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Probing Phosphorus Efficient Low Phytic Acid Content Soybean Genotypes with Phosphorus Starvation in Hydroponics Growth System

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Abstract Phosphorus is an essential nutrient required for soybean growth but is bound in phytic acid which causes negative effects on both the environment as well as the animal nutrition. Lowering of phytic acid levels is associated with reduced agronomic characteristics, and relatively little information is available on the response of soybean plants to phosphorus (P) starvation. In this study, we evaluated the effects of different P starvation concentrations on the phytic acid content, growth, and yield of seven mutant genotypes along with the unirradiated control, JS-335, in a hydroponics growth system. The low phytic acid containing mutant genotypes, IR-JS-101, IR-DS-118, and IR-V-101, showed a relatively high growth rate in low P concentration containing nutrient solution (2 μM), whereas the high P concentration (50 μM) favored the growth of IR-DS-111 and IR-DS-115 mutant genotypes containing moderate phytate levels. The mutant genotypes with high phytic acid content, IR-DS-122, IR-DS-114, and JS-335, responded well under P starvation and did not have any significant effect on the growth and yield of plants. Moreover, the reduction of P concentration in nutrient solution from 50 to 2 μM also reduced the phytic acid content in the seeds of all the soybean genotypes under study. The desirable agronomic performance of low phytic acid containing mutant genotype IR-DS-118 reported in this study suggested it to be a P-efficient genotype which could be considered for agricultural practices under P limiting soils.

Keywords Genotypes · Hydroponics · PCA · Phytic acid · P starvation

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Abbreviations

PCA	Principal component analysis
PA	Phytic acid
SDW	Seed dry weight
SHDW	Shoot dry weight
SY	Seed yield
KOH	Potassium hydroxide

Introduction

Soybean (*Glycine max* (L.) Merrill) seeds have been long considered as a rich source of protein and oil for food as well as feed purposes. The seeds also contain large quantities of phytic acid which is a major phosphorus (P) reserve and accounts for 67 to 78 % of total seed P [1]. Although this compound fulfills the requirement of P and *myo*-inositol during seed germination, it poses serious problems due to its negative impact on the environment and the animal nutrition [2]. Phytic acid forms complexes with the mineral cations, such as Fe^{3+}, Zn^{2+}, Ca^{2+}, and Mg^{2+}, thereby reducing their bioavailability to the non-ruminant animals which are deficient of sufficient phytase in their digestive tract [3]. The excreted waste of these animals contains undigested phytic acid which is a leading source of P and the most common cause of eutrophication in the receiving waters [4].

Phosphorus is one of the most essential nutrients required for the plant growth and development. In the developing soybean seeds, P is bound in phytic acid and is rendered unavailable to the monogastric animals [2]. Thus, the efforts are being directed towards producing low phytic acid soybean varieties for use as a high available inorganic phosphorus diet and feed source providing enhanced nutrition to both humans and animals. So far, various strategies have been employed for reducing the phytic acid levels in the food crops. These include variations in the pH and ionic strength in order to resolve the complexes formed by phytic acid with minerals and proteins, milling, germination of seeds [5–6], mutations in the *myo*-inositol-1-*phospate synthase* gene [7], overexpression of the phytase enzyme [8], and the silencing of the ABC transporter gene which plays a critical role in the accumulation of phytic acid in soybean seeds [2]. Lowering of phytic acid levels, however, has an adverse effect on the seed germination [9] which strongly limits the plant productivity [10]. The reduction of phytate levels in soybean seeds by altering the phosphorus status of the soil does not have adverse effects on the germination of soybean seeds [11] and may be beneficial as it would reduce the application of P fertilizer and in turn decrease the cost to the farmers. Since there is not much information available concerning the effect of reduced P availability on the productivity of the soybean in addition to the reduced phytic acid content, the present study was designed to enhance our knowledge to fill this gap. Such information also has an added advantage in identifying low phytic acid soybean genotypes having the ability to utilize P more efficiently and also maintain normal/improved growth under low P supply conditions.

Hydroponics is a simple and suitable method to study the effects of a particular nutrient starvation on the plant yield without meddling with other factors viz. changes in concentration of other nutrients, temperature, and pH [12]. This soilless technique also enables to get easy access to all plant tissues and empowers manipulating the nutrient profile of the growth medium as compared to the soil [13]. Thus, in the present study, we have used the hydroponic growth system to explore the effects of P starvation on the phytic acid content and yield of three groups of mutant genotypes varying in phytic acid content by thorough assessment of the parameters viz. number of pods, number of seeds/pod, seed dry weight, shoot dry weight, and seed yield in order to follow-up the P efficient lines.

Materials and Methods

Experimental Material

Based on a previous laboratory analysis of phytic acid content in 136 irradiated lines from 34 bulked mutant populations of soybean by Kumar et al. [14], a total of eight different soybean genotypes (seven mutant populations and one control) were selected for this study for monitoring of their phytic acid levels and growth parameters under different concentrations of P in the hydroponic growth system. Three mutant genotypes of soybean, i.e., IR-JS-101, IR-DS-118, and IR-V-101, were selected for their low phytic acid content (0.47 to 0.81 g/100 g flour), and the two mutant genotypes viz. IR-DS-111 and IR-DS-115 were selected for their moderate phytic acid content (1.62 to 1.63 g/100 g flour). The mutant genotypes, i.e., IR-DS-122 and IR-DS-114, were selected for their high phytic acid content (2.56 to 2.65 g/100 g flour) as against the unirradiated soybean genotype, i.e., JS-335 which was used as a control.

Phosphorus Starvation Experiment

The general workflow for the soybean hydroponics system is shown in Fig. 1. Soybean (*Glycine max* (L.) Merrill) seeds of the selected genotypes were procured from the Division of Genetics, IARI, New Delhi. The seeds were surface sterilized for 5 min using 10 % sodium hypochlorite and then washed ten times with sterile distilled water. The surface-sterilized seeds were planted in a germination tray containing the soil mix (agropit, vermiculite, and river sand in 1:2:1 ratio) and moistened with water and kept in greenhouse under National Phytotron Facility, IARI, New Delhi. The temperature and relative humidity in the greenhouse were maintained at 28/22 °C and 80/60 % (day/night), respectively. After 7 days of sowing, three seedlings of each genotype were transferred onto the thermocol sheets each placed on four hydroponic tanks containing full strength Hoagland solution (HIMEDIA, India). The experiments were performed in triplicate. The Hoagland solution used for the hydroponic growth had the following ion concentrations: 4.5 mM potassium nitrate (KNO_3), 4.5 mM calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$], 2 mM magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 1 mM ammonium nitrate (NH_4NO_3), 0.1 mM iron (Fe-EDTA), 0.046 mM boric acid (H_3BO_3), 0.009 mM manganese chloride ($MnCl_2 \cdot 4H_2O$), 0.0008 mM zinc sulfate ($ZnSO_4 \cdot 7H_2O$), 0.0003 mM copper sulfate ($CuSO_4 \cdot 5H_2O$), 0.0005 mM sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), and 0.5 mM potassium phosphate (KH_2PO_4). The pH of the nutrient solution was adjusted to 6.0 with 3 M KOH. Distilled water was used for the preparation of nutrient solution, and the solution was replaced every 3 to 4 days. The aeration was provided via a plastic tubing fitted to an aquarium pump at one end and Y-connectors fitted to the other. Plastic tubing was extended to the bottom of each hydroponic tank, and the clamps were used to adjust the airflow. Twenty-one days after transferring the seedlings to the hydroponic system and 1 week before the onset of flowering, P starvation treatments were initiated by reducing the P concentration in the nutrient solution to 50, 25, 10, and 2 μM which was provided separately in the four hydroponic tanks. The plants were harvested 75 days after planting, and number of pods and number of seeds/pods were

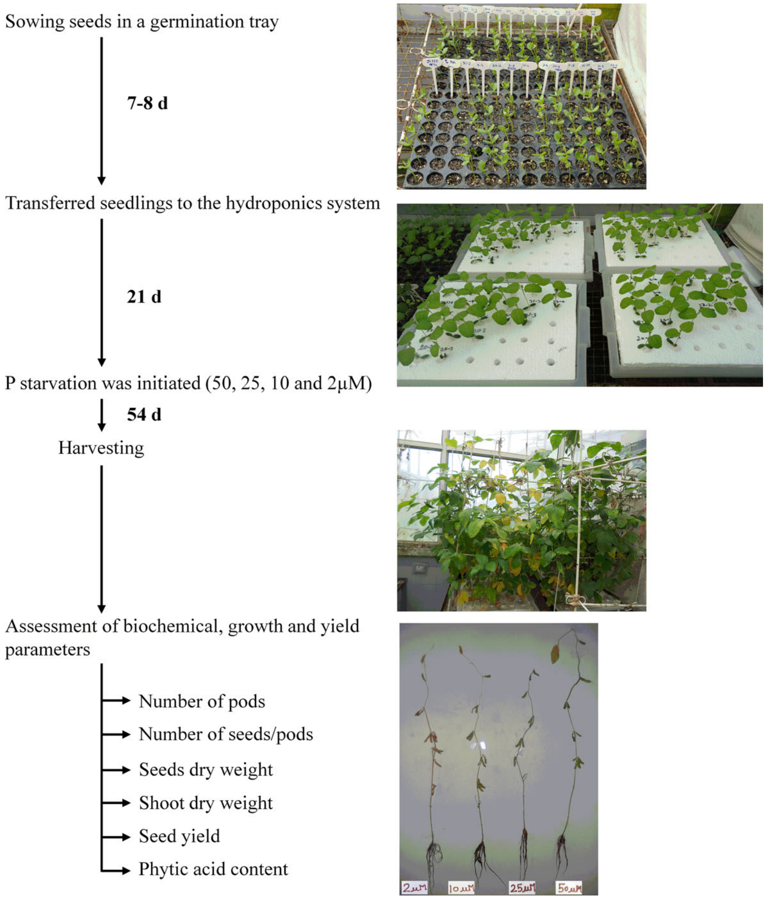

Fig. 1 Schematic representation of the overview of hydroponics growth method. An outline showing the timeline and key steps involved in the method. Time duration is indicated in *bold* on the *right side of the arrows* and images describing the view of the respective steps are provided on the *right side of each step*

recorded. The seed dry weight (SDW), shoot dry weight (SHDW), and seed yield (SY) were recorded by air-drying the shoots and seeds in an oven at 60 °C for 72–96 h, and the seeds were also kept at 4 °C for further biochemical analyses.

Determination of Phytic Acid Content

The phytic acid (PA) content was estimated in the seeds of all the selected soybean genotypes under different P starvation concentrations using the phytic acid (phytate)/total phosphorus assay enzymatic kit (Megazyme International Ltd., Ireland). The content was calculated as followed by Kumar et al. [14].

Statistical Analysis

The graphs were made by calculating the mean ± SD of three experiments. Two-way ANOVA followed by Tukey's multiple comparison test was used for the statistical analysis by using

GraphPad prism software version 6.0. The principle component analysis (PCA) was carried out by using XLSTAT-Pro 7.5 software (Addinsoft, New York, USA).

Results

Response of Plants Growth to P Starvation

The effect of P starvation on the growth and yield of different soybean genotypes is shown in Fig. 2a–e. The P starvation caused chlorosis and little scorching of older leaves, but as such the genotypes did not vary significantly in growth and yield in response to P starvation (Fig. 1). Variable P concentration did not have any significant effect on the growth and yield of IR-JS-

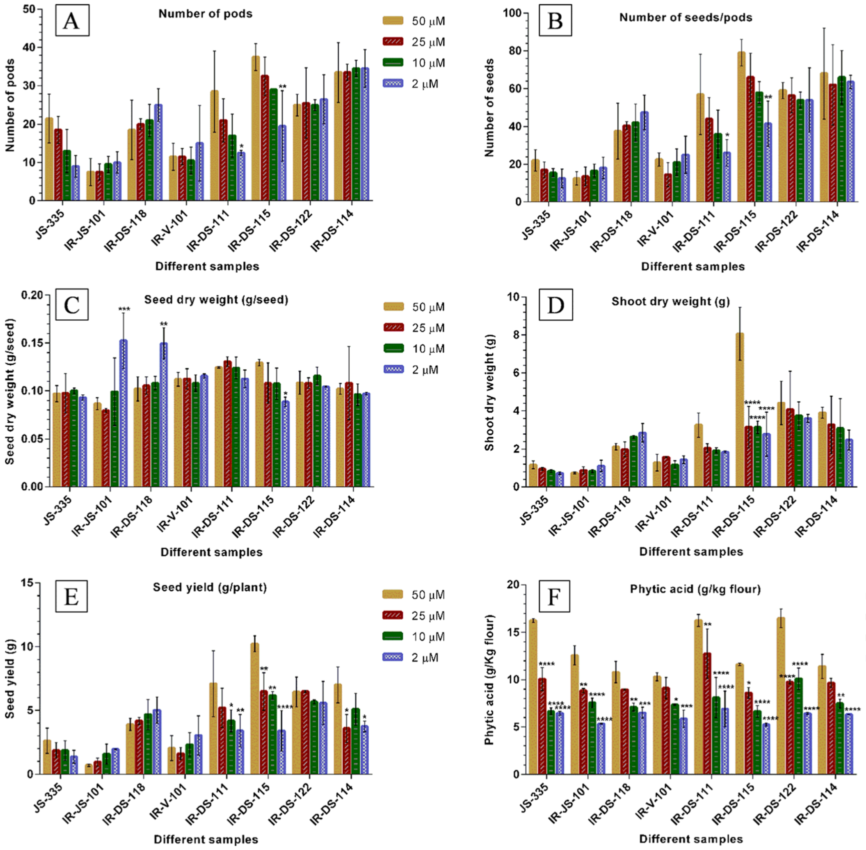

Fig. 2 Assessment of different parameters for growth and yield of mutant soybean genotypes along with the control JS-335 under P starvation conditions. Seven mutant soybean genotypes were selected viz. IR-JS-101, IR-DS-118, IR-V-101, IR-DS-111, IR-DS-115, IR-DS-122, and IR-DS-114, and their responses were assessed at 50, 25, 10, and 2 µM P concentration for **a** number of pods, **b** number of seeds/pods, **c** seed dry weight, **d** shoot dry weight, **e** seed yield, and **f** phytic acid content. The *Y*-axis represents the magnitude of each parameter and *X*-axis represents the different samples. *Bar graphs* show means ± SD of three experiments. Significance was evaluated within samples of each genotype at 2, 10, and 25 µM P concentration compared to 50 µM P concentration. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$)

101, IR-DS-118, and IR-V-101 genotypes except the SDW, which showed a 1.76- ($p < 0.001$) and 1.47-fold ($p < 0.01$) increase, respectively, at 2 μM P concentration compared to 50 μM P concentration. Interestingly, at 2 μM P concentration, the IR-DS-118 genotype showed a significant increase in the number of seeds/pod, SDW, and SY by 3.8- ($p < 005$), 1.66- ($p < 0.01$), and 3.72-fold ($p < 0.05$), respectively, as compared to unirradiated control JS-335. This might indicate that low P concentration (2 μM) favored the growth and yield of this mutant genotype (Fig. 2c). In IR-DS-115 genotype, we observed a significant decrease in number of pods, number of seeds/pod, SDW, SHDW, and SY by 1.9- ($p < 0.01$), 1.88- ($p < 0.01$), 1.46- ($p < 0.05$), 2.90- ($p < 0.0001$), and 3.01-fold ($p < 0.0001$), respectively, at 2 μM P concentration as compared with 50 μM P concentration (Fig. 2a–e). Similar results were obtained for the mutant genotype IR-DS-111, which showed a 2.23- ($p < 0.05$), 2.19- ($p < 0.05$), and 2.09-fold ($p < 0.01$) decrease in number of pods, number of seeds/pod, and SY, respectively, at 2 μM P concentration as compared with 50 μM P concentration (Fig. 2a, b, and e). Further, genotypes with moderate phytic acid content (IR-DS-115 and IR-DS-111) showed a significant increase in the number of pods, number of seeds/pod, SHDW, and SY as compared with the unirradiated control JS-335 but their growth and yield showed a reduction under low P concentration compared with high P concentration treatment. The results suggested that the mutant genotypes with moderate phytic acid content did not respond well under inadequate P concentration. In contrast to the genotypes, JS-335, IR-DS-122, and IR-DS-114 responded well to the low P concentrations in the nutrient medium and did not reveal any significant negative effect on the yield parameters viz. number of pods, number of seeds/pod, SDW, SHDW, and SY (Fig. 2a–e). In addition, high phytic acid content genotypes (IR-DS-122 and IR-DS-114) also showed a significant increase in number of pods, number of seeds/pod, SHDW, and SY as compared with unirradiated control JS-335 when the P concentrations were changed from 50 to 2 μM in nutrient medium. The results thus suggested that the mutant genotypes with high phytic acid content contained adequate P reserves for their growth under inadequate P concentration.

Phytic Acid Content Under P Starved Soybean Seeds

Seeds of different soybean genotypes exhibited a lot of variation in PA content under P starvation conditions. PA content showed a progressive reduction in all three mutant genotype categories when the P concentrations were reduced from 50 to 2 μM in the nutrient medium (Fig. 2f). The magnitude of variation in phytic acid levels for different genotype categories was however different. In low PA content mutant genotypes, the fold reduction in phytic acid levels of IR-JS-101 was 1.42- ($p < 0.01$), 1.67- ($p < 0.0001$), and 2.36-fold ($p < 0.0001$), whereas in IR-DS-118, it was 1.21-, 1.52- ($p < 0.01$), and 1.66-fold ($p < 0.001$) at the 25, 10, and 2 μM P concentrations, respectively, as compared to 50 μM P concentration. The IR-V-101 showed 1.13-, 1.40- ($p < 0.05$), and 1.76-fold ($p < 0.001$) reduction at the 25, 10, and 2 μM P concentrations, respectively, compared to 50 μM P concentration (Fig. 2f). The phytic acid profiles of moderate PA content containing mutant genotypes showed that in IR-DS-111, the content reduction was 1.27- ($p < 0.01$), 2.00- ($p < 0.0001$), and 2.36-fold ($p < 0.0001$), whereas in IR-DS-115, it was 1.35- ($p < 0.05$), 1.75- ($p < 0.0001$), and 2.21-fold ($p < 0.0001$) at the 25, 10, and 2 μM P concentrations, respectively, compared to 50 μM P concentration (Fig. 2f). For high PA content mutant genotypes, the reduction was 1.69- ($p < 0.0001$), 1.63- ($p < 0.0001$), and 2.57-fold ($p < 0.0001$) in IR-DS-122, whereas 1.18-, 1.52- ($p < 0.01$), and 1.81-fold ($p < 0.0001$) reduction was observed in IR-DS-114 at the 25, 10, and 2 μM P concentration, respectively, compared to 50 μM P concentration (Fig. 2f). The control JS-335 showed the similar pattern of reduction by 1.62- ($p < 0.0001$), 2.44- ($p < 0.0001$), and 2.52-fold ($p < 0.0001$) at the 25, 10, and 2 μM P concentration, respectively, compared to 50 μM P concentration (Fig. 2f). The results showed that the PA content of all the mutant genotype categories as well as the control was the lowest at 2 μM P concentration. Further, IR-DS-118 and IR-V-101 genotypes showed a significant decrease in PA content with 0.66- ($p < 0.0001$) and 0.63-fold ($p < 0.0001$), respectively, at 50 μM P concentration, whereas no significant variation in all the mutant genotypes was observed at 2, 10, and 25 μM P concentration as compared to unirradiated control JS-335 (Fig. 2f).

It could thus be inferred from the results that IR-DS-118 genotype not only showed reduced phytic acid levels under low P concentration but also a decreased phytic acid level at high P concentration when compared to unirradiated control JS-335. Moreover, the increased growth and yield parameters under low P concentration conditions further strengthen its potential as a P-efficient genotype.

Principal Component Analysis

The sources of variation within the different soybean genotype categories were investigated by using PCA in which the observations were the different P concentrations, i.e., 2, 10, 25, and 50 μM, and variables were the values of different soybean genotypes for each of the selected growth parameters. The scree plots between eigenvalues and principal components (F1–F4) for the number of pods, number of seeds/pod, and PA content showed that principal component 1 (F1) described the largest variation contributing 85.9, 92, and 64 %, followed by F2 (13.5, 7.7, and 20.1 %), F3 (0.4, 0.3, and 10.8 %), and F4 (0.2, 0.1, and 5.2 %) components, respectively, as shown in Fig. 3a, b, and d. The other yield parameters, i.e., SDW, SHDW, and

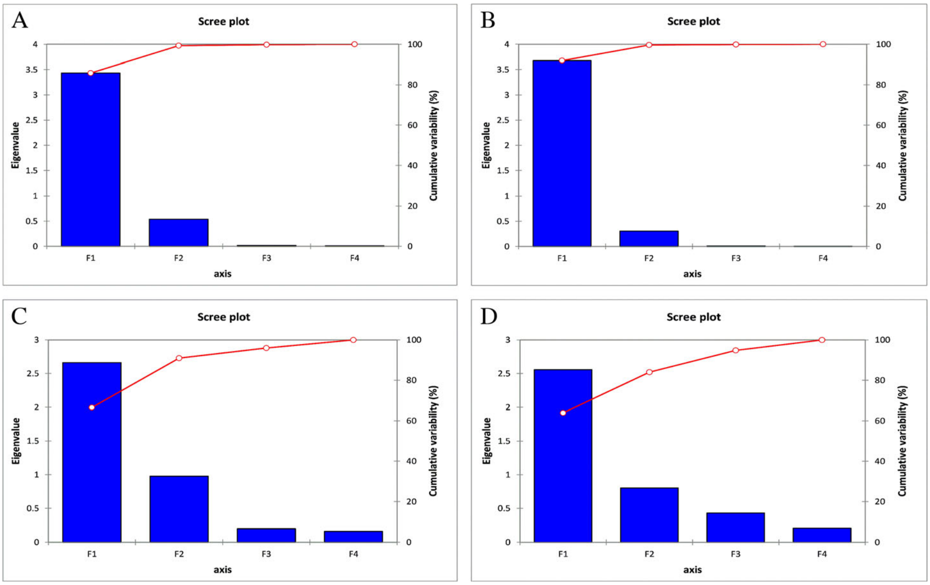

Fig. 3 Scree plots showing the principal components (F1–F4), eigenvalues, and cumulative variability for different parameters as generated by principal component analysis. **a** Number of pods, **b** number of seeds/pods, **c** seed dry weight, shoot dry weight, and seed yield, and **d** phytic acid content. The components 1 and 2 caused major variability while 3 and 4 contributed partially

SY exhibited an identical scree plot generated by PCA analysis which showed that the largest variation was described by F1 (66.6 %) followed by F2 (24.4 %), F3 (5 %), and F4 (4 %) components (Fig. 3c). These results inferred that F1 and F2 best described the sources of variation between the different P concentrations. Further, biplots between F1 and F2 components were redrawn for the number of pods, number of seeds/pod, SDW, SHDW, SY, and PA content with blue dots highlighting the different soybean genotypes and red dots indicating the different P concentrations (Fig. 4). Among the biplots, the SDW, SHDW, and SY exhibited identical biplots and thus represented as a single figure (Fig. 4c). The biplots inferred the grouping of different mutant genotypes in separate zones around the origin. The low PA content mutant genotypes were found loaded in the cluster around low P concentration, while moderate PA content mutant genotypes were found loaded in the cluster around high P concentration (Fig. 4). The high PA content mutant genotypes, however, showed an irregular behavior (Fig. 4).

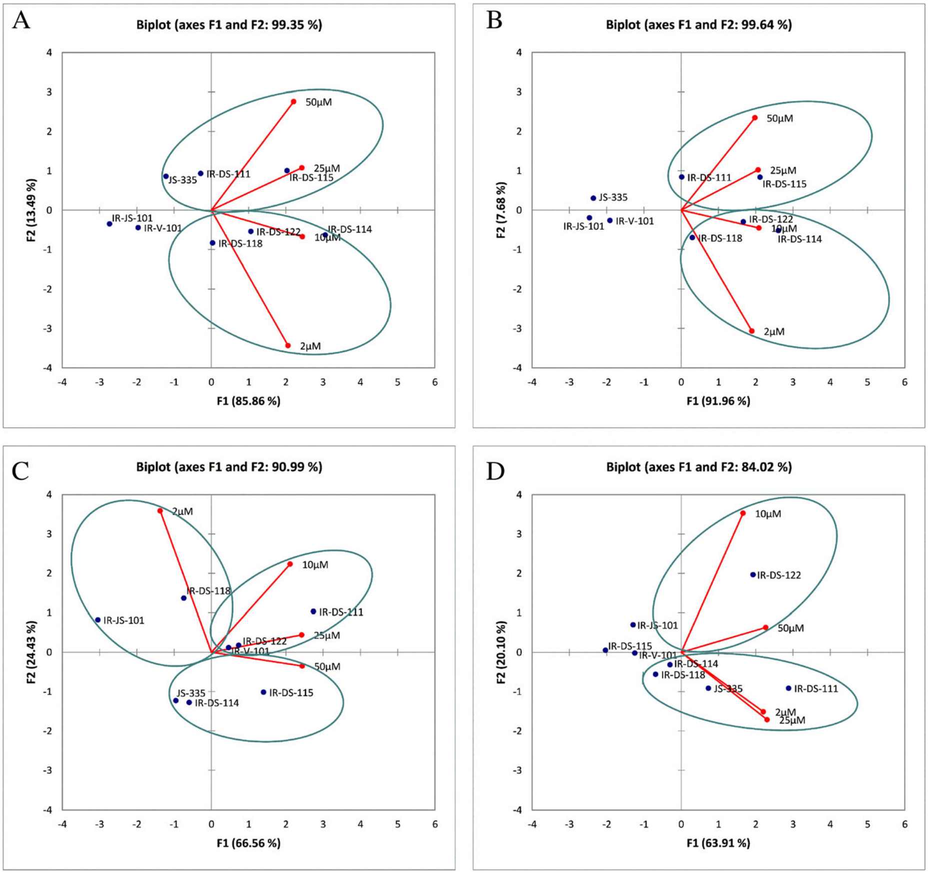

Fig. 4 Biplots between components 1 and 2 for different parameters as generated by principal component analysis. **a** Number of pods, **b** number of seeds/pods, **c** seed dry weight, shoot dry weight, and seed yield, and **d** phytic acid content. The data showed the four different P concentrations, i.e., 50, 25, 10, and 2 µM in *red dots* and all the selected soybean genotypes in *blue dots*

Discussion

The primary objective of this study was to determine the effects of reduced external P supply on PA content and yield as well as growth parameters of mutant genotypes of soybean categorized as per their PA content. A progressive reduction in phytic acid levels was observed in the low, moderate, and high PA content containing mutant genotypes as well as in the control (JS-335), when the P concentrations in the nutrient medium were reduced from 50 to 2 μM. This might be explained by the fact that P bound in phytic acid in the developing soybean seeds is rendered available upon reduction of P concentrations in nutrient solution leading to decreased phytic acid levels. Raboy and Dickinson [15] also showed a decrease in phytic acid P levels among the seeds of *Glycine max* and *G. soja* upon the reduction of P concentrations in nutrient solution from 50 to 2 mg L^{-1}. Li et al. [16] and Mollers et al. [17] have also reported the reduction in phytic acid levels under low P availability conditions. Further, PCA analysis also provided support and refined the present results by representing that IR-DS-114, IR-DS-118, IR-DS-111, and JS-335 were distributed in the cluster around 2 and 25 μM P concentrations, while IR-DS-122 was distributed in the cluster around 10 and 50 μM P concentrations (Fig. 4d). IR-JS-101 also made significant contribution at 50 μM P concentration based on its distance from the origin (Fig. 4d). In this respect, it might be inferred that IR-DS-118, the low phytic acid mutant genotype, could be the desired genotype which made significant contribution at low P concentration, i.e., 2 μM.

Furthermore, the deficient P supply did not have a significant effect on the yield of genotypes containing low PA content as assessed by the number of pods, number of seeds/pod, SDW (g/seed), SHDW (g), and SY (g/plant). Also, the SDW showed significant increase in the genotypes IR-DS-118 and IR-JS-101 upon decreasing the P concentration of nutrient solution from 50 to 2 μM (Fig. 2c), which might be indicative of the fact that these two mutant genotypes containing low phytic acid content are using P more efficiently compared to other mutant genotypes. This is supported by a previous study which also showed that the plants that uptake P efficiently with quick growth might accumulate less P in the plants [18]. The PCA analysis results also verified the same as the IR-DS-118 genotype which was represented in the cluster around 2 μM P concentration in the biplots (Fig. 4a–c). The IR-JS-101 also made the significant contribution at 2 μM P concentration based on its distance from the origin (Fig. 4a–c). The present findings corroborated with a previous study which reported a similar growth of roots and shoots from soybean seeds containing low and medium phytic acid P [11].

On the other hand, the mutant genotypes containing the moderate PA content, i.e., IR-DS-111 and IR-DS-115, showed significant decrease in yield with the decrease in P concentration in the nutrient solution from 50 to 2 μM (Fig. 2a–e), which indicated the possibility of IR-DS-111 and IR- DS-115 being P sensitive genotypes. This was also supported by PCA analysis which showed both IR-DS-115 and IR-DS-111 in the cluster around 25 and 50 μM P concentrations in the biplots (Fig. 4a–c). The reduced yield of mutant genotypes with moderate PA content obtained in this study was found to be consistent with the previous reports which also showed a reduction in yield of barley [19], tomato [12], and soybean [15] in response to P deficiency. The high PA content containing mutant genotypes, i.e., IR-DS-122 and IR-DS-114, did not have a significant effect on the yield with the increase in P concentration of nutrient solution from 2 to 50 μM (Fig. 2a–e), which could be explained by the fact that mutant genotypes with high PA content contain adequate P reserve to support constant growth rate but high P accumulation at a relatively low P supply [18]. The PCA analysis also supported the results and showed an irregular representation of high PA content mutant genotypes in the clusters around different P concentrations in the biplots (Fig. 4a–c). Raboy et al. [11] also documented that soybean seeds possess much more reserved P than required for germination and early seedling growth. Moreover, a positive relationship has also been reported between available P level and phytic acid content in the mature seeds of rice, wheat, and soybean plants [20–22].

With the maximum phytic acid level of 10.8 g/kg flour, the number of pods—25, number of seeds/pod—48, SDW—0.15 g/seed, SHDW—2.85 g, and SY—5.03 g/plant; the selected mutant genotype, i.e. IR-DS-118, in the present study, has shown a consistent yield and a consistent reduction in the phytic acid levels under low P concentrations in the nutrient solution, which reflects upon its promising P utilizing efficiency. The exact mechanism behind this behavior needs further elucidation. These features are desirable from an agronomic perspective and thus warrant a next level investigation for confirming P efficient status of this mutant.

Conclusions

The mutant genotypes under study thus showed three patterns of response to P starvation in hydroponic growth system. The low PA content mutant genotypes showed an increased growth and yield upon decreasing the P concentration in nutrient solution while moderate PA content mutant genotypes showed decreased growth and yield upon decreasing the P concentration in nutrient solution. In contrast, the high PA content mutant genotypes along with the control soybean genotype, i.e. JS-335, showed no significant alteration in their growth and yield with the decrease in P concentration in nutrient solution. This data provides a basis for the selection of P-efficient genotype, i.e. IR-DS-118, which showed reduced PA level and increased growth and yield under low P supply. Perhaps the testing of the identified mutant under low P soil conditions is further required which might provide a more comprehensive picture of its role as a P-efficient genotype.

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Conflict of Interest The authors declare that they have no competing interests.

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