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Research Article

Improving the Bioavailability of Seed Phosphorous in Low Phytic Acid Soybean Mutants

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Abstract:
Phytic acid, the heat stable anti-nutritional factor forms 75% of the total Phosphorous (P) in soybean seeds. It acts as strong chelating agent binding to metal ions reducing the bioavailability of Fe, Zn, Mg and Ca in human and non-ruminant livestock. In the present study, 106 soybean germplasm lines were screened to estimate the seed phytate. It ranged from 0.16 to 4.741mg per g soy flour. High yielding, low phytate cultivar were selected and subjected to 250 Gy gamma ray irradiation. In M_3 generation, mutants having phytic acid content ranged from 0.075 to 2.58 mg/g of soy flour were identified. These mutants have shown as much as 50% or more reduction in seed phytate compared to control. Although low phytic acid line had much higher inorganic 'P' concentrations than seed of the normal lines, the balance between protein and oil content was not altered. Since, corn-soy and soymeal are commonly fed to livestock; reducing phytate content would contribute to increased bioavailability of 'P' in these livestock feeds.

Keywords: Soybean, Gamma rays, Phytic acid, Inorganic P, seed oil, protein

Introduction

Soybean [*Glycine max* (L.) Merill] has been recognized as valuable source of high quality protein and oil. In India, it has emerged as an important oilseed crop. It is cultivated over an estimated area of 9.75 million ha. with a production of 10.05 million tons contributing nearly 40 per cent of the total oilseed produced in India. As an important dietary source of protein, fat, fiber, minerals and vitamins, soybean provides many bioactive components such as phytoestrogens with potential benefits for human health (Messina 1999). Meanwhile, other components present in soybean like trypsin inhibitors and phytic acid can act as anti-nutritional factors which interfere with protein digestion or chelate nutritionally essential elements including Ca, Zn and Fe (Hurrell, 2003; Mohammed *et al*., 1991). Majority of the phosphorus in the seeds of higher plant is stored as *myo*-inositol 1,2,3,4,5,6 hexakisphosphate, otherwise termed phytic acid or phytate (Reddy *et al*., 1989). Phytase is an enzyme that breaks down phytate, releasing inorganic phosphorus and *myo*inositol. Non-ruminant animals such as poultry and swine lack gastric phytase activity and are unable to efficiently utilize phytate phosphorus (Oltmans *et al*., 2004).

Given the potential importance of phytate in the seed, a dramatic reduction in seed phytate level could impact seed viability and quality. Low phytic acid (lpa) mutants were reported in soybean, maize, barley and rice. They are useful in reducing problems associated with mineral malnutrition due to high seed phytic acid. Plants having low phytic acid, produce seeds that have normal levels of total 'P.' Therefore, these mutations do not affect the ability of plant to take up 'P' and transport it to a developing seed. Instead, lpa mutants block the ability of a seed to synthesize 'P' into phytic acid. Phytate content of soybean seeds could be reduced to one-third the normal amount by growing maternal plants on low phosphate soil (Rayboy *et al*., 1984). In nutritionally induced low-phytate soybean seeds, germination and viability were not compromised. A mutant line with reduced phytate P and increased inorganic P was developed by chemical mutagenesis (Wilcox *et al*., 2000). Two soybean mutants that fall in the '*lpa1*' class have been identified (Wilcox *et al*., 2000; Hitz *et al*., 2002). Other mutants causing accumulation of inositol phosphate intermediates have been designated as '*lpa*2' found that low phytate was controlled by recessive alleles at two independent loci (pha1 and pha2) and exhibit duplicate dominant epistasis (Oltmans *et al*., 2004; Rayboy *et al*., 1984).

A few reports pertaining to the genotypic variability of phytic acid in soybean have been reported. However, information on gamma rays induced mutagenesis for the phytic acid in soybean seeds is scarce. In the present studies, apart from investigating the variability of phytic acid among Indian cultivars and germplasm resources of soybean, we also attempted to induce mutations for low phytic acid content in a popular variety, NRC-37, using gamma rays.

Material and Methods

A total of 106 soybean lines comprising released varieties and germplasm resources were considered for the phytic acid and inorganic phosphorous estimation.

They were grown at the Experimental facility, Gamma field, Trombay, during 2010 rainy season following augmented design. All the agronomic practices were followed to raise ideal healthy crop. Seeds of NRC-37, a leading soybean variety were subjected to 250 Gy gamma rays and were sown in the field along with control. The M_1 plants were harvested individually and forwarded to M_2 generation as plant to row progeny. Large number of M_2 plants was screened for morphological variation, including plant height, flower color, leaf shape and earliness. Individual plant progenies of selected M_2 plants were grown as M_3 generation along with parent. True breeding lines with uniform family characters were selected and chosen for the biochemical analysis. Morphological and yield characters such as plant height, number of pods, yield per plant and seed index were recorded.

Determination of Phytic Acid: The assay of phytic acid is based on modified colorimetric method (Vaintraub, and Lapteva, 1988). About 30 mg of ground seed sample was used for extraction of phytic acid in 0.2 N HCl buffer and kept overnight. Crude acid extracts were transferred to fresh tubes containing 20 mg NaCl. The contents were shaken at 350 rpm for 20 min. to dissolve the salt and were allowed to settle at –20°C for 20 min. The mixtures were centrifuged at 8000 rpm at 10°C for 20 min. and clear supernatant was diluted 25 times by mixing with distilled ddH_2O. 750 µl of this diluted sample were combined with 250 µl of modified Wade reagent (0.03% $FeCl3.6H_2O$ + 0.3% sulfosalicylic acid) in a eppendorf tube, thoroughly mixed on a vortex, and centrifuged at 8000 rpm at 10°C for 10 min. A series of calibration standards containing 0, 0.5, 1, 1.5, 2, 3, 4, 5, 7.5, 10 and 12 µg ml^{-1} phytic acid-P were prepared from sodium phytate (Sigma, St. Louis, MO). Absorbance of color reaction products for both samples and standards were read at 500 nm on a UV-Vis spectrophotometer (Jasco, Japan), and calculation of sample phytic acid-P content was estimated by the method described by Latta and Eskin, (1980).

Determination of Inorganic Phosphorous: Inorganic P was estimated colorimetrically following extraction of 30-50 mg of a ground sample in 12.5% (v/v) TCA and 25mM $MgCl_2$ buffer (Chen *et al*., 1956). Overnight incubated samples were centrifuged at 10,000 rpm and supernatant was diluted 1:2 with distilled water. A 100 µl of the diluted sample was mixed with Chen's reagent and incubated in water bath at 50°C for 1h. After incubation, samples were cooled and absorbance was taken at 660nm in a UV-Vis spectrophotometer (Jasco, Japan). A standard curve was plotted by taking the absorbance of known amount of disodium hydrogen phosphate. Based on the calibration curve of the standard inorganic P, the respective OD value of a sample was converted to concentration of inorganic P and expressed in mg/g of soy flour.

Estimation of oil and protein content: Oil content of soybean varieties/germplasm resources was estimated by solvent extraction method (AACC, 1976) using soxhlet apparatus. The nitrogen content of the seed was determined by micro-kjeldahl method (AOCS, 1984) and the amount of total protein was calculated from nitrogen content using a conversion factor 6.25.

Statistical analysis: Analysis of variance was calculated for the yield and biochemical traits using standard statistical procedures. Summary statistics and simple correlation coefficients were calculated between yield and seed quality parameters using PAST software (Hammer *et al*., 2001).

Results and Discussion

As many as 106 germplasm lines were screened for eight traits during rainy season, 2010. Among the genotypes evaluated, TCS-269 recorded highest number of pods per plant (41) with a seed yield of 22.3g. Phytic acid and inorganic 'P' was estimated in 106 germplasm lines following modified colorimetric method. The results showed that phytic acid ranged from 0.16 to 4.74 mg/g (Table 1); while the inorganic 'P' ranged from 0.024 to 2.10 mg/g (Fig. 1). Soybean genotype TCS 244 recorded the lowest phytic acid of 0.16 mg/g with high protein content (54.73%). Variation in the sensitivity of the cultivars to the effects of growing conditions may be attributed to genotypic variation in 'P' uptake by crop plants as a consequence of changes in root surface area and rhizosphere acidification. The accumulation of phytic acid would also depend on factors that affect uptake of 'P' such as differential status of soils, soil pH, temperature and P mineralizing microorganisms in the soils (Israel *et al*., 2007).

The oil content among the germplasm lines ranged from 13.94 to 19.22%, while protein content ranged from 44.2 to 54.73%. Although there was negative correlation between them but no significant difference was noticed in low phytic acid or inorganic 'P' lines for these traits. Changes in protein concentration were associated with 'P' nutrition and not with the phytic acid of the lines. Total oil percentage in the seed of the low and normal phytic acid lines was similar and relatively stable between deficient and excessive 'P' levels (Israel *et al*., 2007).

Improvement in either single or few economic traits and quality characters can be achieved with the help of induced mutations within the shortest possible time. With the successful use of gamma rays in crop improvement, we also adopted this approach in inducing

low phytic acid mutations. A successful ruling variety, NRC-37 was irradiated with gamma rays at 250 Gy and 62 high yielding; well adapted and stable mutants were subjected to phytic acid and inorganic 'P' estimation (Table 3). Among the mutants screened, yield ranged from 2.45 to 26 g per plant. The range for phytic acid among the NRC-37 mutants was 0.076 to 2.22 mg/g; while, inorganic 'P' ranged from 1.16 to 2.84 mg/g as against 2.22 mg/g and1.65 mg/g in the control respectively (Table 3). The mean values for phytic acid (1.05 mg/g) were much less than the control (2.22 mg/g). Low phytic acid mutant (NRC-47) recorded 0.076 mg/g against 2.226 mg/g in the control. It also registered 1.916 mg/g of inorganic 'P'. The other promising low phytic acid mutant (NRC-34) had 0.10 mg/g with 2.803 mg/g of inorganic 'P' (Fig 1). The reduced phytate and increased inorganic 'P' for the low phytate lines was expected on the basis of previous studies (Wilcox *et al*., 2000). There was no significant difference in emergence among low phytate lines (Oltmans *et al*., 2005). The seedling emergence and vigor was good and comparable with control plants. Based on several studies, phytic acid accumulates gradually during seed development and Inorganic 'P' concentrations decreases during seed development and total 'P' levels remain relatively consistent
(Rayboy *et al*., 2000).
Correlation studies stated that oil and protein were negatively correlated (-0.076); seed size and yield were also negatively correlated (-0.132). Phytic acid and inorganic 'P' were negatively correlated (-0.012), while, phytic acid and seed protein content (-0.105) and inorganic 'P' with oil content (-0.109) were also negatively correlated (Table 2). The genetic variability for the inorganic 'P' and phytic acid indicated that low phytic acid lines had high inorganic P and vice versa and this trend was also documented by earlier reports. As evident in germplasm lines, even mutants also recorded negative correlation between phytic acid and inorganic 'P' (-0.345).

Soybean lines developed using these mutants or similar genetic resources would represent an improved source of 'P' for animal feeds. Use of such seeds would reduce the need for 'P' supplementation of feeds. With the advancement of molecular technologies, development of new marker systems or extending the existing marker resources for identifying 'lpa' mutant would be of great interest towards marker-assisted breeding.

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Table 1.Evaluation of soybean genotypes for *per se* performance, oil, protein and seed phosphorous content

Genotype	Plant height (cm)	*No. of Pods/pl*	Yield(g/pl)	Seed Index (g)	Oil (%)	Protein (%)	Inorganic 'P' (mg/g)	Phytic acid (mg/g)
TCS 201	60.20	48.80	12.60	13.50	16.48	47.52	0.582	2.155
TCS 201A	55.00	34.00	8.20	13.20	17.28	45.70	0.348	1.350
TCS 217	33.80	38.40	7.50	14.50	13.94	49.50	0.441	1.451
TCS 219	34.00	21.30	8.30	14.00	18.49	50.25	1.937	1.464
TCS 220	29.00	25.00	8.00	13.30	18.71	49.61	0.452	1.606
TCS 221	28.10	22.20	3.20	8.00	17.20	45.30	0.430	1.456
TCS 222	41.40	55.20	8.10	11.38	18.03	49.83	0.178	1.684
TCS 228	30.40	57.60	14.80	16.14	19.22	45.70	0.605	1.335
TCS 232	34.00	49.60	11.10	16.10	17.56	45.39	0.024	1.590
TCS 233	35.00	38.00	12.50	16.10	16.52	48.40	0.053	1.553
TCS 234	31.80	32.40	6.30	14.13	18.63	47.52	0.101	1.457
TCS 243	38.00	45.00	18.30	13.90	18.57	49.11	0.967	1.075
TCS 244	30.00	18.00	2.80	15.60	15.83	54.73	0.476	0.160
TCS 247	42.80	23.80	5.80	11.80	14.77	48.62	0.973	1.619
TCS 248	34.20	30.40	8.60	13.80	18.43	44.20	0.777	1.226
TCS 253	43.80	43.20	11.80	13.20	18.13	46.95	0.776	1.372
TCS 254	24.00	22.00	10.10	10.00	15.38	46.15	0.855	1.699
TCS 255B	35.00	32.00	12.60	8.60	18.20	46.20	0.921	1.483
TCS 256	52.00	116.00	20.90	11.20	18.93	48.30	0.648	1.490
TCS 258	30.60	25.00	5.70	15.80	15.65	54.25	1.067	1.708
TCS 259	18.20	29.20	9.70	16.20	17.88	51.46	0.825	0.870
TCS 267	35.00	28.00	13.10	15.50	16.73	49.45	1.273	1.463
TCS 269	22.40	41.30	22.30	15.80	18.01	51.97	0.743	1.546
TCS 270	33.80	34.00	14.40	13.80	17.84	47.88	0.622	1.331
TCS 271	34.00	35.00	15.60	14.95	17.43	51.87	1.286	1.269
TCS 278C	42.60	72.80	15.80	13.30	14.93	50.24	1.944	1.397
TCS 279	41.90	43.00	15.10	9.52	17.01	47.24	1.864	1.443
TCS 291	45.60	72.80	12.80	10.00	15.71	46.38	1.796	1.490
TCS 306	35.00	23.00	6.70	15.73	15.02	51.96	2.106	1.417
Range	18.2-78.5	18-116	1.9-180.8	7.0-19.6	13.33-20.68	44.08-54.86	0.024-2.663	0.126-3.534
Mean	35.8	38.62	12.79	13.42	16.89	49.12	1.019	1.810
S.E	0.972	1.466	1.73	0.249	0.147	0.246	0.067	0.045
S.D	9.82	14.80	17.52	2.51	1.48	2.48	0.67	0.45

Table 2. Correlation among the yield and seed quality parameters with seed phosphorous content in soybean genotypes.

Traits	Yield (g/pl)	Seed Index (g)	Oil (%)	Protein (%)	Inorganic 'P' (mg/g)
Seed Index (g)	-0.132				
Oil (%)	0.0911	0.285			
Protein (%)	-0.088	0.304	-0.076		
Inorganic P (mg/g)	0.206	-0.072	-0.109	0.149	
Phytic acid (mg/g)	0.021	0.077	0.081	-0.105	-0.012

Table 3. Yield, phytic acid and inorganic phosphorous content in NRC-37 mutants of soybean in M_3 generation

NRC-37 mutants	Yield (g/pl)	Inorganic 'P' (mg/g)	Phytic acid (mg/g)
NRC 1	15.84	2.381	1.434
NRC 5	14.2	2.336	0.776
NRC 7	12.35	2.427	0.780
NRC 9	14.05	2.688	0.832
NRC 13	14.86	2.298	0.780
NRC 14	15.24	2.127	0.527
NRC 16	22.75	2.208	0.870
NRC 17	11.66	2.088	0.563
NRC 18	14.6	2.532	0.824
NRC 19	21.4	1.921	0.700
NRC 20	25.1	2.842	0.408
NRC 21	13.5	2.551	0.738
NRC 22	11.84	2.305	0.790
NRC 24	16.7	1.976	0.953
NRC 25	17.14	2.399	0.882
NRC 26	25.96	2.641	0.875
NRC 27	7.72	1.573	0.870
NRC 28	8.1	2.056	0.880
NRC 33	2.45	1.570	0.721
NRC 34	9.9	2.803	0.100
NRC 47	11.83	1.916	0.076
NRC 48	11.6	1.950	0.849
NRC 52	31.5	1.707	0.507
NRC 66	19.36	1.509	0.805
NRC 68	19.1	1.677	0.900
NRC 77	27.2	2.138	0.700
NRC 88	15.3	1.797	0.910
NRC-37 (Control)	7.8	1.65	2.226
Range	2.45-42.6	1.16-2.84	0.07-2.22
Mean	18.35	1.99	1.05
S.E	0.93	0.048	0.047
S.D	7.52	0.389	0.386

Fig. 1 Genetic variability for seed phosphorous content among soybean genotypes and NRC-37 mutant derivatives