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**Research Article**

# Distribution of phytic acid and allelic variations in inositol monophosphatase gene in chickpea (*Cicer arietinum* L.)

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**Abstract**
Phytic acid (PA) is an anti-nutritional factor, which chelates divalent cations, limiting their bioavailability. In this study, 31 Indian cultivars of different agroclimatic zones and 52 germplasm lines representing 21 chickpea growing areas worldwide were evaluated for variability in PA content. Over two consecutive growing seasons, a wide variation in PA content (8.81-21.97 mg/g) was observed. ANOVA for PA content in both cultivars and non-cultivated accessions indicated significant Genotype X Environment interactions, yet, genotypes with consistently low (<13 mg/g) and high (>17 mg/g) PA were identified. Furthermore, using a bulked segregant analysis approach, the transcriptional regulation through repeat length variation in *inositol monophosphatase* (*CaIMP*) gene associated with PA content was demonstrated in two $F_2$ populations segregating for its alleles. Both the *CaIMP* alleles (shorter repeat, $NCPGR_{170}$ and longer repeat, $NCPGR_{200}$) were equally distributed in 26 popular *desi* chickpea cultivars. This information will help selecting germplasm for developing nutritionally improved chickpea cultivars.

**Keywords**
5'UTR; Antinutrient; Bulked segregant analysis; *CaIMP,* Chickpea, microsatellite repeat; Phytic acid, repeat length variation; SSR

## Introduction

Chickpea (*Cicer arietinum* L.) is an important grain legume of family Fabaceae grown worldwide in around 12.65 million hectares area and the production of about 12.09 million tonnes (FAOSTAT 2016). It ranks second among the grain legumes in terms of global production, and India is the largest producer with an annual production of around 7.82 million tonnes, representing 65% of world's chickpea production covering an area of 8.39 million hectares (FAOSTAT, 2016). Chickpea is nutritionally important grain legume, particularly in vegetarian diets, as its 80% of the total dry grain mass is constituted of proteins and carbohydrates and it is also free of cholesterol (Geervani 1991; Wood and Grusak 2007; Chibbar *et al*. 2010). The carbohydrate content in chickpea is around 60g/100g, which is higher than other pulses. The total fat content ranges from 2.7-6.48%, most of which is polyunsaturated. It is an important source of proteins with protein percent (of the total dry seed mass) ranging from 17-22% (before dehulling) and 25.3-28.9% (after dehulling). It is also rich in dietary fiber (18-22 g/100g raw chickpea seeds) (Jukanti *et al*. 2012). In addition, it isalsoa good source of minerals with an average of 3.0–14.3 mg of iron, 2.2–20 mg of zinc, 49–53mg calcium, per 100 g edible portion (Patterson *et al*.1997; Wood and Grusak 2007; Ray *et al*. 2014). Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate; IP6; PA)is a potent chelating agent of divalent mineral ions like calcium, zinc, and iron, and reduces their bioavailability. In plants, phytic acid is the primary reservoir of phosphates in seeds and is mainly stored in globoids as phytate (Otegui *et al*. 2002). It is also involved in the formation of complexes with protein, negatively affecting protein solubility, enzymatic degradation, gastric absorption and the malting process (Bilgiçli *et al*. 2006; Dai *et al*. 2007). Due to lack of phytase enzyme, PA cannot be dephosphorylated in digestive tract of monogastric animals, and excreted phytate is one of the reasons for environmental phosphate pollution as it causes eutrophication of aquatic ecosystem (Raboy2009). The level of PA ranges from 5–50 mg/g (0.5–5% w/w) in edible legumes, nuts, cereals, and oil seeds (Graf *et al*. 1987).

Because of phytic acid's negative impact on food and/or feed, several breeding efforts have been made to develop low-phytic-acid lines (Cichy and Raboy 2009; Raboy 2009). However, with a few exceptions, most of these lines showed negative impact on yield and plant performance (Raboy *et al*. 2015). PA also plays important role in regulating various biotic and abiotic stresses (Meis *et al*.2003; Oltmans *et al*. 2005; Naidoo *et al*. 2012). Inositol monophospatase (IMP) gene

involved in phytic acid biosynthetic pathway was recently found to be associated with phytic acid content in chickpea grains (Joshi Saha and Reddy 2015; Dwivedi *et al*. 2017). Allelic variations in the length of a microsatellite repeat (NCPGR90) was found to be present in the promoter of this gene that regulated the expression of *CaIMP* either transcriptionally (Joshi Saha and Reddy 2015) or translationally (Dwivedi *et al.*2017).

Recently there has been a renewed interest in phytate biology following discovery of many novel roles of phytic acid and its derivatives in several organism including plants (Williams *et al*. 2015; Scherer *et al*. 2016; Joshi-Saha and Reddy 2016). Considering the important role of PA in nutrition as well as plant biology, there is a need to study its distribution in nutritionally important grain legumes. There are very few reports on the estimation of phytic acid content in chickpea; moreover, only a limited number of genotypes have been analysed till date (Duhan *et al*.1989; Chitra *et al*. 1995; Jukanti *et al*. 2012). Large collections of chickpea germplasm are now available (Upadhyaya *et al*. 2011). There is a need to characterize such germplasm collections for various traits for their better utilization in the breeding programs. In addition, the cultivars that are popularly grown in a region should also be characterized with respect to the nutritive value for their better popularization in the community. Therefore, the present study was undertaken with the objective to characterize 31 chickpea cultivars adapted to different agro climatic zones in India and 52 germplasm lines representing 21 chickpea growing areas of the world with respect to variability in phytic acid content and also to identify genotypes having high and low phytic acid content useful for breeding programs. Furthermore, the expression of *CaIMP* was studied in two $F_2$ segregating populations, for its alleles to confirm the transcriptional regulation of this gene. In addition, the distribution of allelic variations in *CaIMP* was studied in popular *desi* cultivars of the Indian subcontinent for their better utilization in the future crossing programs for developing nutritionally improved chickpea genotypes.

**Material and Methods**

Eighty-three genotypes of chickpea including 31 cultivars and 52 accessions of both *desi* and *kabuli* types adapted to various agro-climatic zones were analyzed in the present study(Table1, Table2).

The genotypes were grown in a randomized complete block design (RCBD) with two replications at the Experimental and Gamma field facility, Bhabha Atomic Research Centre, Trombay Mumbai (19˚ 03″ N, 72˚ 93″ E) during the month of October–November and were harvested during month of February–March. The genotypes were grown in two growing seasons (*Rabi* 2012–2013 and *Rabi* 2013–2014). The crop was raised in 2m rows with 30 cm × 10 cm spacing between rows and between plants respectively. Standard agronomic practices were carried out to raise a successful crop.

Well-dried seeds from at least five single harvests of each genotype per replication were pooled for the analysis. The seeds were ground into a fine powder and passed through a mesh of 0.5mm sieve size to obtain a homogenous powder. Phytic acid content was measured according to the previously reported method (Joshi-Saha and Reddy 2015).

The replicated data set was subjected to analysis of variance using General linear Model (GLM) in Minitab ver 17.1.0 statistical software. Genotypic variance (Vg), phenotypic variance (Vp) and error variance (Ve) were calculated using pooled ANOVA table. Phenotypic coefficient of variation (PCV %) and genotypic coefficient of variation (GCV %) were calculated as $GCV\ \% = \sqrt{Vg}/x \times 100$ and $PCV(\%) = \sqrt{Vp}/x \times 100$(Singh and Chaudhary 1985). Broad sense heritability ($H^2$) was calculated as the ratio of Vg and total phenotypic variance (Vp = Vg + Ve). Genetic advance were calculated as GA (%) = K x σP x $H^2$x100, where K is selection differential at 5% =2.06, σP= phenotypic standard deviation, and $H^2$= broad sense heritability (Badigannavar *et al.* 2016). Genetic advance over mean (%GAM) was calculated as percentage of genetic advance over the mean (Badigannavar *et al.* 2016). Genetic parameters (GCV%, PCV%, $H^{2,}$ GA and GAM) in $F_2$ population were calculated as described earlier (Dhole and Reddy 2011).Based on two year's phytic acid content, a cluster analysis was performed using Euclidean distances. A dendrogram was constructed using the Unweighted pair group method with Arithmetic Averages (UPGMA) algorithm on NTSYS-pc version 2.1 software (Rohlf 1990).

DNA was extracted from fresh leaf tissues of one-month-old field-grown plants using CTAB method (Doyle and Doyle 1987) and quantified using Nano Drop™. The allelic variation for the simple sequence repeat marker (NCPGR90) present in the promoter region of *CaIMP* (Joshi-Saha and Reddy 2015) was analysed using the primer pair NCPGR90F: 5′-TAGCATACCATTGTCAACCA-3′;NCPGR90R:5′-AGAGCACATACGGTTTTGT-3′. PCR cycle consisted of an initial denaturation at 94˚C for 4 min followed by 40 cycles of 94˚C for 30 s, 55˚C for 45 s, and 72˚C for 50 s, followed by a final extension of 10 min at 72˚C.

Total RNA was isolated from leaf sample collected from laboratory grown 10 days old chickpea seedlings or one-month old plants (in case of $F_2$ population) grown in field conditions using Nucleopore RNA Sure plant RNA isolation kit according to the manufacturer's protocol. Two micrograms of DNA seI-treated RNA was used for cDNA synthesis using Revert Aid First strand cDNA synthesis kit (Fermentas) and random hexamer primer as per manufacturer's instructions. Reverse Transcriptase (RT) PCR was carried out to check the repeat length variation using primer pair NCPGR125F: 5′-CGGTTTTGTGTATGGTGAGT-3′ and RTCa700R1:5′-CGTTCCTTTGTGTTGGACGT-3′. Quantitative real-time PCR for inositol monophosphatase gene was performed using 2 different primer pairs, Primer Pair 1 (PP1): (RTCa700F1: 5′-CACATCAAAATGGTTGACAATG-3′; RTCa700R1: 5′-CGTTCCTTTGTGTTGGACGT-3′) (Joshi-Saha and Reddy 2015) and Primer Pair 2 (PP2): (CaIMP9F: 5′-AGCGTGTAGCTGCTTCAAACC-3′; CaIMP10R: 5′-GTTTGGCGCAGAGCATCA-3′) (Dwivedi *et al*., 2017). Elongation factor 1-alpha (EF1α; EF1αF: 5′-CTGTAACAAGATGGATGCCAC-3′; EF1αR: 5′-CAGTCAAGGTTAGTGGACCT-3′) was used as control housekeeping gene. Real time PCR was performed as described previously (Joshi-Saha and Reddy 2015). The expression of *CaIMP* mRNA was normalized to the expression ofEF1α and relative changes in gene expression were calculated using the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001). For RNA isolation, leaf samples were pooled from at least 5 plants of each genotype. For expression analysis in $F_2$ population, leaf samples were pooled from 10 plants of each genotype ($H_{170}$: Homozygous for 170 bp allele, $H_{200}$: Homozygous for 200 bp allele and Het: Hetrozygous with one 170bp and other 200 bp allele). Two independent experiments each with three technical replicates of each sample were analyzed in the real-time PCR analysis.

**Results and Discussion**

The present study was aimed to evaluate phytic acid content in 83 (66 *desi* and 17 *kabuli* biotypes) chickpea accessions, of which 52 were germplasm accessions representing 21 diverse geographical locations of chickpea growing areas of the world and constitute a part of a minicore collection (Upadhyaya *et al*. 2011). In addition, 31 chickpea cultivars adapted to different agro-climatic zones of India, which is the largest chickpea producer in the world, were also analyzed for the PA content in two growing seasons. Over the combined two years, a wide variation for phytic acid content was observed (range in 2013: 8.81 (ICC-8740) –21.97(ICC-8151)mg/g, mean 15.26mg/g, range in 2014: 10.47(ICC-5504) –21.6 (ICC-1923)mg/g, mean 14.84mg/g). Overall, the phytic acid content of *desi* (2013: 15.34 mg/g; 2014: 15.05 mg/g) and *kabuli*(2013: 14.98 mg/g; 2014:14.03 mg/g) biotypes did not differ significantly from each other (t test: 2013: p=0.65; 2014: p=0.15).

Thephytic acid content present in legumes generally ranges from 5–50 mg/g (Graf *et al*. 1987). A previous study has reported a range of 7.7–12.3 mg/g in 13 *desi* and 5.4–11.4 mg/g in 3 *kabuli* chickpea genotypes (Chitra *et al.*1995). The present study is in concurrence with this report as one of the genotype (ICCV2) was common to both the studies and showed similar phytic acid content of 11.4mg/g in the previous study and 12.17 mg/g and 11.21 mg/g in 2013 and 2014 respectively in the present study. Ten chickpea genotypes grown in dry land production area in western Canada contained phytic acid ranging from 3.8–9.0 mg/g (Bueckert *et al.*2011), while 10 commercially grown cultivars of USA showed a range of 5.8–13.6 mg/g (Thavarajah and Thavarajah 2012). The overall wider variation in the present study could be due to the use of more diverse group of accessions, genotypic differences as well as environmental variations, as the genotypes are from wide range of agro-climatic zones. Analysis of variance for phytic acid content indicates significant genotypic variation as well as Genotype X Environment (GXE) interactions for this trait in both cultivars and non-cultivated accessions **(**Table 1, Table 3a**)**.The phytic acid content was found to be influenced by environmental conditions; with hot and dry weather conditions reducing the PA content as compared to the wetter environment (Bueckert *et al*. 2011).In the present study the phytic acid distribution range was wider in case of germplasm accessions as compared to the cultivated varieties (Table 3b).This could be due to limited adaptability of cultivars to a particular region with respect to the non-cultivated accessions representing a wider geographical distribution.

Parameters that estimate genetic variability were also calculated. Among the genotypes, the value of genetic coefficient of variation (27.93%) and phenotypic coefficient of variation (28.97%) were close to each other, and a high heritability (0.92) for the trait was estimated (Table 4).While, in $F_2$ populations derived from two crosses ICC-1052 X ICC-8950 and ICC-1052 X ICC-14778 between parents having contrasting PA contents showed wider difference between GCV% (12.34% and 12.41% for the two crosses respectively) and PCV% (15.08% and 14.26% for the two crosses respectively). The heritability for PA content was high (0.67 and 0.76 for the two crosses respectively) in both the $F_2$ populations (Table 4).These genetic parameters (GCV% and PCV%) also indicated that both genotype and environment

contributes significantly to the trait, yet high heritability with moderate GCV and genetic advance suggest that selection may be effective in early generations for these traits (Chen *et al*. 1996; Kumar *et al*. 2014).Indeed, in $F_2$ population derived from two crosses ICC-1052 X ICC-8950 and ICC-1052 X ICC-14778 between parents having contrasting PA contents (ICC-1052: 14.6±1.3 mg/g; ICC-8950: 18.9±1.8 mg/g; ICC-14778: 17.4±1.6 mg/g),the distribution of phytic acid in $F_{2:3}$ seeds of individual $F_2$ plants showed continuous variation that fitted into normal distribution with transgressive segregants(Fig.1a,b, c, d).

A dendrogram was constructed based on phytic acid content of two seasons using Euclidean distances and UPGMA clustering. The analysis grouped the 83 genotypes in six clusters (Fig. 2, Table 5). Of the 6 clusters, cluster IA and cluster IV contained the genotypes with low (range: 11.12-14.19 mg/g, cluster average: 13.12 mg/g) and high PA (range: 16.79-20.02 mg/g, cluster average: 18.20 mg/g) respectively in both the years of cultivation. While, cluster IB and cluster II consisted of genotypes with similar cluster averages and ranges (cluster average: 15.15 mg/g and 14.9 mg/g respectively; cluster range: 14.73-16.15 mg/g and 13.15-16.24 mg/g respectively).However, genotypes in both these clusters showed significant G X E interactions. Cluster V and VI were small with only 2 genotypes in each cluster. Both the clusters contained high cluster average of 16.93 mg/g and 16.10 mg/g respectively; however, genotypes in these two clusters also showed significant G X E interactions with variations with respect to the year of cultivation. Cluster III consisted of a single OTU ICC 8740, a *kabuli* biotype from Afghanistan with lowest average PA content of 10.49 mg/g. The eight black seeded genotypes (ICC-4418, ICC-2507, ICC-6306, ICC-1052, ICC-4814, ICC-8522, ICC-3776, ICC-12537) were grouped in Cluster I. Sixteen out of thirty-one cultivars were grouped together in cluster II having low to moderate average PA content. Interestingly, eleven out of eighteen genotypes clustered in group IV were also grouped together in a previous genetic diversity study using retrotransposon amplified fragment length polymorphism (REMAP), inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers, suggesting a common genetic base of these genotypes (Joshi-Saha and Reddy 2014).

Despite a significant environmental effect, genotypes having low (<13 mg/g), moderate (13-17 mg/g) and high (>17 mg/g) phytic acid consistently for both the seasons were identified. Among the cultivars ICCV2, Vijay and JG63 had consistently low PA, cultivars RSG 44, Vaibhav, ICCV37, JG6, Phule G5, JG11, JG315, JG14 had consistantly moderate and cultivars RSG888, JGG1and JG16 had consistently high PA content (Table 1). Similarly, among non-cultivated accessions ICC-4918, ICC-5504,ICC-8350, ICC-8740, ICC-11879, ICC-13124, ICC-15510, IC-268928, and IC-268989had consistently low PA while, ICC- 2263, ICC-3776, ICC-4814, ICC-6263, ICC-7184, ICC-7819, ICC-8522, ICC-12928, ICC-13441, ICC-14051, ICC-14799 had consistently moderate and ICC-1164, ICC-2242, ICC-2720, ICC-3325, ICC-8058, ICC-9586, ICC-12155, ICC-12947, ICC-14669, ICC-14778, ICC-15612, ICC-16207, and IC-269010had consistently high PA content (Table 2).

Alleles of inositol monophoshatase gene with variation in $(CT)_n$ repeat in its promoter region were previously found to be associated with phytic acid content in chickpea (Joshi Saha and Reddy 2015; Dwivedi *et al.*2017). However, conclusion regarding the influence of repeat length variation on transcription of this gene is variable. Therefore, we studied the expression of *CaIMP* gene in four genotypes differing in their repeats (ICC-867, ICC-3325, ICC-12299 and ICC-4814), using two sets of primers reported earlier. As reported previously (Joshi Saha and Reddy 2015), the genotypes having smaller repeats (ICC-867, ICC3-325, ICC-12299) showed higher expression relative to the genotype having longer repeat (ICC-4814)with both the primer pairs (Fig. 3a, b). However, the fold change based on the primer pair CaIMP9F/10R (Dwivedi *et al*. 2017) was lessas compared to primer pair RTCa700F1/RTCa700R. Further analysis of melt curve of the qRT-PCR products indicates a single sharp peak for amplification product for primer pair RTCa700F1/R1 while a broad peak merging with background fluorescence was observed in case of CaIMP9F/10R primer pair (Fig 4 a,b).To ascertain the effect of repeat length on transcription of CaIMP, the expression of *CaIMP* was also analysed in the segregating $F_2$ population of two crosses between genotypes(ICC-1052 X ICC-8950 and ICC-1052 X ICC-14778). The parental genotypes were previously shown to have contrasting repeat length variations in the 5'UTR of the *CaIMP*gene (Joshi Saha and Reddy 2015).The genotypes having shorter repeat (ICC-8950 and ICC-14778) showed higher expression of *CaIMP* with respect to the genotype having longer repeat (ICC-1052)(Fig. 5a).A bulked segregant analysis approach was used for studying the expression analysis of *CaIMP* from pooled samples of the segregants from two populations genotyped for the repeat length variation. A higher expression of *CaIMP* was observed in the $F_2$segregantswith alleles either homozygous for 170bp repeat ($Ho_{170}$) or heterozygous ($Het_{170/200}$) in comparison with the segregants having homozygous longer repeats ($Ho_{200}$) (Fig. 5b).These results indicate that repeat

length variation in the promoter region regulates the expression of *CaIMP* gene. Additionally, we also studied the allelic distribution of *CaIMP*in 26 popular *desi* cultivars of India, which is the largest chickpea producing country in the world. Both the alleles (shorter repeat, $NCPGR_{170}$ and long repeat, $NCPGR_{200}$) were almost equally distributed (Table 6).The expansion of the $(CT)_n$ repeat in 5' UTR of *CaIMP*is evolutionarily recent in *Cicer arietinum* (Joshi Saha and Reddy 2015). Seven wild accessions belonging to the primary gene pool of *Cicer* (*C. reticulatum* and *C. echinospermum*) were found to have shorter repeat ($NCPGR_{170}$) (Dwivedi *et al*. 2017).*C. reticulatum* is also considered as the wild progenitor of cultivated chickpea, *C. arietinum* (Ladizinsky and Adler 1976). Of the 71 traditional landraces with geographical locations outside Indian subcontinent, more than 80% were found to have the longer allele (Dwivedi *et al.*2017). In the same study, among the 38 *kabuli* genotypes, the distribution of alleles was skewed with more *kabuli* genotypes (n=33) having longer repeat ($NCPGR_{200}$), whereas the distribution of longer and shorter repeats ($NCPGR_{170}$) was almost equal among 33 *desi* genotypes. In the present study, an equal distribution of both the alleles in *desi* Indian cultivars suggests possibility of introgression from primary gene pool as well as from genotypes outside the Indian sub continent.In addition, 4 genotypes (ICCV10, JG315, JG218 and GCP105) showed another allele $NCPGR_{190}$.

In the present investigation, influence of environmental variations on phytic acid content in chickpea revealed that despite environmental influence, PA content was consistent over two seasons in few genotypes. The genotypes with consistently low and/or moderate phytic acid, particularly the accessions and cultivars well adapted to a particular agroclimatic zone (Table 1 and Table 2), will be useful in breeding programs for developing nutritionally improved chickpeas. In addition, the transcription of *inositol monophosphatase* gene was found to be regulated by the repeat length variation in its promoter region, with genotypes having smaller repeats showing higher transcription as compare with that of longer repeat. The shorter repeat length was previously found to be associated with drought tolerance in chickpea (Joshi Saha and Reddy 2015). In the segregating $F_2$ populations, it was possible to identify the $F_2$segregants with shorter repeats and low-to-moderate PA content, which will be useful in developing nutritionally improved chickpea cultivars that are not compromised in their drought tolerance.

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**Table 1.Cultivars used in the present study and their phytic acid contents in two seasons**

| S. No. | Cultivar | Biotype[a] | Pedigree | Phytic acid content (mg/g) | |
|---|---|---|---|---|---|
| | | | | ***Rabi* 2012-13** | ***Rabi* 2013-14** |
| 1 | ICCV2 | K | [((K850*GW5/6)*P48)*(L550*Guamchil 2)] | 12.18 | 11.21 |
| 2 | ICCV10 | D | P1231*P1265 | 16.00 | 11.17 |
| 3 | DCP 92-3 | D | Selection from local germplasm | 16.37 | 13.32 |
| 4 | Vijay | D | P 127*Annegeri 1 | 12.93 | 12.73 |
| 5 | JAKI- 9218 | D | (ICCV37*GW5/7)*ICCV107 | 14.43 | 11.95 |
| 6 | RSG 44 | D | JG62*F496 | 13.28 | 14.36 |
| 7 | RSG 888 | D | RSG44*E100Y | 19.05 | 16.50 |
| 8 | KAK 2 | K | (ICCC2*Surutato77)*ICC7344-ICCX 870026-PB-PB-14P-BP-62 AK-7AK-BAK | 16.24 | 11.69 |
| 9 | PUSA 391 | D | ICC 3935*PUSA 256 | 17.32 | 15.17 |
| 10 | Vaibhav | D | Selection from GP ICCV 91106 | 14.73 | 13.02 |
| 11 | ICCV 37 | D | (P 418* JG62)*P1630 | 16.36 | 15.28 |
| 12 | JG 130 | D | (Phule G 5*Narsinghpurbold)*j JG74. | 17.94 | 13.99 |
| 13 | JG 6 | D | (ICCV 10*K 850)*(H 208*RS 11) | 15.57 | 13.69 |
| 14 | Phule G 5 | D | B 110* N 31 | 16.49 | 13.65 |
| 15 | Virat | K | (ICC7676*ICCC32)*(ICCC49*FLIP-82-IC)*ICCV3 | 14.43 | 11.77 |
| 16 | JG 11 | D | [(Phule G 5* Narsinghpurbold )*ICCCX-860263-BF-BP-91 BP | 14.43 | 15.02 |
| 17 | Vishal | D | K 850*ICCL 80074 | 16.31 | 13.02 |
| 18 | JG 315 | D | Selection from WR 315 | 15.41 | 15.80 |
| 19 | JG 16 | D | ICCC4*ICCV10 | 16.45 | 19.57 |
| 20 | GG 3 | D | NA | 14.86 | 12.25 |
| 21 | JGG 1 | D | Selection from germplasm | 20.74 | 16.61 |
| 22 | JG 218 | D | ICCV4*P1353 | 17.14 | 13.87 |
| 23 | GCP 105 | D | ICCL84224*Annegeri 1 | 17.23 | 11.45 |
| 24 | JGK 1 | K | (ICCV2*Surutato77)*ICC 7344 | 15.65 | 11.36 |
| 25 | GG 2 | K | JG1258*BDN 9-3 | 15.83 | 10.47 |
| 26 | GCP 101 | D | GCP 2*ICCV2 | 16.74 | 11.58 |
| 27 | Vihar | K | (ICCC32*ICCL80004)/(ICCC49*FLIP82-886)*ICCV3 | 16.92 | 12.03 |
| 28 | JG 12 | D | NA | 16.27 | 17.42 |
| 29 | JG 14 | D | [(GW5/7*P 327)*ICCL83149] | 14.87 | 13.51 |
| 30 | JG 63 | D | Single plant selection from JG 62 | 11.97 | 13.21 |
| 31 | Phule G 12 | D | GW 5/7 * Ceylon 2 | 17.45 | 14.73 |

a. D: *Desi*, K: *Kabuli*

**Table 2.Germplasmused in the present study and their phytic acid contents in two seasons**

| Sr. No. | Accession | Biotype[a] | Seed color | Origin | Phytic acid content (mg/g) | |
|---|---|---|---|---|---|---|
| | | | | | ***Rabi* 2012-13** | ***Rabi* 2013-14** |
| 1 | ICC-867 | D | Dark brown | India | 13.90 | 16.63 |
| 2 | ICC-1052 | D | Black | Pakistan | 12.13 | 15.37 |
| 3 | ICC-1164 | K | Light orange | Nigeria | 17.45 | 18.74 |
| 4 | ICC-1923 | D | Yellow | India | 13.90 | 21.60 |
| 5 | ICC-2242 | D | Brown | India | 19.89 | 20.15 |
| 6 | ICC-2263 | K | Brown | Iran | 16.34 | 15.94 |
| 7 | ICC-2507 | D | Black | Iran | 12.35 | 14.30 |
| 8 | ICC-2720 | D | Yellow brown | Iran | 17.50 | 16.76 |
| 9 | ICC-2990 | D | Brownish beige | Iran | 12.35 | 15.37 |
| 10 | ICC-3325 | D | Brown | Cyprus | 17.45 | 18.91 |
| 11 | ICC-3421 | K | Beige | Israel | 12.97 | 14.48 |
| 12 | ICC-3776 | D | Black | Iran | 14.97 | 16.01 |
| 13 | ICC-4418 | D | Black | Iran | 14.26 | 11.21 |
| 14 | ICC-4814 | D | Black | Iran | 13.77 | 14.83 |
| 15 | ICC-4918 | D | Light brown | India | 12.00 | 12.02 |
| 16 | ICC-5434 | D | Yellow brown | India | 15.90 | 18.73 |
| 17 | ICC-5504 | D | Light brown | Mexico | 11.78 | 10.47 |
| 18 | ICC-6263 | K | Beige | Russia and CIS | 14.08 | 13.77 |
| 19 | ICC-6306 | D | Black | Russia and CIS | 12.62 | 14.83 |
| 20 | ICC-7184 | D | Dark brown | Turkey | 14.48 | 14.95 |
| 21 | ICC-7308 | K | Beige | Peru | 14.08 | 10.80 |
| 22 | ICC-7819 | D | Brownish beige | Iran | 16.16 | 14.66 |
| 23 | ICC-8058 | K | Beige | Iran | 17.01 | 16.58 |
| 24 | ICC-8151 | K | Beige | USA | 21.97 | 11.73 |
| 25 | ICC-8350 | D | Orange brown | India | 13.55 | 12.43 |
| 26 | ICC-8522 | D | Black | Italy | 15.15 | 15.43 |
| 27 | ICC-8740 | K | Beige | Afghanistan | 8.81 | 12.17 |
| 28 | ICC-8950 | D | Yellow brown | India | 11.73 | 20.51 |
| 29 | ICC-9586 | D | Brown | India | 19.89 | 19.76 |
| 30 | ICC-11764 | K | Beige | Chili | 14.97 | 12.79 |
| 31 | ICC-11879 | K | Beige | Turkey | 13.42 | 13.11 |
| 32 | ICC-12155 | K | Yellow brown | Bangladesh | 19.18 | 18.29 |
| 33 | ICC-12299 | D | Yellow brown | Nepal | 19.62 | 11.10 |
| 34 | ICC-12537 | D | Black | Ethiopia | 13.86 | 18.44 |
| 35 | ICC-12928 | D | Yellow brown | India | 13.86 | 13.73 |
| 36 | ICC-12947 | D | Yellow brown | India | 17.58 | 19.00 |
| 37 | ICC-13124 | D | Light brown | India | 12.26 | 13.32 |
| 38 | ICC-13441 | K | White beige | Iran | 14.26 | 15.98 |
| 39 | ICC-14051 | D | Brown | Ethiopia | 14.17 | 15.58 |
| 40 | ICC-14098 | D | Yellow brown | Ethiopia | 16.57 | 14.43 |
| 41 | ICC-14669 | D | Yellow brown | India | 17.76 | 20.24 |
| 42 | ICC-14778 | D | Brown | India | 17.59 | 17.89 |
| 43 | ICC-14799 | D | Yellow brown | India | 14.17 | 16.08 |
| 44 | ICC-14815 | D | Yellow brown | India | 11.73 | 15.15 |
| 45 | ICC-15510 | D | Yellow brown | Morocco | 13.37 | 13.17 |
| 46 | ICC-15612 | D | Yellow brown | Tanzania | 17.59 | 19.20 |
| 47 | ICC-15697 | K | Beige | Syria | 11.68 | 13.59 |
| 48 | ICC-16207 | D | Yellow brown | Myanmar | 19.01 | 20.29 |
| 49 | IC-268928 | D | Dark brown | NBPGR | 12.80 | 12.73 |
| 50 | IC-268936 | D | Dark brown | NBPGR | 13.15 | 13.91 |
| 51 | IC-268989 | D | Green | NBPGR | 11.29 | 12.51 |
| 52 | IC-269010 | D | Dark brown | NBPGR | 17.58 | 16.61 |

a. D: *Desi*, K: *Kabuli*

**Table 3a.Analysis of variance for phytic acid content in cultivars and non-cultivated accessions**

| Source | df | MS | F-value | P-value |
|---|---|---|---|---|
| Genotype | 82 | 28.28 | 13.61*** | 0.00 |
| Year | 1 | 19.08 | 14.21*** | 0.00 |
| Genotype X Year | 82 | 9.347 | 6.96*** | 0.00 |
| error | 166 | 1.343 | | |
| CV% 7.65 | | | | |
| $R^2$ 91.11 | | | | |

***Level of significance at 0.001.

**Table 3b.Diversity for phytic acid content in chickpea cultivars and germplasm accessions**

| | PA content (mg/g) Year 2013 | | PA content (mg/g) Year 2014 | |
|---|---|---|---|---|
| **Genotypes (n)*** | **Range** | **Mean** | **Range** | **Mean** |
| **Varieties (31)** | 12.0 – 20.7 | 15.8 | 10.5 – 19.6 | 13.6 |
| **Accessions (52)** | 8.8 – 22.0 | 14.9 | 10.5 – 21.6 | 15.5 |

***** Number of genotypes in each category

**Table 4.Estimation of genetic variability parameters for phytic acid content**

| Populations | Vg | Vp | GCV (%) | PCV (%) | $H^2$ | GA | GAM (%) |
|---|---|---|---|---|---|---|---|
| Genotypes | 17.62 | 18.94 | 27.93 | 28.97 | 0.92 | 8.78 | 58.45 |
| $F_2$ (ICC 1502 X ICC 8950) | 4.53 | 6.76 | 12.34 | 15.08 | 0.67 | 3.59 | 20.82 |
| $F_2$ (ICC 1502 X ICC 14778) | 5.50 | 7.26 | 12.41 | 14.26 | 0.76 | 4.22 | 22.33 |

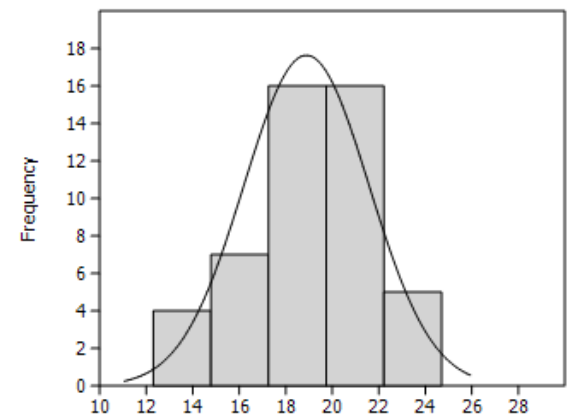
Vg: genotypic variance, Vp: phenotypic variance, GCV: genetic coefficient of variation, PCV: phenotypic coefficient of variation, $H^2$: Broad sense Heritability, GA:genetic advance; GAM: genetic advance over mean

**Table 5.Grouping of 83 chickpea genotypes based on phytic acid content**

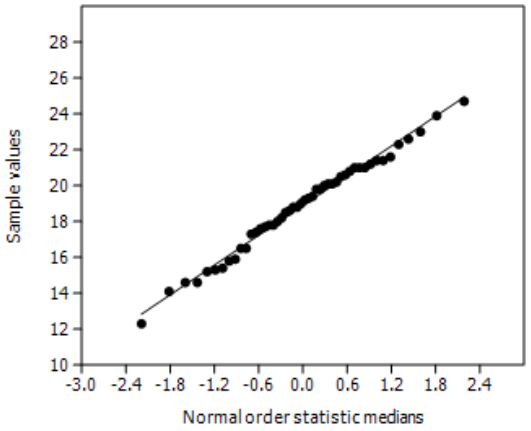
| Cluster Number | Number of Genotypes | Name of Genotypes |
|---|---|---|
| IA | 30 | ICCV2, ICC-4918, ICC-5504, Vijay, IC-268928, ICC-11879, ICC-11510, ICC-8350, Vaibhav, ICC-11764, JG-14, ICC-6263, ICC-12928, JAKI-9218, Virat, GG3, ICC-4418, ICC-7308, RSG444, ICC-3421, IC-268936, ICC-2507, ICC-6306, ICC-1052, ICC-2990, ICC-14815, JG63, ICC-13124, ICC-15697, IC-268989 |
| IB | 11 | JG11, ICC-7184, ICC-4814, ICC-867, ICC-13441, ICC-14799ICC-14051, JG315, ICC-8522, ICC-3776, ICC-12537, |
| II | 19 | ICCV10, JGK1, KAK2, GG3, GCP105, GCP101, Vihar, DCP92-3, Vishal, PhuleG5, JG6, PUSA391, Phule G12, JG130, JG218, ICCV37, ICC-2263, ICC-7819, ICC-14098 |
| III | 1 | ICC-8740 |
| IV | 18 | RSG888, ICC-12155, JGG1, ICC-2242, ICC-9586, ICC-16207, JG16, ICC-5434, ICC-1164, ICC-3325, ICC-12947, ICC-15612, ICC-14778, ICC-14669, JG12, ICC-2720, ICC-269010, ICC-8058 |
| V | 2 | ICC-1923, ICC-8950 |
| VI | 2 | ICC-8151, ICC12299 |

**Table 6.Distribution of *CaIMP* alleles among popular *desi* cultivars of India**

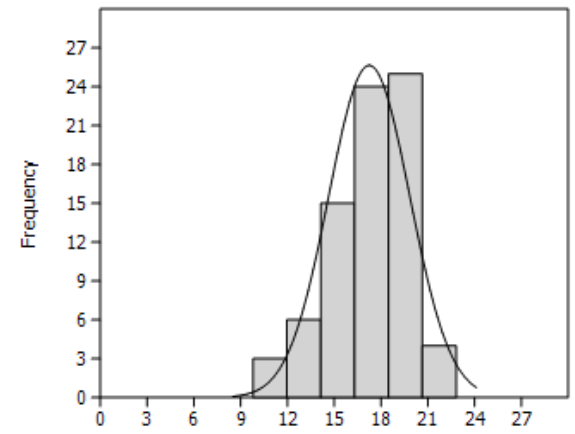
| Sr. No. | Cultivar | Allele (bp) |
|---|---|---|
| 1 | ICC 4948 (G130) | 170 |
| 2 | ICC 15996 (ICCV10) | 190 |
| 3 | DCP 92-3 | 170 |
| 4 | Vijay | 170 |
| 5 | JAKI 9218 | 170 |
| 6 | RSG 44 | 170 |
| 7 | RSG888 | 170 |
| 8 | Pusa 391 | 170 |
| 9 | vaibhav | 200 |
| 10 | ICCV37 | 170 |
| 11 | JG 130 | 200 |
| 12 | JG6 | 200 |
| 13 | Phule G 12 | 170 |
| 14 | Phule G 5 | 200 |
| 15 | JG 11 | 200 |
| 16 | Vishal | 200 |
| 17 | JG 315 | 190 |
| 18 | JG 16 (SAKI 9516) | 200 |
| 19 | GG3 | 200 |
| 20 | JGG1 | 170 |
| 21 | JG218 | 190 |
| 22 | GCP 105 | 190 |
| 23 | GCP 101 | 200 |
| 24 | JG 12 | 200 |
| 25 | JG 14 | 170 |
| 26 | JG 63 | 170 |



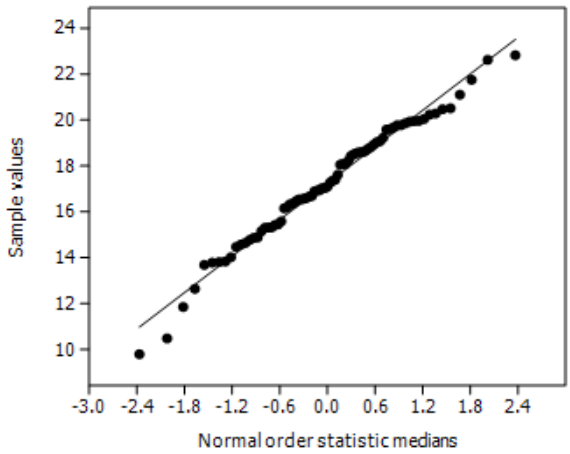

**1a**




**1b**




**1c**




**1d**

**Fig. 1a.** Frequency distribution of phytic acid content in the $F_{2:3}$ population of the cross ICC- 1052 X ICC-14778**1b.**Normality plot showing the fit of data to normal distribution with transgressive segregant on either side of the distribution**1c.** Frequency distribution of phytic acid content in the $F_2$ population of the cross ICC-1052 X ICC-8950**1d.**Normality plot showing the fit of data to normal distribution with transgressive segregant on either side of the distribution.

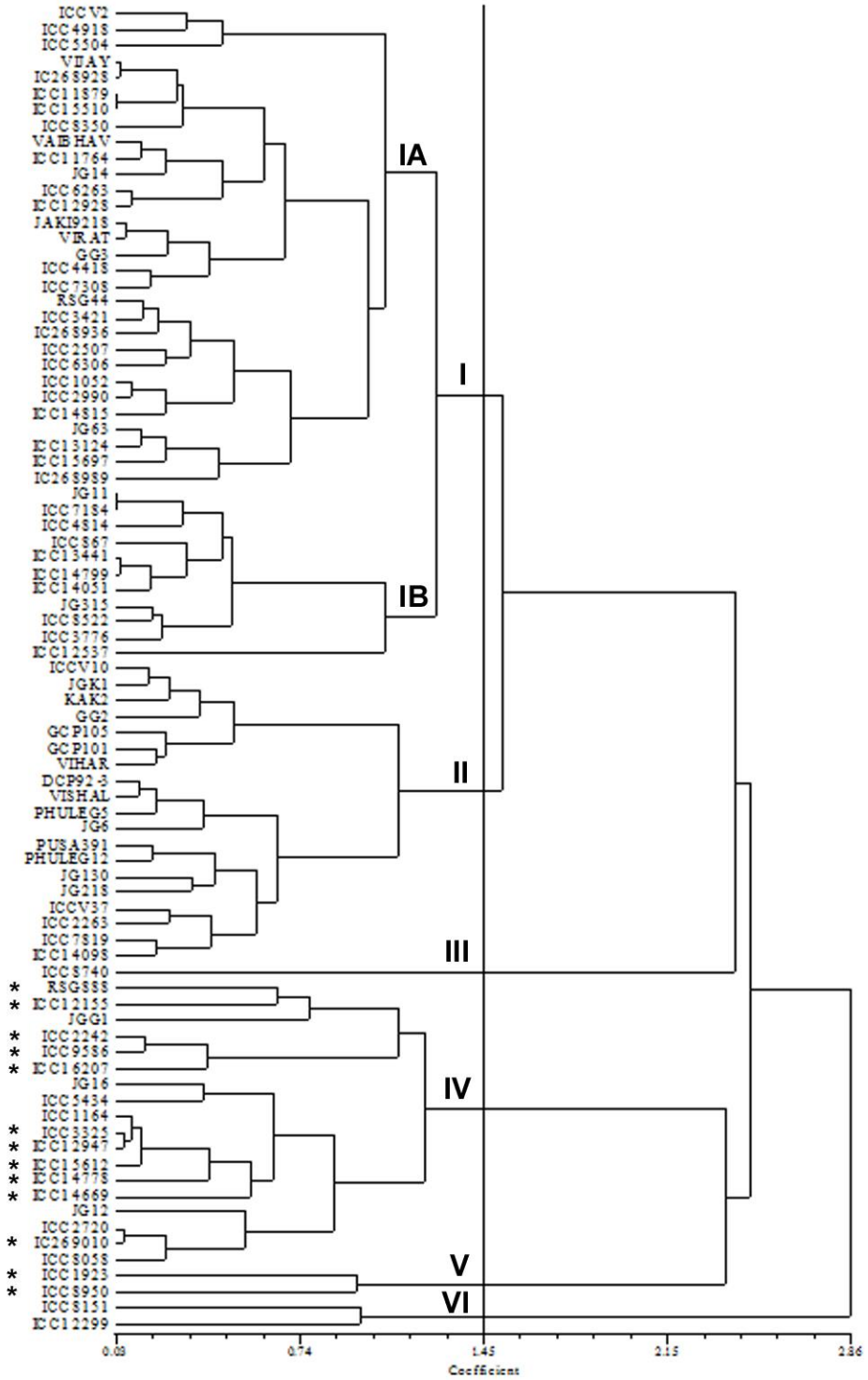



**Fig. 2.**Dendrogram based on Euclidian distance and UPGMA clustering, showing clustering of chickpea genotypes based on phytic acid content of two growing seasons. Asterisks (*) represent the genotypespreviously clustered together in a genetic diversity study using REMAP, ISSR and SSR marker analysis (Joshi-Saha and Reddy 2014)

**3a**


**3b**

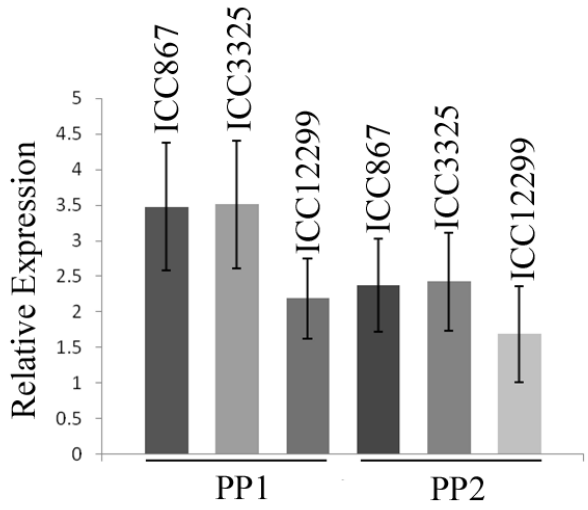
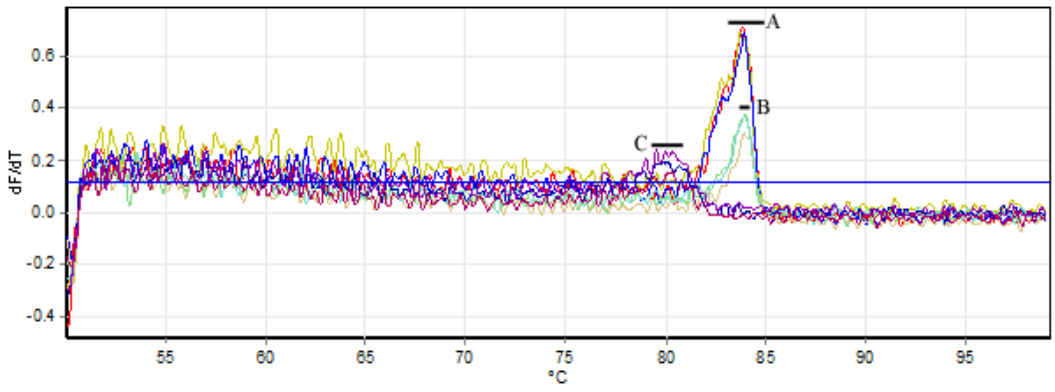



**Fig. 3a.**RT-PCR showing amplification of housekeeping gene Elongation factor 1-alpha (EF1α) (lanes 1-4) in genotypes ICC 867, ICC 3325, ICC 4814 and ICC 12299 respectively and CaIMP repeat length variation in the 5' UTR of these genotypes (Lanes 9-12). Lanes 5-8 and Lanes 13-16 are –RT controls of Lanes 1-4 and Lanes 9-12 respectively. Lane 17 is PCR negative control, while Lane M is 100 bp ladder as marker.
**3b.**Relative expression of CaIMP in genotypes having shorter repeat in its 5' UTR with respect to ICC 4814 (with longer repeat) as control, measured using two different primer pairs: PP1 (RTCa700 F1/R1; Joshi Saha and Reddy 2015) and PP2 (CaIMP9F/10R; Dwivedi*et al*. 2017)

**4a**




**4b**

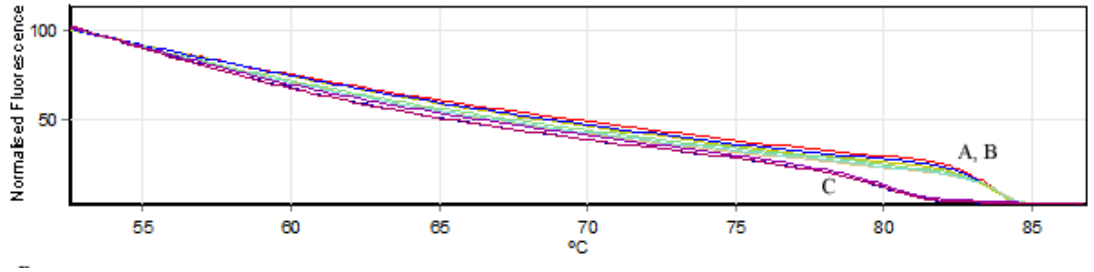
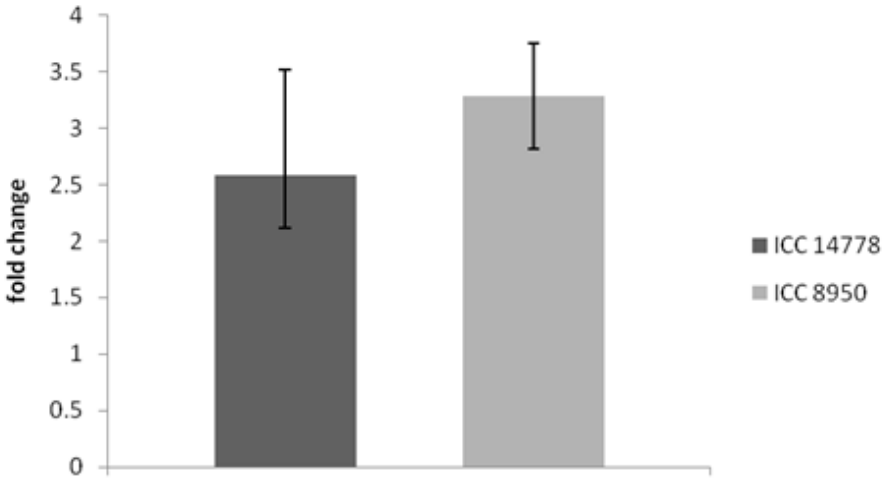



**Fig. 4a.**High resolution melt curve **4b.** Melt curve analysis, of qRT-PCR to check the primer pairs used in the present study. For the ease of visualization, melt curve for only one genotype (ICC 867) is displayed above in triplicate. A. Housekeeping gene (Elongation factor 1-alpha (EF1α), B. *CaIMP* using primer pair 1 (RTCa700 F1/R1; Joshi Saha and Reddy 2015) and C. *CaIMP* using primer pair 2 (CaIMP9F/10R; Dwivedi*et al*. 2017)

**5a**




**5b**

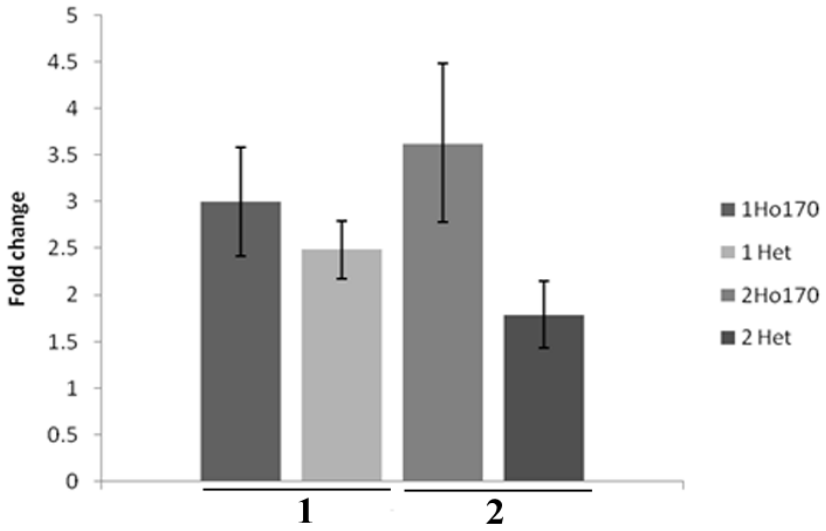



**Fig. 5a.** Relative expression *CaIMP* in genotypes ICC-14778 and ICC-8950 having shorter repeat (170bp allele) in their 5' UTR with respect to genotype ICC-1052 having a longer repeat (200bp allele)5**b.**qRT PCR showing relative expression of *CaIMP* of $F_2$ bulked segregants either homozygous for 170 bp allele ($Ho_{170}$) or hetrozygous ($Het_{170/200}$) relative to those having alleles homozygous for 200 bp alleles ($Ho_{200}$). 1. Segregants from cross ICC-1052 X ICC- 14778; 2. Segregants from cross ICC-1052 X ICC- 8950