 a	0.27 c	10.33 bc	4.23 g	44.56 c	15.47 h	38.32 e	17.22 g
4	NH776	9.15 cd	11.70 bc	25.50 b	21.50 de	1.04 ab	0.61 bc	10.85 b	6.45 f	62.18 b	19.67 f	43.57 bc	19.17 f
5	*NH210*	9.10 cd	9.90 cd	23.50 c	13.62 h	1.29 a	0.40 c	11.55 a	2.21 h	32.84 d	8.89 h	44.61 b	14.73 h
6	Rasi	11.50 bcd	13.30 ab	22.50 cd	18.70 g	0.97 ab	0.59 bc	9.30 d	6.34 f	61.12 b	20.00 f	43.26 c	17.50 g
	P x G LSD<0.05	2.605		1.634		0.523		0.632		2.184		1.275	

‡Means within columns followed by the same letter are not significantly different at $P \leq 0.05$.

Table 3: Root and shoot dry weight of N22 (wild type), two gain of function mutants and one loss of function mutant (in italics) on 40th day in GS (glass with soil) method (Experiment 4).

S.No	Mutant	Root dry weight (g/plant)		Shoot dry weight (g/plant)	
		60 P	low P	60 P	low P
1	N22	0.04‡ d	0.03 e	0.75 c	0.12 e
2	NH686	0.07 a	0.06 c	1.37 a	0.62 d
3	NH787	0.07 a	0.06 c	1.34 a	0.62 d
4	*NH359*	0.06 b	0.01 f	1.28 b	0.07 f
	P x G LSD<0.05	0.002		0.045	

‡Means within columns followed by the same letter are not significantly different at $P \leq 0.05$.

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Supplementary Table 1: Seed yield of N22 and 8 mutants used in this study and grown in low P (Olsen 1.8) field for four years.

S.No	Line	Yield/plant (g)	
		Normal	**low P**
1	N22 (wild type)	6.77 d‡	0.80 h
2	NH686	8.99 a	4.21 e
3	NH787	8.97 a	4.17 e
4	NH363	8.93 ab	3.98 e
5	NH776	7.69 c	2.43 f
6	NH767	7.48 c	2.90 g
7	*NH210*	8.75 ab	0.47 i
8	*NH162*	8.66 b	0.37 j
9	*NH359*	7.65 c	0 j
	P x G LSD<0.05	0.293	

Please note that in normal P (60Kg/ha) all mutants yield significantly more than N22 but in low P, 5 mutants yield significantly more and are classified as gain of function mutants for tolerance to low P and three mutants (*in italics*) which yield significantly less or do not produce seeds are classified as loss of function for tolerance to low P in field. *NH359* does not flower in low P field and dies by 60 days after transplanting.

‡Means within columns followed by the same letter are not significantly different at $P \leq 0.05$.

Supplementary Table 2: Effect of P presence and absence in medium on root and shoot P concentration in H, HS, PS methods in N22 and NH787.

S.No	Method	Mutant	Root P concentration (µg/g DW)		Shoot P concentration (µg/g DW)	
			+P	-P	+P	-P
1	Hydroponics	N22	42.86 f	9.16 h	44.73 f	9.93 h
		NH787	89.56 d	31.33 g	72.43 e	28.30 fgh
	P x G LSD<0.05		8.076		21.689	
2	Sand	N22	24.86 g	6.49 h	39.71 fg	9.65 h
		NH787	69.16 e	25.89 g	48.30 f	19.91gh
	P x G LSD<0.05		8.076		21.689	
3	Pots	N22	319.00 b	101.67 c	820.70 b	178.30 d
		NH787	629.33 a	315.00 b	1016.7 a	425.00 c
	P x G LSD<0.05		8.076		21.689	

Supplementary Table 3: Effect of P deficiency on root and shoot dry weight on 40th day in glass with soil in 20P, 30P, 40P and 50P (Experiment 4).

S.No	Mutant	Root dry weight (g/plant)				Shoot dry weight (g/plant)			
		20P	30P	40P	50P	20P	30P	40P	50P
1	N22	0.03 j	0.03 j	0.04 i	0.04 i	0.26 i	0.32 hi	0.40 gh	0.54 e
2	NH686	0.06 de	0.06 c	0.06 b	0.07 a	0.75 d	0.89 c	0.98 b	1.13 a
3	NH787	0.06 e	0.06 cd	0.06 b	0.07 a	0.75 d	0.89 c	0.99 b	1.06 ab
4	*NH359*	0.04 h	0.04 h	0.05 g	0.05 f	0.30 i	0.44 fg	0.51 ef	0.71 d
	P x G LSD<0.05	0.002				0.088			

P = Kg/ha P