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RESEARCH ARTICLE

Enumerating the phytic acid content in maize germplasm and formulation of reference set to enhance the breeding for low phytic acid

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Abstract Phytic acid (Myoinositol 1, 2, 3, 4, 5, 6 hexakisphosphate) is a ubiquitous compound present in plants. It is an important constituent in seed reducing the bioavailability of phosphorous and mineral nutrients when fed to monogastric animals like swine, poultry, fish etc. Hence, identification of maize germplasm with reduced phytic acid content is imperative to formulate the breeding programs to evolve low phytate lines. Towards this, three hundred and thirty-eight maize germplasm accessions available at Department of Millets, TNAU, were raised and screened for phytic acid content which varied from 2.77 to 16.70 mg/g of seed. Based on the variability present, a reference set with fifty-eight genotypes for phytic acid was formulated. The reference set was formed with random genotypes selected from the base population to follow a normal distribution (skewness; 0.17, kurtosis; 0.61 and K–S test for normality $D_n = 0.70$) for phytic acid. The non-significant difference between the means of the base and the reference ensured the entire representation of the base in the formulated reference for phytic acid. Among all the lines in the reference set, the lowest phytic acid content were observed in the lines UMI-113 (2.77 mg/g) followed by UMI-300-1 (3.17 mg/g), UMI-467 (5.50 mg/g) and UMI-158 (6.58 mg/g) could be used as donors for low phytic acid in breeding programs. The principal component analysis for studying the extent of variability in the reference, revealed six major principal components that exhibited 80.40% of variation with flowering traits, ear height and phytic acid as a major contributor for variability. The characters namely plant stand, germination percentage, kernel yield, ear length, ear diameter and number of kernels per row were found to be positively correlated with the phytic acid and this emphasizes the negative pleiotropic effects of low phytic acid lines in germination and seed set. Thus this formulated reference set enables the breeders to handle minimum population for further grouping the genotypes to analyse their heterotic potential combined with low phytic acid.

Keywords Phytic acid · Reference set · Maize · Skewness · Kurtosis and Kolmogorov–Smirnov (K–S) test

Introduction

Maize (*Zea mays* L.) is a versatile crop grown over a range of agro climatic zones. Most of the maize traded is used for food and industrial purposes. The poultry industry is heavily dependent on maize as it forms 50–60% of the input required for broiler feed and 25–35% of the input required for layer feed. Maize is the preferred source of energy in feed when compared with other substitutes due to its availability, higher energy and price economics (Rouf shah et al. 2016).

Among the major components in maize viz., carotenoids, tocopherols, minerals, phytic acid, anthocyanin and other phenolic compounds, phytic acid forms a compound of profound interest due to number of issues concerning the nutritional quality. It is virtually a ubiquitous component of plant seeds, supplying both phosphate and cations during germination. However, phytic acid is

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considered as an anti-nutrient (Raboy 2003) when it is used as a feed to human and other mono-gastric animals such as swine, poultry and fish. The monogastric animals lack phytase required for separating phosphorous from phytic acid and additionally, the phosphorous released from undigested phytic acid causes environmental pollution such as eutrophication.

Phytic acid (Myoinositol, 1, 2, 3, 4, 5, 6 hexakisphosphate) comprises most of the storage form of phosphorous in cereal grains and its negatively charged nature, causes chelation of minerals such as iron and zinc (Fredlund et al. 2006). This makes them unavailable for absorption during digestion causing micronutrient mal-nutrition (Brinch-Pederson et al. 2002). Two strategies can be adopted to reduce this antinutritional factor. One is to exploit the induced variation by mutation and second is to utilize the naturally occurring variability in the germplasm (Sparvoli and Cominelli 2015). Since the induced mutations were found to produce abnormal seed development in maize, screening of germplasm and identification of low phytate lines is crucial to maintain the phosphorous homeostasis.

In order to restrain the difficulties in handling a large number of germplasm, a reference set can be formulated for phytic acid. A reference set is a pool of germplasm accessions with highest possible variability for a specific trait. As reported by Upadhyay et al. (2008), the utilization of germplasm resources for any particular trait is less than one percent. This could eventually narrow the genetic base of the lines screened and developed. Hence to overcome this, development of a reference set incorporating all the variability for the trait of interest without testing alleles is imperative.

The reference set will enable the breeders to handle, manage and explore a minimum population representing the maximum diversity of source germplasm. In view of this, a reference set for phytic acid was developed and studied to identify the potential prebreeding materials.

Materials and methods

Screening maize germplasm for phytic acid content

The germplasm accessions available at Department of Millets, Tamil Nadu Agricultural University, Coimbatore were screened for phytic acid content using the method suggested by Davies and Reid (1979). Well powdered seeds in three biological replicates were subjected for analysis. Sodium phytate was used as a standard and run along with each batch of samples in series of concentrations to calculate the phytic acid content of the samples from the standard graph.

Methodology standardized for phytic acid estimation

Handful of randomly selected seeds were ground to a fine powder. 0.5 g of the finely grounded powder was added to 10 ml of 0.5 M HNO_3 in a 50 ml centrifuge tube with a magnetic bead and was kept in a magnetic stirrer for 3 h. After 3 h of shaking, the extract was filtered through a Whatman filter Paper No. 1 and 0.2 ml of the filtered extract was taken in microcentrifuge tubes. To this extract, 0.2 ml of freshly prepared ferrous ammonium sulphate was added and kept in a boiling water bath for 20 min. The tubes were cooled to room temperature and 1 ml of iso-amyl alcohol followed by 20 µl of ammonium thiocyanate were added. The tubes were then shaken well and centrifuged at 3000 rpm for 10 min at 4 °C. Finally, 0.2 ml of the supernatant were taken in microtitre plates and the color developed was read at 460 nm. The standard stock solution was prepared by dissolving 50 mg of sodium phytate (from rice) in 20 ml of distilled water and the volume was made up to 100 ml with distilled water. The working solution (0.5 mg/ml phytate concentration) is now prepared by taking 1 part of the stock with 9 parts of distilled water. A series of standards were prepared from this working solution (Table 1) and run along with the samples for estimation of the phytic acid content.

Table 1 Standard series for phytic acid estimation using Davies and Reid method

Concentration (mg/ml)	Working standard (ml)	0.5 M HNO_3 (ml)	Total volume (ml)
0.5	0.2	0	0.2
0.25	0.1	0.1	0.2
0.125	0.05	0.15	0.2
0.1	0.04	0.16	0.2
0.05	0.02	0.18	0.2
0.025	0.01	0.19	0.2
0	0	0.2	0.2

Table 2 Genotypes categorized based on phytic acid content in base population

S. nos.	Phytic acid range (mg/g)	Number of genotypes
1.	Below 3	1
3.	5–6	3
4	7–7.9	9
5.	8–8.9	26
6	9–9.9	290
7.	10–11	4
8.	12–16	4
9.	Above 16	1
	Total	338

Table 3 Descriptive statistics for phytic acid content in the base population and in the reference set formulated

Statistics	Statistical measures in 338 inbred germplasms	Statistical measures in the reference set of 58 inbred germplasms
Mean	9.543	10.01
SE	0.048	0.38
Median	9.673	9.83
Mode	9.489	15.07
SD	0.888	2.92
Sample variance	0.789	8.57
Kurtosis	27.377	0.61
Skewness	0.031	0.17
Range	13.926	13.93
Minimum	2.774	2.77
Maximum	16.700	16.70

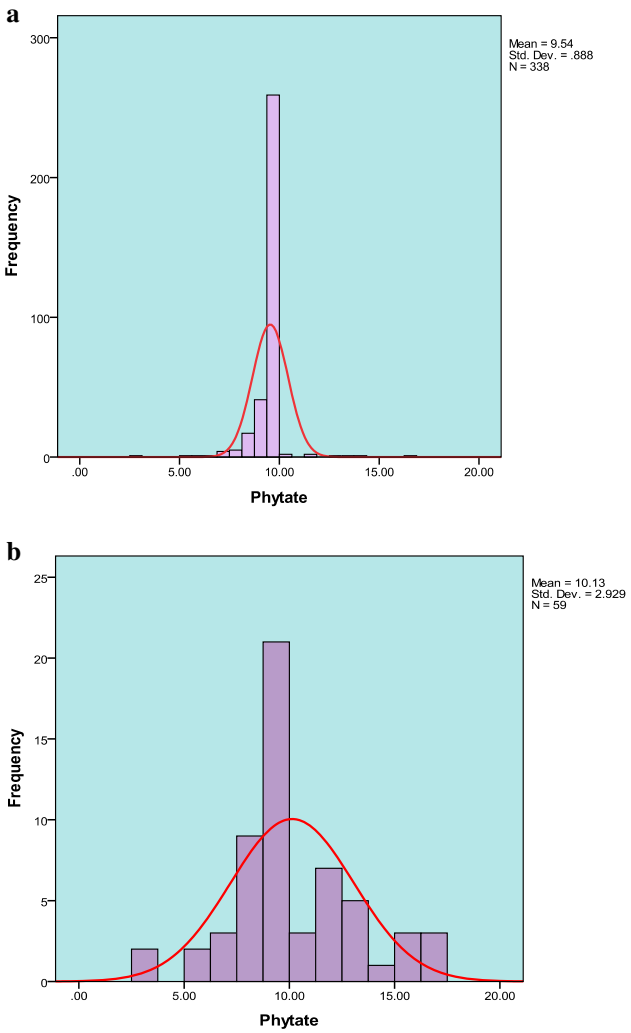

Fig. 1 **a** Distribution of phytate in base population, **b** distribution of phytate in reference population

Table 4 Genotypes categorized based on phytic acid content in reference set

S. nos.	Phytic acid range (mg/g)	Number of genotypes	Germplasm accessions
1	Up to 3	1	UMI 113
2	3–6	3	UMI 300-1, UMI 467, In 6
3	6–9	17	In 12, UMI 158, UMI 1100, In 3, UMI 447, UMI 1031, UMI 170-4, UMI 1124, UMI 1017, UMI 510-1-2, UMI 1004, UMI 1030, UMI 1013-1, UMI 351, UMI 504, UMI 1054, UMI 1105
4	9–12	25	UMI 919-1, UMI 960-1, Box No. 1137-6, UMI 346-2 RS, UMI 338-1, UMI 163-3, UMI 550, UMI 262, UMI 1156, UMI 1101, UMI 614A, UMI 823, UMI 135, UMI 1027, UMI 679, UMI 1036, UMI 1112, UMI 363, UMI 1005-1, UMI 507, UMI 612, UMI 51 WS, UMI 260, UMI 375, UMI 1009-2
5	12–15	6	UMI 687-1, UMI 955-2, UMI 607, UMI 1113, UMI 1126-1, UMI 1110-1, UMI 304
6	15–18	6	UMI 161, UMI 857-1, UMI 473-1, UMI 779, UMI 265, UMI 265

Table 5 Kolmogorov–Smirnov test

D_n	PA	GP	PS	DT	DS	ASI	PH	EH	LP	LE	CW	EL	ED	KR	RC	TW	KY	The Kolmogorov table value ($D_{n,\alpha}$) (n = population size)
Traits																		
The highest difference between the S(n) and F(x) the cumulative normal distribution function	**0.70**	3.02	2.48	1.65	2.37	**0.78**	1.89	**0.65**	**0.73**	**0.83**	2.66	**0.69**	**0.90**	**0.47**	**0.70**	**0.45**	2.48	**1.36**

The bolded ones are the traits that have a lesser value than the K–S table value $D_{n,\alpha}$. These traits are normally distributed

[a]Sn = the sample value divided the population size

Table 6 Paired t test between the base and the reference set

	Base pop	Reference set
Mean	10.01	9.36
Variance	7.96	4.36
Observations	58	58
Hypothesized mean difference	0	
df	57	
t Stat	2.91	
P(T ≤ t) one-tail	0.0026	
t critical one-tail	1.6720	ns

GP germination percentage (%), *PS* final plant stand (%), *DT* days to 50% tasseling (days), *DS* days to 50% silking (days), *ASI* anthesis-silking interval (days), *PH* plant height (cm), *EH* ear height (cm), *LP* number of leaves per plant (count), *LE* number of leaves above the upper most ear (count), *CW* cob weight (g), *EL* ear length (cm), *ED* ear diameter (cm), *KR* number of kernels per row (cm), *RC* number of rows per cob (count), *TW* test weight (g), *KY* single plant kernel yield (g), *PA* phytic acid content (mg/g), *ns* non significant, *0* zero mean difference

Raising the reference set germplasm for field evaluation and estimation of phytic acid content

The 338 inbreds were raised in the field during, Kharif 2015 and the phytate content of all the inbreds were estimated. The 338 inbreds were grouped based on the range of their phytic acid content and random genotypes from each group were selected to form a reference set of 58 genotypes (Table 2). All the germplasm in the reference set were raised during summer 2016, by following the Augmented Block Design-I with two agronomically superior inbreds as checks viz., UMI 70-1 and UMI-285. Observations were taken on seventeen traits viz., germination percentage (%), final plant stand (%), plant height (cm), days to 50% tasseling, days to 50% silking, anthesis-silking interval (days), ear height (cm), number of leaves per plant (count), number of leaves above the upper most ear (count), cob weight (g), ear length (cm), ear diameter (cm), number of kernels per row (count), number of rows per cob (count), test weight (g), single plant kernel yield (g) and phytic acid content (mg/g) in the grains.

Statistical analysis

The distribution of the genotypes in the base and the reference set in the curve were analysed by using SPSS and their significance was tested by using paired t test for the conformation of null difference in means between reference set and the base (Upadhyay et al. 2008). The skewness, kurtosis of the curve and the Kolmogorov–Smirnov test for normality were calculated to ensure the normal distribution of the genotypes selected. Also in order to

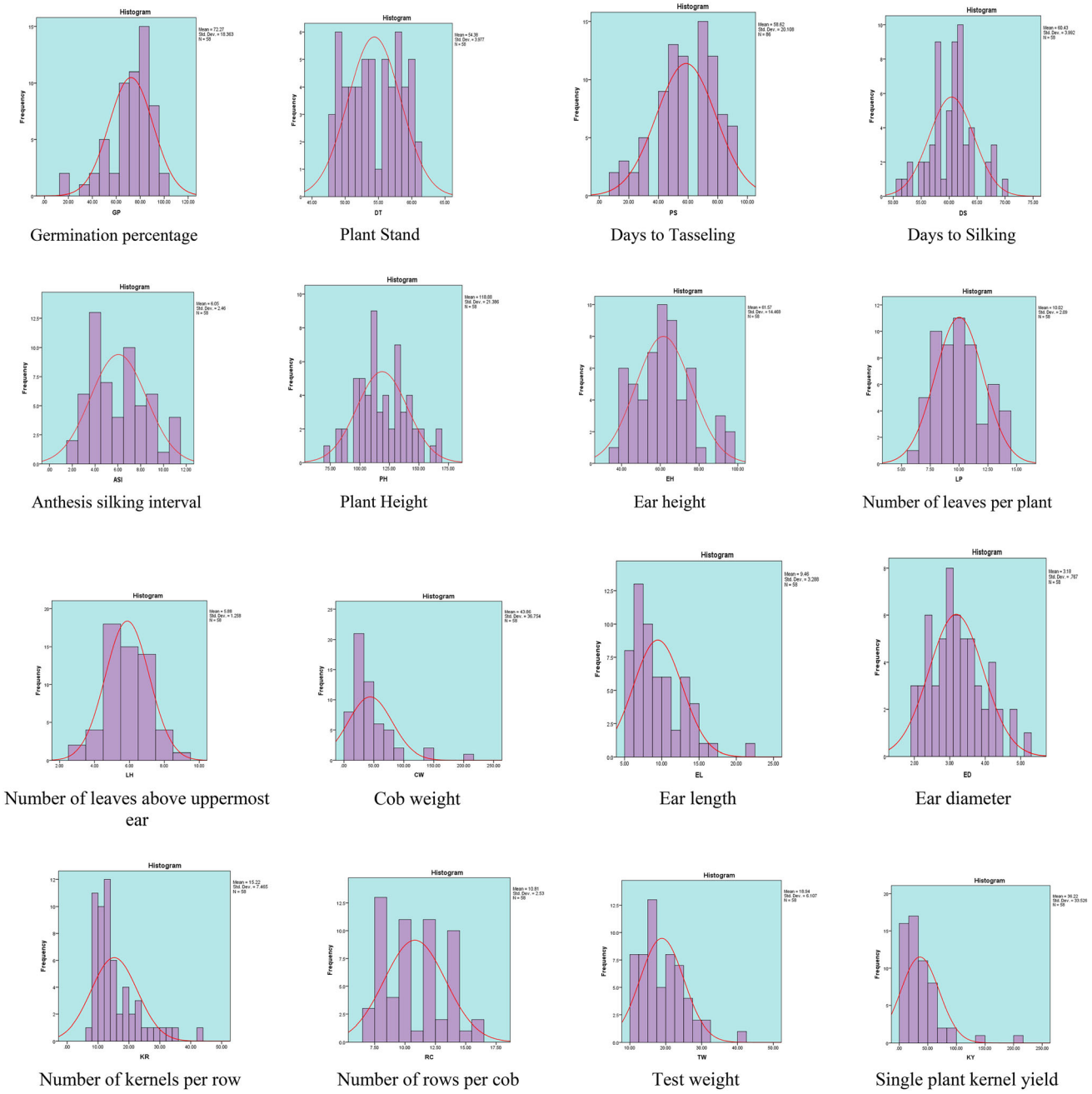

Fig. 2 The histograms of the morphological traits characterized in the reference set

understand the variability and correlation among the traits recorded, Principal component analysis was done by STAR (Statistical tool for Agricultural Research) from IRRI and the analysis for the augmented block design was done from the Statistical Package of Augmented Designs (SPAD) from IASRI.

Results and discussion

Formulation of reference set

The reference set is formulated in such a way that the trait distribution among the selected genotypes is perfectly normal (Upadhyay et al. 2008). The phytic acid content in 338 germplasm accessions ranged from 2.77 to 16.70 mg/g of seed. Similar results were reported by Suresh Kumar

Table 7 Skewness and kurtosis of morphological traits in the reference set

S. nos.	Traits	Skewness	Kurtosis
1.	GP	− 1.18	1.47
2.	PS	− 0.60	− 0.03
3.	DT	− 0.0084	− 1.292
4.	DS	0.00284	0.30
5.	ASI	0.39	− 0.78
6.	PH	0.18	− 0.32
7.	EH	0.45	− 0.053
8.	LP	0.29	− 0.73**
9.	LE	0.016	− 0.079
10.	CW	2.55	8.095
11.	EL	1.196**	1.56**
12.	ED	0.50**	− 0.22
13.	KR	1.67	2.93**
14.	RC	0.26	− 1.09**
15.	TW	1.03	1.35
16.	KY	2.78	10.54

*5 percent significant; **1 percent significant

et al. (2014), Chiangmai et al. (2011) and Shi et al. (2003). The base population were grouped into nine different classes based on their phytic acid content (Table 2). Among the 338 germplasm, UMI-113 showed the lowest phytic acid of 2.77 mg/g and six genotypes recorded a phytic acid below 5 mg/g. The line, UMI-265 showed the highest phytic acid content of 16.70 mg/g. The skewness of the base population for the phytic acid was observed to be 0.03 and the kurtosis was 27.37 (Tables 3, 4). The non-significant value of the skewness indicated the normal distribution for phytic acid in the curve. On contrary, the kurtosis was significant and was found to be greater than 1, depicting a leptokurtic curve of the phytic acid in the base population (Fig. 1a).

It shows that the randomly selected base population had a lot of outliers for the phytic acid content (Ashwini et al. 2011). This implies that the base population had most of the lines with phytic acid in a medium range of 9.0–9.9 mg/g. This can also be well understood from the value of mode (9.48) in the base population (Tables 2, 3). To form a reference set, the base population were grouped into nine different classes based on their phytic acid and genotypes were selected randomly from each of the classes based on their numbers, to form a normal distribution for phytic acid content. The randomly selected genotypes in the reference set consisted of 58 genotypes. The phytic acid content in the formulated reference set followed normal distribution with skewness and kurtosis of 0.17 and 0.61 respectively, with a standard bell shaped curve (Fig. 1 b). Non significance for both skewness and kurtosis in the curve showed that the population has equally distributed

Table 8 ANOVA: analysis of variance

Source of variation	*df*	GP	PS	DT	DS	ASI	PH	EH	LP
Blocks unadjusted	2	916.80	264.40	30.50	30.56	0.41	757	311.60	0.009
Treatments adjusted	59	304.30**	1305.40**	16.83	20.76	6.82	775	253.40*	4.668*
Control	1	33.70**	19.30 **	10.67	4.17	1.50	37.64*	60.20*	10.66*
Blocks adjusted	2	69.88**	17.60 **	1.17	1.17	0.17	59.00	8.70	0.167
Treatments unadjusted	59	335.2	313.70	17.81	21.76	6.82	798.00	263.60	4.66
Augmented	57	337.20	309.6 **	15.82	15.93	6.05	457.00	78.50*	26.20*
Control versus augmented	1	524.50**	844.50**	138.45*	371.38*	56.32*	427.93*	1336.08*	92.51*
Error	2	0.10	0.10	4.17	7.17	0.50	45.00	2.70	0.167

Source of variation	LE	CW	EL	ED	KR	RC	TW	KY	PA
Blocks unadjusted	1.52	2087	11.22	0.75	17.10	6.87	141.69	1455	10.77
Treatments adjusted	1.86	20.26*	11.38	7.73	64.70*	8.70	45.13*	1831*	7.37*
Control	2.66	9447.00	25.88*	59.62*	262.90*	25.00*	103.16*	0.05	0.37
Blocks adjusted	3.00	85.27*	0.28	0.03	0.7	0.67	0.54	0.35	0.46
Treatments unadjusted	1.89	2314.00	11.75	0.76	65.20	8.91	37.90	1879	7.72
Augmented	9.49	12.19	13.45	0.58	39.89*	9.60	103.16**	1124*	7.96*
Control versus augmented	115.23*	451.82*	63.43*	6.11*	293.90*	215.79*	46.74*	46,811**	7.26
Error	0.167	111.00	0.80	0.09	1.40	0.67	0.73	57.00	0.23

*5 percent significant; **1 percent significant

Table 9 Standard errors of differences and critical differences

	GP	PS	DT	DS	ASI	PH	EH	LP	LE	CW	EL	ED	KR	RC	TW	KY	PA
Standard errors																	
SE between two control treatments	0.24	0.30	1.7	2.2	0.05	5.5	1.3	0.33	0.33	8.6	0.73	0.25	0.97	0.67	0.7	6.2	0.39
SE of two augmented treatments of same block	0.42	0.51	2.9	3.8	1.00	9.5	2.3	0.58	0.58	14.90	1.27	0.44	1.67	1.15	1.2	10.7	0.67
SE of two augmented treatments of different blocks	0.52	0.63	3.5	4.6	1.22	11.6	2.8	0.71	0.71	18.20	1.55	0.54	2.05	1.41	1.5	13.1	0.82
SE of augmented treatment and control	0.38	0.47	2.6	3.5	0.91	8.7	2.1	0.53	0.53	13.60	1.16	0.40	1.53	1.05	1.1	9.8	0.61
Critical difference																	
Between two control treatments	2.4	2.9	17	22	5.7	54	13	3.3	3.3	85	7.3	2.5	9.6	6.6	6.9	61	3.9
Two augmented treatments of same block	4.2	5.1	29	38	9.9	94	23	5.7	5.7	148	12.6	4.4	16.6	11.50	12.0	106	6.7
Two augmented treatments of different blocks	5.1	6.2	35	46	12.2	116	28	7.0	7.0	181	15.4	5.3	20.3	14.0	14.7	130	8.2
Augmented treatment and control	3.8	4.7	26	35	9.1	86	21	5.2	5.2	135	11.5	4.0	15.1	10.50	11.0	97	6.1

GP germination percentage (%), *PS* final plant stand (%), *DT* days to 50% tasseling (days), *DS* days to 50% silking (days), *ASI* anthesis-silking interval (days), *PH* plant height (cm), *EH* ear height (cm), *LP* number of leaves per plant (count), *LE* number of leaves above the upper most ear (count), *CW* cob weight (g), *EL* ear length (cm), *ED* ear diameter (cm), *KR* number of kernels per row (cm), *RC* number of rows per cob (count), *TW* test weight (g), *KY* single plant kernel yield (g), *PA* phytic acid content (mg/g)

Table 10 The principal component eigen values

Statistics	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15	PC16	PC17
SD	2.3819	1.5709	1.3186	1.2704	1.0775	1.0066	0.9238	0.8381	0.7866	0.5875	0.5298	0.4520	0.3728	0.3407	0.2082	0.1706	0
Proportion of variance	0.3337	0.1452	0.1023	0.0949	0.0683	0.0596	0.0502	0.0413	0.0364	0.0203	0.0165	0.0120	0.0082	0.0068	0.0026	0.0017	0
Cumulative proportion	0.3337	0.4789	0.5812	0.6761	0.7444	0.8040	0.8542	0.8955	0.9319	0.9522	0.9687	0.9807	0.9889	0.9957	0.9983	1.0000	1
Eigen values	**5.6733**	**2.4677**	**1.7386**	**1.6139**	**1.1611**	**1.0132**	0.8534	0.7024	0.6187	0.3452	0.2807	0.2043	0.1390	0.1161	0.0434	0.0291	0

Reliable eigen values greater than 1 are bolded

Table 11 The eigen vectors for six PC's

Variables	PC1	PC2	PC3	PC4	PC5	PC6
PS	0.0691	− 0.1389	0.5249	− 0.0349	0.0747	− 0.3809
DT	0.1930	0.3301	0.0490	0.3855	0.2424	− 0.3977
DS	0.1611	0.3358	0.3608	0.3626	0.2935	− 0.0142
ASI	− 0.0398	0.0334	0.5300	− 0.0108	0.1038	0.6187
PH	− 0.2509	0.3455	− 0.0699	− 0.3706	0.1439	− 0.0791
EH	− 0.1566	0.3560	− 0.1164	− 0.3801	0.3552	0.0619
LP	− 0.0901	0.5249	0.1467	− 0.1075	− 0.2346	0.0822
LE	− 0.1166	0.3916	0.0970	− 0.0227	− 0.5675	− 0.1331
CW	− 0.3909	− 0.0747	0.0360	0.0319	0.1936	− 0.1370
EL	− 0.3650	− 0.0761	0.0886	0.0865	− 0.0162	− 0.1268
ED	− 0.3447	− 0.0009	0.0621	0.1424	− 0.0362	− 0.0483
KR	− 0.3564	− 0.0817	0.0210	0.0989	0.0263	− 0.2180
RC	− 0.2309	− 0.0272	0.0833	0.3223	− 0.4374	0.0666
TW	− 0.2608	− 0.1011	0.1703	0.0924	0.1397	0.3012
KY	− 0.3891	− 0.0849	0.0182	0.0253	0.1971	− 0.1211
PA	0.1177	− 0.1197	0.2799	− 0.3159	− 0.0313	− 0.2158
GP	0.0524	− 0.1798	0.3655	− 0.4163	− 0.1563	− 0.1953

phytic acid ranging from 2.77 to 16.70 mg/g (Table 4). The Kolmogorov–Smirnov test stated by Anderay Kolmogorov and Nikolai Smirnov for analyzing the normal distribution of the data also revealed the goodness of fit (Table 5) for the formulated reference set in a continuous normal distribution for phytic acid ($D_{n,\ \alpha} > D_n$, Blythe and Merhaut 2007). In order to check whether the reference set represents the whole diversity of the base population, a *paired t test* was carried out between the mean values of the base and reference set. Since there was no significant difference between the two means (Table 6), it is concluded that, a reference set for phytic acid has been formulated (Upadhyay et al. 2008). This set can be further used in low phytate breeding programs.

Distribution of morphological traits in the reference set

The reference set was characterized for sixteen morphological traits other than phytic acid. The traits viz, anthesis silking interval, ear height, number of leaves per plant, number of leaves above the uppermost ear, ear length, ear diameter, number of kernels per row, number of rows per cob and test weight were found to establish a normal distribution by the Kolmogorov–Smirnov test ($D_{n,\ \alpha} > D_n$, Table 5). The histograms of morphological traits are depicted in the Fig. 2. In order to cognize the symmetry of the curves, skewness and kurtosis were also studied. The skewness for all these traits were non-significant with an exception for ear length and ear diameter. This indicated that most of the traits had no skewness and portrayed a symmetrical distribution. The trait, ear diameter had a significant skewness of 0.5 (Table 7) which was fairly symmetrical whereas ear length had a higher significant skewness of 1.196. This indicates that the reference set for phytic acid was positively skewed for ear length. Deploying the relative size of the tails, kurtosis was found to be significant for number leaves per plant, ear length, number of kernels per row and number of rows per cob (Table 7). Based on these values, the traits cob weight and single plant kernel yield were found to be leptokurtic and other traits revealed a platykurtic curve except for the trait, number of kernels per row which was mesokurtic (Kim 2013). Hence it could be understood that this formulated reference set for phytic acid also had a higher variability for other observed traits.

Variability in the formulated reference set

The significance of the entries versus control in the ANOVA ensured a wide range of variability for phytic acid and other traits. The non-significance of the blocks for all the traits revealed the homogeneity of the experimental field (Table 8). The standard errors and critical differences were also calculated for comparison of the adjusted means in the blocks (Table 9). In order to understand the variability of the characterized reference set, a principal component analysis was performed. The total exhibited variations were categorized to seventeen principal components and out of which the first six principal components

Table 12 Correlation matrix of the traits observed in the reference set

	PS	DT	DS	ASI	PH	EH	LP	LE	CW
PS	1.0000	0.0770	0.1978	0.2096	− 0.1840	− 0.2640*	− 0.1037	− 0.0359	− 0.0528
DT		1.0000	0.8177**	− 0.2355*	− 0.1513	− 0.0410	0.1664	0.0483	− 0.3619**
DS			1.0000	0.3669**	− 0.1577	− 0.0067	0.2675*	0.0600	− 0.3052*
ASI				1.0000	− 0.0217	0.0549	0.1827	0.0233	0.0696
PH					1.0000	0.7901**	0.5554**	0.3910**	0.5071**
EH						1.0000	0.4092**	0.1875	0.2827*
LP							1.0000	0.6797**	0.0594
LE								1.0000	0.0950
CW									1.0000
EL									
ED									
KR									
RC									
TW									
KY									
PA									
GP									

	EL	ED	KR	RC	TW	KY	PA	GP
PS	− 0.0013	− 0.1094	− 0.0503	− 0.1647	0.0164	− 0.0678	**0.2336***	**0.3644****
DT	− 0.3812**	− 0.2324*	− 0.3359**	− 0.1594	− 0.3124*	− 0.3723**	− 0.0096	− 0.2042
DS	− 0.2825*	− 0.2036	− 0.2867*	− 0.1023	− 0.1668	− 0.3265*	0.0271	− 0.1059
ASI	0.1390	0.0314	0.0588	0.0848	0.2233*	0.0505	0.0613	0.1513
PH	0.3668**	0.4329**	0.3277*	0.0712	0.2298*	**0.5034****	− 0.1258	− 0.0281
EH	0.1867	0.1679	0.2077	− 0.0809	0.0922	**0.2969****	− 0.0881	− 0.0718
LP	0.0594	0.1806	0.0429	0.1076	0.0711	0.0377	− 0.0943	− 0.0921
LE	0.2482*	0.2016	0.1798	0.2862*	− 0.0109	0.0758	− 0.1158	− 0.0797
CW	0.8218**	0.7794**	0.8478**	0.3927**	0.5718**	**0.9616****	− 0.2003	− 0.0955
EL	1.0000	0.6051**	0.8920**	0.4793**	0.4633**	**0.8107****	**0.2189***	− 0.0869
ED		1.0000	0.5995**	0.5828**	0.6105**	**0.7396****	**0.2196***	− 0.0643
KR			1.0000	0.4587**	0.3205*	**0.8311****	**0.2315***	− 0.0985
RC				1.0000	0.3536**	**0.4167****	0.1479	− 0.0794
TW					1.0000	**0.5966****	0.0972	− 0.0759
KY						1.0000	**0.2322***	− 0.1057
PA							1.0000	**0.3442****
GP								1.0000

Significant correlated traits have been indicated and bolded ones are the traits that are discussed in the paper

GP germination percentage (%), *PS* final plant stand (%), *DT* days to 50% tasseling (days), *DS* days to 50% silking (days), *ASI* anthesis-silking interval (days), *PH* plant height (cm), *EH* ear height (cm), *LP* number of leaves per plant (count), *LE* number of leaves above the upper most ear (count), *CW* cob weight (g), *EL* ear length (cm), *ED* ear diameter (cm), *KR* number of kernels per row (cm), *RC* number of rows per cob (count), *TW* test weight (g), *KY* single plant kernel yield (g), *PA* phytic acid content (mg/g)

*– 5 percent significant and ** – 1 percent significant

were reliable with Eigen values above 1 accounting for 80.40% of the total variation (Table 10). The first principal component contributed 33.37% of the variability and this component was observed to be strongly correlated to three characters viz., Days to 50% tasseling, Days to 50% silking and phytic acid (Table 11). This suggests that these three components contribute to the maximum variation and they vary together (Gireesh et al. 2015). The second principal component contributed 14.52% of the total variation. It comprised of the key traits such as number of leaves per plant, ear height, plant height and ear length. The third and the fourth principal component contributed 10.23% and

Table 13 Mean performance of the reference set (Summer 2016)

Genotypes	GP 1	PS 2	DT 3	DS 4	ASI 5	PH 6	EH 7	LP 8	LE 9	CW 10	EL 11	ED 12	KR 13	RC 14	TW 15	KY 16	PA 17
Box no. 1137-6	41.67	8.33	58.00	63.00	5.00	140.00	95.00	13.00	7.00	19.10	6.40	2.50	15.00	8.00	12.10	14.52	9.33
In 3	33.33	25.00	56.00	61.00	5.00	134.00	76.00	14.00	6.00	14.30	6.10	2.30	8.00	8.00	15.00	9.60	7.51
In 6	16.67	16.67	56.00	58.00	2.00	160.00	63.00	13.00	8.00	61.70	8.60	4.10	13.00	14.00	28.10	51.14	5.59
In 12	83.33	66.67	50.00	57.00	7.00	140.00	65.00	11.00	7.00	146.50	14.50	4.80	28.00	14.00	23.80	93.29	6.26
UMI 113	66.67	33.33	58.00	61.00	3.00	138.00	89.00	10.00	6.00	41.30	9.00	4.00	13.00	13.00	21.00	35.49	2.77
UMI 135	50.00	50.00	57.00	61.00	4.00	99.00	45.00	8.00	5.00	33.40	8.40	4.30	11.00	14.00	19.40	29.87	9.91
UMI 158	83.33	75.00	53.00	60.00	7.00	110.00	55.00	9.00	6.00	95.10	16.40	3.40	35.00	12.00	20.90	87.78	6.78
UMI 161	75.00	50.00	49.00	58.00	9.00	110.00	60.00	9.00	5.00	85.50	12.10	4.20	15.00	13.00	40.40	78.78	15.07
UMI 163-3	75.00	66.67	51.00	60.00	9.00	135.00	65.00	10.00	6.00	146.60	15.30	4.80	30.00	14.00	32.10	134.82	9.53
UMI 170-4	83.33	50.00	52.00	58.00	6.00	130.00	40.00	10.00	6.00	39.30	10.70	3.30	11.00	14.00	16.10	24.79	8.04
UMI 260	83.33	41.66	51.00	61.00	10.00	140.00	95.00	8.00	3.00	26.60	7.30	2.10	9.00	10.00	21.60	19.44	11.57
UMI 262	83.33	41.67	49.00	53.00	4.00	110.00	40.00	9.00	7.00	34.20	9.40	4.20	14.00	14.00	13.40	26.26	9.82
UMI 265	100.00	66.67	53.00	60.00	7.00	167.00	92.00	12.00	7.00	12.40	8.00	2.00	8.00	7.00	17.40	9.74	16.70
UMI 300-1	50.00	33.33	56.00	64.00	8.00	119.00	76.00	10.00	7.00	59.50	14.00	3.20	23.00	12.00	22.90	63.20	3.17
UMI 304	50.00	41.67	50.00	58.00	8.00	116.00	65.00	9.00	6.00	81.10	14.00	3.60	23.00	12.00	23.80	65.68	13.89
UMI 338-1	66.67	58.33	50.00	61.00	11.00	98.00	53.00	8.00	5.00	21.10	6.30	2.30	9.00	7.00	18.30	11.52	9.51
UMI 346-2 RS	83.33	75.00	48.00	51.00	3.00	105.00	45.00	8.00	5.00	23.30	9.50	2.00	9.00	8.00	25.10	18.07	9.37
UMI 351	66.67	50.00	59.00	63.00	4.00	123.00	69.00	9.00	5.00	64.40	11.00	4.00	21.00	14.00	17.60	51.74	8.89
UMI 363	91.67	66.67	48.00	55.00	7.00	98.00	45.00	8.00	5.00	26.30	7.00	3.30	10.00	10.00	21.00	21.00	10.48
UMI 375	83.33	58.33	49.00	60.00	11.00	132.00	70.00	11.00	6.00	23.10	7.10	3.40	12.00	11.00	14.20	18.74	11.81
UMI 447	75.00	58.33	49.00	58.00	9.00	152.00	75.00	13.00	8.00	36.90	13.50	3.10	19.00	12.00	22.60	51.52	7.78
UMI 467	66.67	58.33	52.00	61.00	9.00	125.00	69.00	11.00	7.00	69.60	12.90	3.00	27.00	12.00	14.80	47.95	5.50
UMI 473-1	91.67	58.33	57.00	62.00	5.00	130.00	64.00	12.00	7.00	19.50	8.00	2.80	11.00	9.00	16.50	16.33	15.37
UMI 504	66.67	33.33	48.00	57.00	9.00	153.00	83.00	14.00	9.00	78.40	11.00	4.40	19.00	14.00	22.25	59.18	8.90
UMI 507	75.00	75.00	60.00	67.00	7.00	142.00	74.00	13.00	7.00	36.80	12.50	3.20	13.00	9.00	13.80	16.14	11.26
UMI 510-1-2	83.33	58.33	52.00	58.00	6.00	88.00	45.00	7.00	5.00	39.50	12.70	2.30	20.00	12.00	14.30	34.32	8.48
UMI 51 WS	83.33	16.67	50.00	56.00	6.00	83.00	42.00	7.00	5.00	42.70	11.00	3.10	24.00	14.00	12.45	41.83	11.55
UMI 550	75.00	50.00	58.00	62.00	4.00	109.00	55.00	10.00	7.00	14.30	7.00	2.60	11.00	8.00	11.30	9.94	9.80
UMI 607	75.00	66.67	60.00	63.00	3.00	113.00	55.00	10.00	6.00	29.50	7.60	3.20	19.00	12.00	15.23	36.47	12.60
UMI 612	100.00	33.33	58.00	62.00	4.00	146.00	75.00	12.00	7.00	51.60	8.20	3.80	13.00	14.00	26.00	47.30	11.41
UMI 614A	66.67	33.33	59.00	62.00	3.00	85.00	42.00	8.00	5.00	19.20	6.00	2.30	7.00	8.00	11.36	6.36	9.88
UMI 679	75.00	66.67	60.00	62.00	2.00	113.00	55.00	8.00	5.00	16.10	7.30	2.00	12.00	8.00	11.10	10.65	9.95
UMI 687-1	91.67	50.00	53.00	61.00	8.00	111.00	63.00	9.00	6.00	20.50	5.60	2.20	8.00	8.00	11.90	7.61	12.48
UMI 779	83.33	83.33	60.00	64.00	4.00	114.00	60.00	9.00	6.00	21.60	5.70	2.70	9.00	12.00	12.30	13.28	16.41
UMI 823	91.67	75.00	49.00	52.00	3.00	110.00	61.00	8.00	5.00	19.30	7.00	2.70	11.00	10.00	15.35	16.88	9.9

Table 13 continued

Genotypes	GP 1	PS 2	DT 3	DS 4	ASI 5	PH 6	EH 7	LP 8	LE 9	CW 10	EL 11	ED 12	KR 13	RC 14	TW 15	KY 16	PA 17
UMI 857-1	91.67	66.67	54.00	61.00	7.00	95.00	50.00	8.00	4.00	16.40	5.90	3.00	9.00	10.00	15.00	13.50	15.07
UMI 919-1	66.67	50.00	58.00	62.00	4.00	72.00	40.00	6.00	4.00	28.70	6.30	2.80	11.00	9.00	20.10	19.89	9.17
UMI 955-2	58.33	50.00	49.00	53.00	4.00	123.00	65.00	9.00	6.00	76.80	14.80	3.40	32.00	12.00	13.50	51.84	12.51
UMI 960-1	16.67	50.00	57.00	61.00	4.00	100.00	52.00	7.00	5.00	14.20	8.60	2.20	8.00	8.00	15.40	9.85	9.28
UMI 1004	58.33	75.00	61.00	68.00	7.00	101.00	58.00	8.00	5.00	25.20	9.20	2.90	12.00	8.00	18.30	17.56	8.69
UMI 1005-1	91.67	66.67	54.00	58.00	4.00	131.00	60.00	10.00	8.00	38.10	12.50	3.60	17.00	10.00	24.20	41.14	11.02
UMI 1009-2	75.00	25.00	57.00	62.00	5.00	122.00	65.00	11.00	5.00	30.50	10.00	3.00	16.00	10.00	16.30	26.08	11.86
UMI 1013-1	83.33	66.67	56.00	67.00	11.00	130.00	60.00	13.00	5.00	24.30	7.00	3.00	13.00	10.00	15.20	19.76	8.87
UMI 1017	83.33	50.00	51.00	55.00	4.00	105.00	63.00	7.00	4.00	41.20	8.00	3.60	14.00	10.00	26.20	36.68	8.38
UMI 1027	66.67	41.67	53.00	56.00	3.00	116.00	64.00	10.00	5.00	36.10	8.00	3.80	14.00	12.00	20.10	33.76	9.94
UMI 1030	50.00	58.33	59.00	64.00	5.00	125.00	59.00	13.00	6.00	23.10	6.20	2.90	10.00	8.00	16.20	16.42	8.74
UMI 1031	83.33	41.67	54.00	59.00	5.00	135.00	63.00	14.00	7.00	24.00	7.50	2.90	12.00	8.00	14.50	19.21	7.99
UMI 1036	83.33	75.00	59.00	70.00	11.00	96.00	50.00	11.00	3.00	54.60	9.00	3.70	14.00	10.00	28.60	40.04	9.97
UMI 1054	66.67	66.67	58.00	62.00	4.00	100.00	65.00	10.00	6.00	12.70	6.00	2.30	13.00	8.00	10.20	10.60	8.94
UMI 1100	83.33	58.33	53.00	60.00	7.00	100.00	40.00	11.00	6.00	27.00	7.00	3.20	10.00	10.00	26.00	23.47	7.25
UMI 1101	91.67	41.67	55.00	64.00	9.00	110.00	57.00	11.00	7.00	39.90	12.00	3.50	13.00	10.00	18.30	23.79	9.87
UMI 1105	41.67	58.33	51.00	58.00	7.00	165.00	75.00	11.00	7.00	212.30	21.30	5.20	43.00	16.00	30.70	202.34	8.99
UMI 1110-1	50.00	58.33	54.00	62.00	8.00	80.00	36.00	9.00	5.00	21.00	7.00	2.70	9.00	8.00	24.10	17.35	13.59
UMI 1112	75.00	50.00	56.00	62.00	6.00	130.00	90.00	10.00	5.00	24.00	7.10	2.90	10.00	7.00	15.60	21.00	10.42
UMI 1113	75.00	41.66	54.00	58.00	4.00	124.00	66.00	14.00	7.00	77.70	13.00	3.60	22.00	16.00	24.10	74.10	12.62
UMI 1124	66.67	41.67	60.00	68.00	8.00	146.00	70.00	10.00	6.00	63.20	9.10	4.10	13.00	15.00	22.30	56.40	8.20
UMI 1126-1	91.67	75.00	61.00	68.00	7.00	107.00	48.00	11.00	8.00	16.00	6.00	2.40	9.00	9.00	12.80	10.36	12.68
UMI 1156	75.00	8.33	52.00	57.00	5.00	104.00	54.00	7.00	4.00	46.60	10.00	2.60	19.00	12.00	15.20	34.65	9.83
Check																	
UMI-70-1	84.46	41.20	50.00	53.00	3.00	195.33	90.33	9.67	4.67	179.48	14.60	5.22	30.53	4.00	25.00	143.08	9.22
UMI-285	79.72	37.61	48.00	51.00	3.00	161.67	84.00	7.00	3.33	100.12	10.44	3.26	22.44	7.33	17.90	128.27	9.72

GP germination percentage (%), *PS* final plant stand (%), *DT* days to 50% tasseling (days), *DS* days to 50% silking (days), *ASI* anthesis-silking interval (days), *PH* plant height (cm), *EH* ear height (cm), *LP* number of leaves per plant (count), *LE* number of leaves above the upper most ear (count), *CW* cob weight (g), *EL* ear length (cm), *ED* ear diameter (cm), *KR* number of kernels per row (cm), *RC* number of rows per cob (count), *TW* test weight (g), *KY* single plant kernel yield (g), *PA* phytic acid content (mg/g)

9.49%, respectively. The traits anthesis silking interval and plant stand contributed to the major variability in PC3. The flowering traits namely, days to 50% tasseling and days to 50% silking exhibited major contributions in PC4. The last PC 5 and PC6 had captured minimum variations of 6.83% and 5.96%, respectively. The highest contributing trait in PC 5 was ear height while anthesis silking interval contributed to the highest in PC 6 (Table 11). We could hereby observe that the days to 50% tasseling, days to 50% silking, anthesis silking interval, ear height and phytic acid has contributed favorably to all the principal components and thus these traits exhibited a higher variation in the formulated reference set (Aci et al. 2018).

Correlation of the traits observed

Among the seventeen characters analysed, cob weight, plant height, ear height, number of leaves above the uppermost ear, ear diameter, number of rows per cob, test weight and number of kernels per row were found to have significant positive correlation with the kernel yield (Raut et al. 2017). Thus these traits could be used as indicators for selecting higher yielding lines. The phytic acid being an essential concern was observed to be positively correlated with the plant stand and germination percentage. This emphasizes that phytic acid has an essential role in the germination and early establishment of the crop (Bregitzer and Raboy 2006). It was also found to be positively correlated with the kernel yield and yield contributing traits such as ear length, ear diameter and number of kernels per row (Table 12). This elucidates the negative characteristics of the cobs in the low phytic acid lines (Raboy et al. 2000). Thus maintaining the intermediate ranges (5–10 mg/g) of phytic lines is also essential for breeding purposes to focus on combined approaches of low phytic acid and high yield. This formulated reference set will enable us to utilize the maximum variability for phytic acid and yield contributing traits by handling a minimum population.

Prominent pre-breeding lines selected from the reference set

Morphological observations recorded in the reference set are shown in the Table 13. Among the 58 genotypes screened, the lowest phytic acid content was observed in the genotype UMI-113 (2.77 mg/g) followed by UMI-300-1, UMI-467, In-6, In-12 and UMI-158 with a low phytic acid content of 3.17–6.78 mg/g, respectively (Table 12). Most of the lines including the above mentioned low phytic acid lines had a poor yield with shriveled cobs and low number of kernels per row, indicating the role of phytic acid in pollination and seed set (Bregitzer and Raboy 2006). Considering the single plant yield in selection, medium phytic acid lines such as In-12 (6.26 mg/g) and UMI-158 (6.78 mg/g) with an average yield of 93.29 g and 87.78 g, respectively, could be used in low phytate breeding programs (Table 13) without compromising the seed yield traits. The genotypes UMI 113, UMI 300-1, and UMI 467 with the lowest phytic acid content could be used as donors to transfer low phytic acid content to other agronomically superior inbred lines in the process of developing low phytate hybrids.

Conclusion

The formulation of reference set enhances the utilization of genetic resources in crop improvement. This reference set formed for phytic acid can be further extensively phenotyped to identify accessions for other beneficial traits to improve maize breeding. The population size of this reference set representing the entire base population is easily manageable and this could increase the feasibility of exchanging these germplasm lines with other maize breeding institutes to enhance low phytate breeding. Further grouping of the genotypes in the reference set to form heterotic pools for producing hybrids with low phytic acid may enable us to investigate their gene action and combining ability.

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