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Identification of putative low phytic acid mutants and assessment of the total P, phytate P, protein and divalent cations in mutant populations of soybean

Sweta Kumari[1], Sanjay Kumar Lal[2] and Archana Sachdev[1,*]

[1]Division of Biochemistry, Indian Agricultural Research Institute (IARI), New Delhi, India
[2]Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi, India

***Corresponding author: arcs_bio@yahoo.com**

Abstract

Phytic acid (myo-inositol hexakisphosphate) is one of the principal storage forms of phosphorous in the plant tissues. The six attached phosphorus groups on the *myo*-inositol ring form a high density of negative charges allow the molecule to bind a number of mineral cations. There by preventing their absorption in addition to decreasing the phosphorus availability. Also undigested by monogastrics, the unabsorbed phytate is a major cause of phosphorus related water pollution. The objective of this study was to select mutant lines among the soybean mutant populations having low phytic acid content, along with high protein and mineral levels. Following the treatment of 17 soybean genotypes with chemical (Ethyl Methane Sulphonate) and physical (gamma rays) mutagens, 34 mutant populations were developed. The M_2 through M_{10} plants were screened on the basis of their resistance to yellow mosaic virus (YMV). The plants of the M_{10} generation analysed for their phytic acid (PA), protein and mineral levels revealed five mutant lines, IR-JS-101-4, IR-V-101-3, IR-DS-118-2, IR-DS-119-4 and IR-DS-122-2 which showed significant reduction in phytic acid and phytic acid phosphorus (PA-P) contents compared to their parental lines. Seed total phosphorus (P) was significantly and highly correlated with PA and PA-P (r=0.98) among the mutant populations and a significant correlation of total P with PA and PA-P (r=0.52 and 0.48 respectively) was also observed in the five mutant lines showing low phytic acid content. PA and seed protein were positively and significantly correlated (r=0.33) among the mutant population and negatively and significantly correlated (r=0.74) in the low phytic acid lines. A negative but no significant correlation between PA and divalent cations (r= 0.05 for Iron, 0.07 for Zinc, 0.04 for Calcium and 0.09 for Manganese) among the mutant populations but a significant and a negative correlation was however observed between PA and iron (r= 0.8) in the low phytic acid lines. The low phytate mutant thus identified in the present study can be a good germplasm source for breeding low phytic acid (LPA) soybean.

Keywords: Calcium; EMS; Iron; Gamma irradiation; LPA**;** Manganese**;** Mineral; Phytic acid; Soybean; Zinc
Abbreviations: AAS_Atomic Absorption Spectrometry; EMS_Ethyl Methane Sulphonate; LPA_Low Phytic Acid; P_Phosphorus; PA – Phytic Acid

Introduction

Soybean is fast emerging as one of the most economical and nutritious foods in the developing countries, including India, and was recently also described as the 'functional food of the century'. Soybean meal is a very important source of protein commonly used in animal feed world wide. However, its role appears to be limited because of the presence of the anti-nutrient, phytic acid (PA), which interferences the protein and mineral digestion of monogastric animals. Also known as *myo*-inositol-1,2,3,4,5,6-hexakisphosphate (PA), is the main phosphorus storage compound in most of the plant seeds (60 to 80%). Being a strong chelating agent, owing to its structure, it readily binds metal cations such as Fe, Zn, Ca and Mn making them insoluble and unavailable thus resulting in a decrease in their bioavailability. During seed maturation, these phytic acid complexes are accumulated in protein storage vacuoles in inclusions called globoids and often exist in the form of mixed salts, such as Fe, Zn, Ca and Mn in plant seeds (Raboy, 1997). Since PA and its salts are almost indigestible for monogastric animals, its abundance in grain food or feed implicates nutritional and environmental problems (Raboy, 2001). These negative effects have led to intensive breeding programs aimed at reducing the PA content in the seeds of several cultivated plants. Screening, by conventional breeding, for low phytic acid (LPA) mutants, capable of restraining the biosynthesis or the storage of PA in the seeds, is one method to be followed by confirmation of increased P and mineral cation bioavailability in LPA seeds by nutritional trials (Mendoza et al., 1998; Hambidge et al., 2005).

Various efforts have been made in the past decade to generate crops with decreased contents of phytic acid. Mutation breeding through γ-irradiation (Larson et al., 2000; Yuan et al., 2007) or chemically induced mutagenesis (Raboy et al., 2000; Dorsch et al., 2003) has been described for various cereals and legumes and *lpa* mutants have been isolated from several crops, such as barley, by chemical mutagenesis (Larson et al., 1998; Bregitzer and Raboy 2006), soybean by chemical and physical mutagenesis (Wilcox et al., 2000; Hitz et al., 2002; Yuan et al., 2007), wheat by chemical mutagenesis (Guttieri et al., 2004), common bean by chemical mutagenesis, and rice by physical and chemical mutagenesis (Larson et al., 2000; Liu et al., 2007a).

In addition, genetic engineering has been successfully applied to produce low phytic acid (LPA) crops. In soybean, transgenic plants expressing a recombinant fungal phytase (Denbow et al., 1998) or over-expressing the soybean phytase (*GmPhy*) gene (Chiera et al., 2004) were produced; reduction of PA-P in soybean seeds was recently achieved through (partially) silencing of the *myo*-inositol 1-phosphate synthase (*MIPS*) gene using RNAi technology (Nunes et al., 2006).

Table 1. Thirty four bulked mutant M_{10} populations generated by treatment with different doses of γ rays and 0.1% ethyl methane sulphonate (EMS) and their corresponding parental lines (check genotypes).

Serial number	Population	Parental lines	Mutagenic Dose
1	IR-DS-101	DS-9702 (advanced breeding line)	0.25 kGy
2	IR-DS-102	PUSA-9712 (released variety)	0.25 kGy
3	IR-JS-101	JS 335[a] (released variety)	0.25 kGy
4	IR-SL-101	SL 688 (released variety)	0.25 kGy
5	IR-PK-101	PK 1024 (released variety)	0.25 kGy
6	IR-MACS-101	MACS-730 (advanced breeding line)	0.25 kGy
7	IR-V-101	V42 (black seeded)	0.25 kGy
8	IR-DS-103	PUSA-20 (released variety)	0.25 kGy
9	IR-DS-104	PUSA22 (released variety)	0.25 kGy
10	IR-DS-105	PUSA 24 (released variety)	0.25 kGy
11	IR-DS-106	PUSA 37 (released variety)	0.25 kGy
12	IR-DS-107	PUSA 40 (released variety)	0.25 kGy
13	IR-JS-102	JS 335[b] (released variety)	0.25 kGy
14	IR-PK-102	PK1368 (released variety)	0.25 kGy
15	IR-JS-103	JS 335[c] (released variety)	0.25 kGy
16	IR-JS-104	JS 335[d] (released variety)	0.25 kGy
17	IR-DS-108	DS-74 (black seeded; Advanced breeding Line)	0.1 % EMS
18	IR-DS-109	DS-74	0.1 % EMS
19	IR-DS-110	DS-74	0.1 % EMS
20	IR-DS-111	DS-74	0.1 % EMS
21	IR-DS-112	DS-74	0.1 % EMS
22	IR-DS-113	DS-74	0.1 % EMS
23	IR-DS-114	DS-74	0.1 % EMS
24	IR-DS-115	DS-74	0.1 % EMS
25	IR-DS-116	DS-74	0.1 % EMS
26	IR-DS-117	DS-74	0.1 % EMS
27	IR-DS-118	DS-74	0.1 % EMS
28	IR-DS-119	DS-74	0.1 % EMS
29	IR-DS-120	DS-74	0.1 % EMS
30	IR-DS-121	PUSA 20 (released variety)	0.20 kGy
31	IR-DS-122	PUSA 24 (released variety)	0.20 kGy
32	IR-DS-123	PUSA 37 (released variety)	0.20 kGy
33	IR-DS-124	PUSA 40 (released variety)	0.20 kGy
34	IR-DS-125	JS 335[b] (released variety)	0.20 kGy

[a,b,c,d] Original seed lot obtained from different sources.

Ockenden et al. (2006), observed the effects of mutation in the LPA crops, by studying the content of phytic acid in relation to the nutritionally relevant minerals in barley, maize, rice and wheat. However, previous studies have indicated that LPA mutations do not show any systematic pattern of increase or decrease of the mineral contents in maize (Lin et al., 2005), barley (Liu et al., 2007b) and wheat (Guttieri et al., 2006).

Considering the potential impact of environmental factors, the objective of the present study was to analyze the contents of phytic acid, total P, phytate P, protein and divalent cations in the 34 bulked mutant populations and their corresponding parental genotypes. Among the chelating divalent cations iron, zinc, calcium and manganese were particularly selected owing to their importance in human nutrition.

Results and Discussion

Soybean mutant lines from thirty four bulked mutant populations of soybean (Table1) were selected for analysis of phytic acid (PA), phytate P, total P, protein and divalent cations. In the present study 17 different genotypes were used for generating 34 bulked mutant populations, which also served as check genotypes.

Effect of mutagen on phytic acid, phytate P, total P, protein and divalent cations in mutant populations

Extensive variation in the concentration of each of the compound analysed was observed in all the mutant soybean population. Phytic acid content among the genotypes of mutant populations varied from 4.7×10^{-3} kg/kg to 28.1×10^{-3} kg/kg flour while phytate P content varied from 1.3×10^{-3} kg /kg to 7.9×10^{-3} kg /kg of flour**.** The phytic acid content of the check genotypes varied from 17.70×10^{-3}kg /kg to 27.70×10^{-3} kg /kg while phytate P content varied from 4.9×10^{-3} kg/kg to 7.8×10^{-3} kg/kg of flour (Tables 2). Five mutant genotypes IR-JS-101-4, IR-V-101-3, IR-DS-118-2, IR-DS-119-4 and IR-DS-122-2 however showed marked reduction in the phytate and phytate P levels compared to check genotypes (Tables 3). These putative LPA mutant lines showing almost 2 to 3 fold decrease in the phytic acid and phytate P contents were taken for further biochemical analysis which revealed that these were completely distinct from the two LPA mutant lines previously reported, M156 and M766 in soybean (Wilcox et al. 2000). In plant breeding programs, generation of mutant crops by induced mutations has become an important tool for generating novel genetic variations. During the past few years, according to the FAO/IAEA Mutant Variety Database (http://www-mvd.iaea.org/MVD/default.htm) more than 100

Table 2. Range of phytic acid, phytic acid P, total P, protein, iron, zinc, calcium and manganese contents (kg/kg soybean flour) among mutant populations and check genotypes.

Compound	Mutant populations	Check genotypes
PA	4.7×10^{-3} to 28.1×10^{-3}	17.7×10^{-3} to 27.7×10^{-3}
PA-P	1.3×10^{-3} to 7.9×10^{-3}	4.9×10^{-3} to 7.8×10^{-3}
Total P	2.3×10^{-3} to 9.1×10^{-3}	5.7×10^{-3} to 8.3×10^{-3}
Protein	362×10^{-3} to 538×10^{-3}	382×10^{-3} to 514×10^{-3}
Iron	94×10^{-6} to 183×10^{-6}	94×10^{-6} to 164×10^{-6}
Zinc	40×10^{-6} to 68×10^{-6}	46×10^{-6} to 55×10^{-6}
Calcium	2547×10^{-6} to 4668×10^{-6}	2774×10^{-6} to 3982×10^{-6}
Manganese	21×10^{-6} to 38×10^{-6}	21×10^{-6} to 27×10^{-6}

soybean varieties have been developed worldwide. However the *lpa* mutation frequency, although variable among populations, has been more or less within the same magnitude, and also within the range of mutation frequency of similar single gene controlled traits (Van Harten 1998). Although genetic transformation can also be used to reduce phytate content in soybean (Nunes et al. 2006), chemical or physical mutagenesis is technically simpler, more cost-effective and publicly accepted. In the present study, soybean LPA had reduction of phytic acid and PA-P by about 68–80% (Table 3) and previously reported soybean LPA mutants (LR 33, M156, and M733) had a PA-P reduction of about 50–70% (Wilcox et al. 2000; Hitz et al. 2002). The same pattern was observed in studies conducted on rice *lpa2* mutant, homologous orthologous of the maize *lpa1* mutant (Xu et al. 2009), whereas similar barley *lpa* mutations showing more than 90% of reduction in seed phytate were viable (Larson et al. 1998; Bregitzer and Raboy 2006). Among soybean mutant population, a slightly greater variation in total phosphorus and protein content was observed compared to the check genotypes. The range of total P content varied from 2.3×10^{-3} kg/kg to 9.1×10^{-3} kg/kg while protein content varied from 362×10^{-3} kg/kg to 538×10^{-3} kg/kg in the mutant populations (Tables 2). The total P content in the check genotypes varied from 5.7×10^{-3} kg/kg to 8.3×10^{-3} kg/kg while the protein content varied from 382×10^{-3} kg/kg to 514×10^{-3} kg/kg of flour. The genotypic variation in phosphorus content may be attributed to effects of growing conditions as phosphorus uptake by crop plants depends upon the sensitivity of the cultivars to the changes in root surface area and rhizosphere acidification. Israel et al. (2007) reported that total phosphorus accumulation also depends on many factors that affect uptake of phosphorus such as phosphorus mineralizing microorganisms in the soils, differential status of soils, soil pH and temperature. Phytic acid is stored in the form of globular crystalloids in the endosperm, embryo or protein bodies of the cotyledons depending upon the crop plants. In legumes, such as in soybean, it might be thus presumed that the protein content may probably have a correlation with the accumulation of total phosphorus, phytic acid P and phytic acid levels as phytic acid is typically stored as globular crystalloids in the protein storage vacuoles. Among the 34 mutant populations extensive variation in mineral accumulation was observed for iron, zinc, calcium and manganese in the range of 94×10^{-6} kg/kg to 183×10^{-6} kg/kg, 40×10^{-6} kg/kg to 68×10^{-6} kg/kg, 2547×10^{-6} kg/kg to 4668×10^{-6} kg/kg, 21×10^{-6} kg/kg to 38×10^{-6} kg/kg respectively, in contrast to check genotype range from 94×10^{-6} kg /kg to 164×10^{-6} kg/kg, 46×10^{-6} kg/kg to 55×10^{-6} kg/kg, 2774×10^{-6} kg/kg to 3982×10^{-6} kg/kg, 21×10^{-6} kg/kg to 27×10^{-6} kg/kg respectively (Table 2). Previous studies have shown that the uptake of Fe, Zn, Ca and Mn increases in plants under different stress conditions. In soybean the Fe, Zn, Ca and Mn concentrations increased when exposed to acid rain (Wang et al., 2000). The trend observed in the present study, that soybean lines high in one mineral are generally high in others too is similar to the results of Kleese et al. (1968). Contents of iron, zinc, calcium, and manganese in the five LPA soybean mutants and their wild-types are shown in Table 3. On an average, the levels of divalent cations showed significantly higher levels of iron (21%), zinc (+19%), calcium (+26%), and manganese (+20%) compared to the corresponding wild-type. The results are in agreement with the observations made for the rice mutant (Os-lpa-XQZ-1) in a recent study with seed material from three other field experiments/season (Ren et al., 2007 and Frank et al., 2009). It is difficult to conclude whether these observations are a result of pleiotropic effects of the target gene from the intended mutation, or of further mutations independent from the primary mutation event. For example, microsatellite analysis of γ-irradiated rice revealed that out-crosses are responsible for different plant phenotypes (Fu et al., 2007).

Regarding the natural variability of mineral concentrations, both the mean contents of calcium, iron, zinc and manganese in soybean mutant populations, check genotypes as well as the contents in the mutants are within the reported natural range (Raboy et al., 1984; Kirchhoff, 2008).

Correlation among the phytic acid, phytate P, total P, protein and divalent cations in mutant populations and in selected LPA soybean mutants

As shown in Table 4, Seed total P was highly correlated with PA and PA-P (r=0.98) among the soybean mutant populations and seed total P was significantly correlated with PA and PA-P (r=0.52 and 0.48 respectively) in low phytic acid soybean lines (Table 5). PA and protein were positively correlated with seed protein (r=0.33) among soybean mutant populations (Table 4) and negatively and significantly correlated (r=0.74) in low phytic acid soybean lines. Raboy et al. (1984; 1991) also reported that grain phytic acid was highly and positively correlated with variation in grain total protein in wheat and soybean. A close correlation of phytic acid with protein and total P indicates that selection against grain phytic acid would lead to undesirable reductions in both grain total P and protein. For example, in maize it was found that a divergent selection for maize kernel protein content resulted in a two fold divergence in the content of kernel phytic acid. It revealed a positive and quantitative relationship between selection for kernel protein and phytic acid (Raboy et al. 1989). However Morre et al. (1990) found no significant correlation between the contents of grain phytic acid and total protein in the bean seeds. A negative and no significant correlation between PA and divalent cations among mutant populations but significant and negative correlation was found between PA and iron in the soybean mutant lines in the present study. Other than iron, calcium showed a significant and positive correlation and zinc and manganese showed a negative and no significant correlation with phytic acid. The negative and no significant

Table 3. PA, PA-P, total P, protein, iron, zinc, calcium and manganese contents (kg/kg soybean flour) of selected low phytic acid (lpa) mutants from 34 bulked populations.

Mutants	PA ($\times10^{-3}$)	PA-P ($\times10^{-3}$)	Total P ($\times10^{-3}$)	Protein ($\times10^{-3}$)	Iron ($\times10^{-6}$)	Zinc ($\times10^{-6}$)	Calcium ($\times10^{-6}$)	Manganese ($\times10^{-6}$)
IR-JS-101-4	6.0*± 0.54	1.7* ± 0.01	4.3* ± 0.03	510* ± 5	178* ± 4	65*± 0.24	3645 ± 12	29* ± 0.6
Control	22.0± 0.87	6.2 ± 0.03	6.8 ± 0.03	443 ± 8	147 ± 8	51 ± 0.32	2774 ± 18	25 ± 0.5
IR-V-101-3	4.7*± 0.35	1.3* ± 0.03	4.6* ± 0.02	520* ± 12	183* ± 3	53* ± 0.19	3607* ± 18	31*± 0.8
Control	22.7± 0.65	6.4 ± 0.03	6.9 ± 0.02	452 ± 6	149 ± 7	46 ± 0.29	2827 ± 12	25 ± 0.9
IR-DS-118-2	8.1*± 0.68	2.2*± 0.03	4.8* ± 0.03	490 ± 9	152* ± 4	58* ± 0.09	4163* ± 13	30* ± 0.5
Control	25.0± 0.47	7.0 ± 0.04	7.7 ± 0.08	473 ± 6	128 ± 4	52 ± 0.31	3435 ± 11	26 ± 0.4
IR-DS-119-4	7.1*± 0.43	2.0* ± 0.03	4.7*± 0.03	500 ± 3	154* ± 2	62* ± 0.14	4388* ± 43	31* ± 0.5
Control	25.0± 0.47	7.0 ± 0.03	7.7 ± 0.08	473 ± 6	128 ± 4	52 ± 0.31	3435 ± 11	26 ± 0.4
IR-DS-122-2	6.1*± 0.59	1.7 ± 0.03	4.3*± 0.02	500* ± 13	151 ± 4	65* ± 0.19	3879* ± 31	32* ± 0.7
Control	22.5 ± 0.54	6.3 ± 0.02	6.9 ± 0.09	455 ± 9	126 ± 3	53 ± 0.18	3147 ± 06	25 ± 0.4

* indicates values are significant over corresponding control values (check genotypes) at 5% significant level

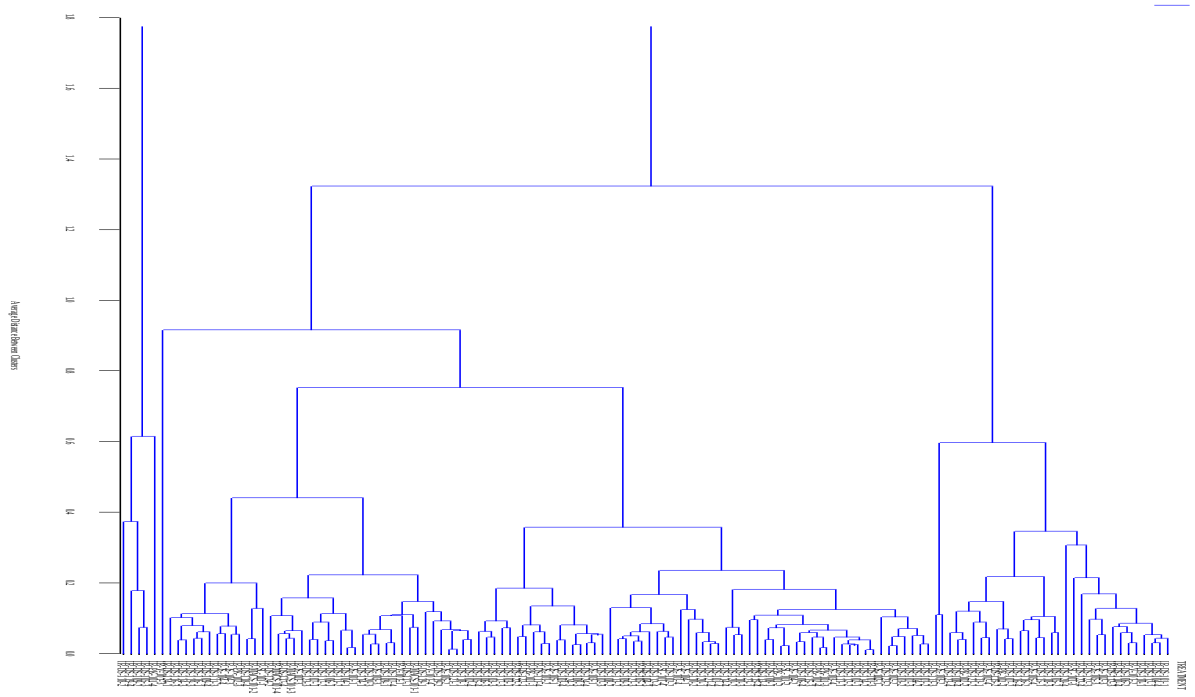

Fig 1. Dendrogram of cluster analysis of 136 mutant lines based on phytic acid, phytate P, total P, protein and divalent cations (iron, zinc, calcium and manganese) in mutant populations. UPGMA clustering method, Squared Euclidean distance. SAS 9.3.

correlation between PA and zinc observed among *lpa* soybean mutants in this study (Table 5) is in consistence with the previous reports from *Glycine soja* lines and in contrast to the soybean lines, where positive and significant correlation was observed (Raboy et al., 1984). For calcium and PA, a correlation of LPA lines, was consistent with positive and significant correlation reported by Raboy et al., 1984 in soybean lines.

Hence, it can be postulated that the total protein and phytic acid content is quantitatively associated and variety-dependent or species-dependent, and it may be feasible to screen the soybean lines with low phytic acid and high protein content by the approach of mutational and conventional breeding.

Cluster analysis

For the mutant population, cluster analysis was performed based on the biochemical parameters, total P, phytate P, protein and divalent cations (iron, zinc, calcium and manganese) and the results were represented by a dendrogram (Fig 1). Figure 1 depicts the dendrogram, which was calculated by commonly used squared Euclidean distance

At 1.34 Euclidean distances, three significant clusters 1, 2 and 3 of 101, 30 and and 5 mutant lines respectively, were generated from the mutant populations (Fig 2). Of the total of five mutant lines selected on the basis of low phytic acid content, the lines IR-JS-101-4 and IR-DS-122-2 were represented in Cluster 1, IR-V-101-3 in Cluster 2 and IR-DS-118-2 and IR-DS-119-4 in Cluster 3.

Materials and Methods

Plant materials

Mature seeds from four randomly selected soybean plants of each of the thirty four bulked mutant populations (4×34 = 136 mutant lines) in M_{10} generation and 17 check genotypes (control/parental lines) (Table 1), were collected from the research fields maintained by the Division of Genetics, IARI, New Delhi, India. The seeds were dried to a final moisture content of 11.5 ± 0.2%, which represented safe moisture value for legume storage. The seed samples were ground in an electric grinder (Tecator, S.No. 2397) to obtain a uniform particle size of less than 0.5 mm for analysis.

Table 4. Simple correlation coefficients among the phytic acid, phytate P, total P, protein, iron, zinc, calcium and manganese of 34 mutant populations.

Traits	PA	PA-P	Total P	Protein	Fe	Zn	Ca	Mn
PA	1	0.997**	0.975**	0.333*	-0.055	-0.071	-0.048	-0.097
PA-P		1	0.975**	0.337*	-0.060	-0.062	-0.039	-0.095
Total P			1	0.314*	-0.071	-0.054	-0.026	-0.098
Protein				1	-0.041	-0.206	-0.173	0.034
Fe					1	0.022	0.078	-0.041
Zn						1	0.026	0.062
Ca							1	-0.021
Mn								1

** and * indicates significant at P=0.01 and P=0.05 respectively.

Table 5. Simple correlation coefficients among the phytic acid, phytate P, total P, protein, iron, zinc, calcium and manganese in selected low phytatic acid soybean lines.

Traits	PA	PA-P	Total P	Protein	Fe	Zn	Ca	Mn
PA	1	0.996**	0.527**	-0.745**	-0.877**	0.244	0.812**	-0.206
PA-P		1	0.489*	-0.736**	-0.870**	0.307*	0.827**	-0.217
Total P			1	-0.362*	-0.514*	-0.639**	0.630**	-0.019
Protein				1	0.934**	-0.350*	-0.767**	-0.038
Fe					1	-0.209	-0.911**	-0.287
Zn						1	0.146	-0.076
Ca							1	0.194
Mn								1

** and * indicates significant at P=0.01 and P=0.05 respectively.

Generation of experimental materials

Two kg seeds of each of the genotypes listed against serial number 1 to 16 and 30 to 34 were irradiated with gamma rays of 0.25 kGy and 0.20 kGy respectively. The genotypes listed

against serial numbers 17 to 29 were stable yellow seeded mutant populations developed from a black seeded parental genotype (check) after treatment with Ethyl Methane Sulphonate (EMS) (Table 1). For this chemical treatment, one kg seed of black seeded check genotype was treated with 0.1 % EMS solution for 4hrs. The treated seed were washed under running water for 12 hours. The irradiated and treated seeds were sown in the field. Delhi being hot-spot for yellow mosaic virus (YMV) disease of soybean, there was high mortality due to YMV as well as due to mutation. One pod each from the surviving plants was bulked together to constitute the next generation. The same process was followed till M_{10} generation.

Analysis of phytic acid, phytate P and total P content in mature seeds of soybean populations

Phytic acid was measured by an assay procedure specific for measuring the released phosphorus, based on the available phosphorus from phytic acid, *myo*-inositol (phosphate)n and monophosphate esters by phytase and alkaline phosphatase using the Phytic Acid/ Total Phosphorus Assay Kit from Megazyme International Ireland Limited.

Phytic acid extraction from soybean flour

The ground seed powder (0.5g) of each sample was thoroughly mixed with 20 ml of 0.66 M HCl in Falcon tubes and shaken at 220 rpm for 16 h in a shaker and centrifuged at 13,000 rpm for 10 min. The crude extract measuring 0.5 ml was mixed with 1.5 ml of 0.75 M NaOH solution for neutralization. The neutralized sample extract was used for the enzymatic dephosphorylation reaction procedure by phytase

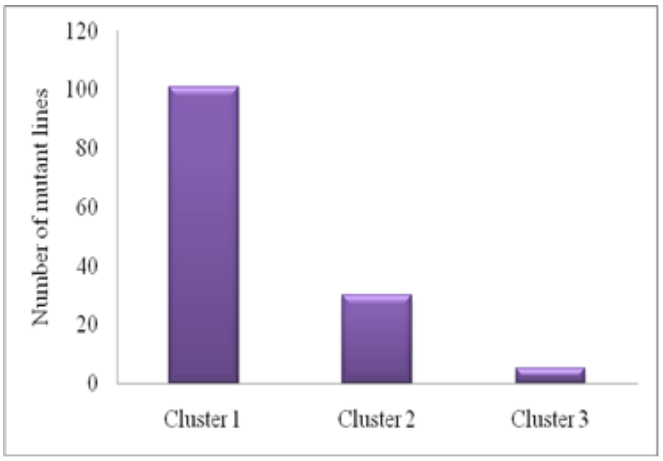

Fig 2. Based on cluster analysis of mutant population, three major cluster of mutant lines, Cluster 1- 101 mutant lines; Cluster 2- 30 mutant lines, and cluster 3- 5 mutant lines.

and alkaline phosphatase enzyme to give total phosphorus.

Enzymatic incubation

To 0.05 ml of the neutralized sample extract, 0.62 ml of distilled water; 0.2 ml of sodium acetate buffer (200mM, pH 5.5) and 0.02 ml of phytase enzyme (12,000 U/ml) were added. The mixture was vortexed and incubated in a water bath for 10 min at 40°C. The subsequent reaction was initiated by the addition of 0.02 ml of distilled water, glycine buffer (400mM, pH 10.4) and alkaline phosphatase (80 U/ml). The mixture was vortexed and incubated in a water bath for 15 min at 40°C. Another set of reaction containing all the above components except phytase and alkaline phosphatase was used a control. After 15 min, the reaction was terminated with 0.3 ml of TCA (50% w/v) and centrifuged at 13,000 rpm for 10 min.

The supernatants were carefully pipetted for colorimetric determination of phosphorus.

Preparation of phosphorus calibration curve

Serial dilutions of phosphorus solution (50 µg/ml-provided in the kit) were made to achieve 0.5 µg P; 2.5 µg P; 5.0 µg P; 7.5 µg P concentrations for a phosphorus calibration curve to be used for absorbance determination.

Colorimetric determination of phosphorus

One ml of each sample as well as phosphorus standard was mixed with 0.50 ml colorimetric reagent (3 volume of 1 M H_2SO_4, 1 volume 2.5% (w/v) ammonium molybdate and 1 volume of 10% (w/v) ascorbic acid) and incubated at 40°C for 1 hr. Absorbance of the color reaction products for both samples and standards were read at 655 nm. Phytic acid concentrations were calculated by using the calibration curve.
Phytate phosphorus was calculated as a difference between the total phosphorus and free phosphorus. Phytic acid was calculated, by assuming that the amount of phosphorus measured exclusively released from phytic acid and this comprises 28.2% of phytic acid.

Determination of Crude protein

Protein (N×6.25) was determined by the micro-Kjeldahl method *(*AOAC, 1990*)*. All nitrogen was converted to ammonia by digestion (0.5 g of sample flour) with 20 ml of concentrated sulphuric acid containing copper sulphate and potassium sulphate as a catalyst. The ammonia released after alkalinisation with sodium hydroxide was steam distilled into boric acid and titrated with hydrochloric acid.

Determination of divalent cations

One gram of ground sample was taken in a 150 ml conical flask. To this, 25-30 ml diacid mixture (HNO_3:$HClO_4$; 9:4 v/v) was added and kept overnight. The mixture was further digested by heating till clear white precipitate settled down at the bottom. The crystals were dissolved by diluting in double distilled water. The contents were filtered through Whatman # 42 filter paper. The filtrate was made up to 50 ml with double distilled water and was used for determination of total iron, zinc and calcium. The samples, which were digested in a diacid solution of HNO_3 and $HClO_4$, were passed through the Atomic Absorption Spectrometry (ECIL, AAS 4141) system and calibrated for iron, zinc, calcium and manganese.

Statistical analysis

All measurements were done in triplicate. For all assays, the data was expressed as mean ± S.D. The data was analyzed using SAS (version 9.3) software program and the correlation coefficients were calculated among all the parameters of mutant populations and the putative low phytic acid mutant. Multiple comparisons were made by LSD (Least Significant Differnce) test at 5% level of significance.

Conclusion

Low phytic acid is one of important studies in soybean to improve promising nutritional lines. Mutants from physical and chemical method can reduce phytic acid level in grain, it means that mineral and protein bioavailability can increase in seed. These lines will be useful in reducing grain phytic acid and improving the nutritional value of soybean and/or processed by-products. Five low phytic acid line such as IR-JS-101-4, IR-V-101-3, IR-DS-118-2, IR-DS-119-4 and IR-DS-122-2 are considered as good donors to transfer traits of LPA, high protein and mineral content into improved varieties in future. These characters will then need to be incorporated into high yield genotypes with disease and insect resistance. These LPA soybean mutant needs further research to analyze whether this mutations affecting the first steps of the biosynthetic pathway (from glucose 6-P to myo-inositol[3]-monophosphate) or mutations perturbing the end of the PA pathway (from myo-inositol[3]-monophosphate to PA) or mutations affecting the transport of PA to the vacuole.

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