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Identification of genomic Regions/genes for high iron and zinc content and cross transferability of SSR markers in mungbean (*Vigna radiata* L.)

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ABSTRACT

Among the legumes, mungbean has highest digestive protein but low micronutrient content like iron and zinc. Biofortification of mungbean has been undertaken to reduce micronutrient malnutrition. The objectives of this study were to identify QTLs for seed Fe and Zn content in F_6 recombinant inbred line (RIL) population (ML776 x Sattya). A large genetic variation and transgressive segregation in RILs were observed for Fe and Zn content. Linkage map was developed which spanned 2919.7cM distance. 17 QTLs (2 for iron and 15 for zinc content) were mapped on four linkage groups; LG 4, LG 6, LG 7 and LG 11 in mungbean. The genomic regions qZn-4-3 and qFe-4-1 on chromosome 4 between PVBR82-BM210 markers; qZn-11-2 and qFe-11-1 on chromosome11 between BM141-BM184 markers, were co-located on the same chromosomal regions for Zn or Fe concentration, which probably were closely linked to each other, or were the same pleiotropic QTLs. The SSR markers associated with QTLs for both high iron and zinc content would also be useful in marker assisted breeding for biofortification in mungbean.

Key words: Biofortification, Micronutrient content, Mungbean, QTLs.

INTRODUCTION

Micronutrient malnutrition is considered as a hidden hunger which is a major problem to health of children, women and poor people (Fletcher *et al.,* 2004; Ghandilyan *et al.,* 2006). Most of the people is suffering from micronutrient deficiency especially iron and zinc because of busy schedule, they never take balance diet (Bouis, 2003). Iron deficiency affects 3.7 billion people and Zinc deficiency affects 49% of the human population (Welch 2002; Brown *et al.,* 2001). Zinc is required as a cofactor in over 300 enzymes and it helps in the formation of DNA binding domain (Palmer and Guerinot, 2009). Zn participates in the synthesis and degradation of nucleic acids, carbohydrates, lipids and proteins (Gelin *et al.,* 2007). Iron is a very important micronutrient to produce RBC (red blood cells) and maintain haemoglobin. Fe deficiency would result in lower haemoglobin (Grotz and Guerinot, 2006). As compared to other deficient micronutrients, the deficiency of iron and zinc is the most prevalent disorder throughout the world (Jeong and Guerinot, 2009; Ghandilyan *et al.,* 2006). Hence, researchers shifted to biofortification which is defined as the process of enhancing micronutrient content in crops to solve malnutrition problem all around the world (Zhu *et al.,* 2007). Biofortification is the best option to enhance the Fe and Zn content with little recurring costs (Chandel *et al.,* 2011). Therefore, to enhance the iron and zinc content in mungbean seed is the best way to alleviate the deficiency of iron and zinc (Singh *et al.,* 2013b)

For developing a variety with high concentration of iron and zinc, it is a foremost thing to identify germplasm with high concentration of both (iron and zinc) micronutrient and to understand their genetic mechanism (Singh *et al.,* 2013a). The quantitative trait loci (QTL) give a powerful genetic approach to characterize the candidate gene and allele mining (Vert *et al.,* 2002). However, few reports are available for identification of QTLs in iron and zinc micronutrient content. Therefore, it is of great importance to study the molecular mechanisms of iron and zinc accumulation in mungbean seed. The direct use of tightly linked markers for QTLs for micronutrients to introgress into crops can reduce the time and cost to develop new cultivars with improved nutritional value.

Through the international, interdisciplinary research programme Harvest Plus, an initiative was developed to alleviate micronutrient malnutrition by increasing the content of Fe and Zn in food crops (Pfeiffer and McClafferty, 2007). Mungbean (*Vigna radiata* L.), an important staple food legume grown, produced and consumed in most of the developing world, is one of the crops targeted for biofortification. Mungbean has been recognized as a nutritionally valuable food for human. Although a cup of beans supplies 24% digestible protein, 25% of the

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recommended daily allowance of iron and 15% of the recommended daily allowance of zinc, the potential exists in mungbean to develop varieties with two to three times the Fe and Zn content (Pfeiffer and McClafferty, 2007). Thus, new mungbean varieties need to be developed with high iron and zinc content which eventually could be improved the nutritional status of vegetarian population

The objectives of this research programme was to identify QTLs controlling high iron and zinc content in mungbean and to investigate iron and zinc concentration using Recombinant Inbred Lines (RILs) derived from contrasting genotypes ML776 x Sattya. The informations generated from this research on SSR markers associated with QTLs for both high iron and zinc content would also be useful in marker assisted breeding for biofortification of mungbean.

MATERIALS AND METHODS

Plant material: This comprised mapping population of 120 F_6 Recombinant Inbred Lines (RIL), developed from two genotypes of Indian mungbean genetic pool. The initial cross ML776 x Sattya was advanced to the F_6 generation by single-seed descent method and seed of RILs was increased for studies during 2013 at the Department of Genetics and Plant Breeding (Pulses section), Chaudhary Charan Singh Haryana Agricultural University (CCSHAU), Hisar, India. The parent ML776 developed by Punjab Agricultural University, Ludhiana, India, having high iron (100.97±2.76 mg/kg) and zinc (34.68±0.93 mg/kg) content, while parent Sattya developed by CCSHAU, containing low iron (38.63±0.69 mg/kg) and zinc (20.92±3.79 mg/kg) concentration.

Estimation of Fe and Zn: Seed harvested from RILs were used for micronutrient analysis. One gram oven dried finely ground powder of seed samples were digested with concentrated di-acid mixture (HNO_3:$HClO_4$; 5:1 v/v) and used for the estimation of iron and zinc contents by Atomic Absorption Spectrophotometer (AAS) ('ZEEnit 700P' Analytik Jena AG, Germany). This experiment was replicated thrice to minimize the experimental error.

Genetic map construction and QTLs detection: Randomly selected 120 RILs covering entire range of micronutrient content were used for identification and mapping of QTLs associated with iron and zinc content. DNA was isolated from the leaf tissues of plants using CTAB method (Saghai-Maroof *et al.,* 1984). The quantity of DNA of each genotype was checked using spectrophotometer and agarose gel electrophoresis using lambda DNA and diluted to 50 nano grams (ng)/ micro litre (µl) final concentrations for further use in Polymerase Chain Reaction (PCR). To carry out polymerase chain reaction (PCR) DNA of 15-20 ng concentrations was used. The known concentration of PCR was carried out at optimized condition with respect to deoxynucleotide triphosphates (dNTPs) (2.5 mM), *Taq* Polymerase (3 units/µl), reverse and forward Primer (10µM), genomic DNA (20 ng/µl).

172 SSR markers were selected from common bean (35 SSR), adzuki bean (120 SSR) and mungbean (17 SSR) for surveying polymorphism between ML776 and Sattya. The RILs were genotyped with 26 polymorphic SSR markers and used to identify QTLs for seed micronutrients (Fe and Zn) accumulation. PCR products from SSR analysis were scored visually for presence or absence of bands; presence scored as (1) and absent scored as (0). Genotypic data were used to construct the linkage map. The linkage map was constructed with MAPMAKER EXP 3.0. Map distances were based on the Kosambi's function (Kosambi, 1994). QTL analysis was done by using Win QTL cartographer version 2.5 by composite interval mapping (CIM). The threshold log likelihood ratio (LOD) score was estimated empirically with 1000 times permutations at a significant level of P = 0.05.

Statistical analysis: The statistical analyses (correlation analysis between traits and test of normality) for phenotypic data were worked out using the SAS 9.3 software (SAS Institute Inc., Cary, USA) while using Microsoft Office Excel 2007 for figures of frequency distribution for Zn and Fe concentration.

RESULTS AND DISCUSSION

Mungbean is an important pulse crop having substantial amounts of low flatulence proteins which makes it indispensable from vegetarian diet consumed by human beings. In mungbean, significant natural variability exists for seed Fe and Zn, making such a goal achievable through plant breeding (Beebe *et al.,* 2000). Nevertheless, there are limited studies on identifying genes and proteins important for iron and zinc regulation in mungbean. The use of QTLs/ molecular markers can reduce the time and cost required to develop new cultivars with improved nutritional content. This research was conducted to target micronutrients (Fe and Zn) content trait for QTL identification for molecular breeding. One of the major prerequisite for initiating a breeding program to develop micronutrient rich genotypes is the identification of donor genotypes with high micronutrient content and bioavailability from available germplasm. A wide range of iron and zinc concentrations was found in mungbean varieties. ML776 had high iron and zinc contents, while Sattya had low iron and low zinc contents making two different sub-groups based on clusters analysis, because high Fe and Zn containing genotypes did not cluster together. Thus these genotypes were used for linkage analysis by developing mapping population to tag QTLs for these micronutrients (Taunk *et al.,* 2012; Aneja *et al.,* 2012). Therefore, a RIL population was developed. The initial cross ML776 x Sattya was advanced to the F_6 generation by single-seed descent method and seed of the RILs was increased for utilization in further studies.

Trait performance and variability for iron and zinc content: Chemical analysis using atomic absorption spectroscopy (AAS) in present study showed that iron content in RILs ranged 9.0-246.1 mg/kg, whereas zinc content varied 19.8-106.1 mg/kg. The maximum iron content was found in RILs no. 104 which had high iron content (246.10 mg/kg), while RILs no. 70 had highest zinc content (106.10 mg/kg) which surpass the parents ML776 and Sattya. The frequency distributions of the iron and zinc showed continuous phenotypic variation in RILs and were not normally distributed with large skewness and kurtosis [Fig. 1 (A, B)]. The't' test for Fe and Zn showed highly significant differences in the RILs. These variability falls within the expected range on the basis of mungbean germplasm screening by earlier researches (Singh *et al.,* 2013; Sarker *et al.,* 2009). Variability in iron and zinc contents in mungbean may due to quantitative inheritance and better uptake (Singh *et al.,* 2013b) and translocation mechanisms of higher iron and zinc in these genotypes, which clearly indicated genetic potential to increase the concentration of these micronutrients (Garcia-Oliveria *et al.,* 2009; Blair *et al.,* 2009; Hacisalihoglu *et al.,* 2004; House *et al.,* 2002; Graham and Welch, 1999; Gregorio *et al.,* 2000).

Genetic map construction and QTLs detection: A powerful forward genetic approach to unravel such polygenic phenomenon is linkage mapping and quantitative trait locus/ loci (QTL) analysis. Linkage analysis is based on the genetic variation which exists between accessions of one plant species and generally performed on genetic segregating populations like F_2, recombinant inbred line (RIL) and DH populations (Koornneef *et al.,* 2004). 26 SSR primers (MBSSRG5, MBSSRG7, Bng95, Bng91, BMd-16, BMd22, CEDG118, PVBR229 , PVBR215, PVBR113, BMd-27, BMd33, BM185, BMd-12, BMd41, BM210, PVBR 269,CEDG279, PVBR82,CEDG248, BM202, BM138, BM184, CEDG008, CEDG111 and BM141) consistently showed polymorphism in the two parents were run on Agarose gel and polyacrylamide gel for genotyping of 120 RILs (Fig. 2).

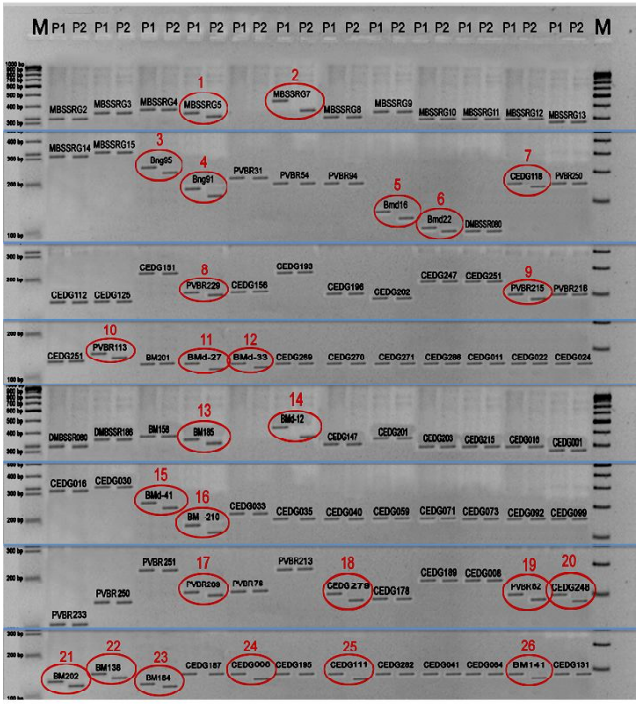

Fig-2: Survey of parental polymorphism between ML776 and Sattya using SSR Primer: 26 out of 172 primers showing polymorphism

Linkage analysis provides knowledge about the genes present in the QTL. It is an unbiased investigation of the genes affecting a particular trait, meaning that genes corresponding to structural as well as regulatory aspects of the process under investigation can be identified.

The genotypic data of all 26 SSR markers was used to construct the linkage map. This map spanned approximately 2919.7cM of genome size (Table 1) and representing four linkage groups; LG 4, LG 6, LG 7 and

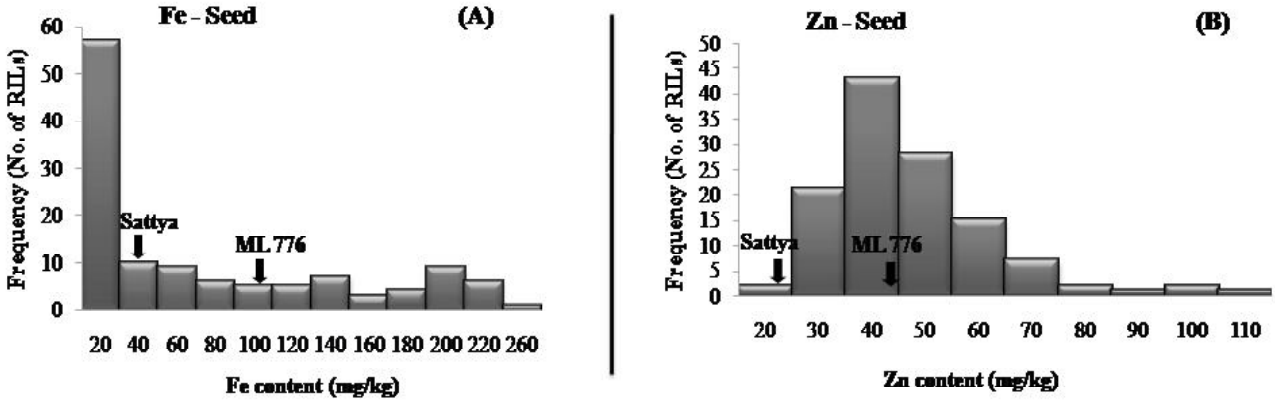

Fig-1: Population frequency distribution for iron (A) and zinc (B) content in seed in ML776 x Sattya RILs of Mungbean

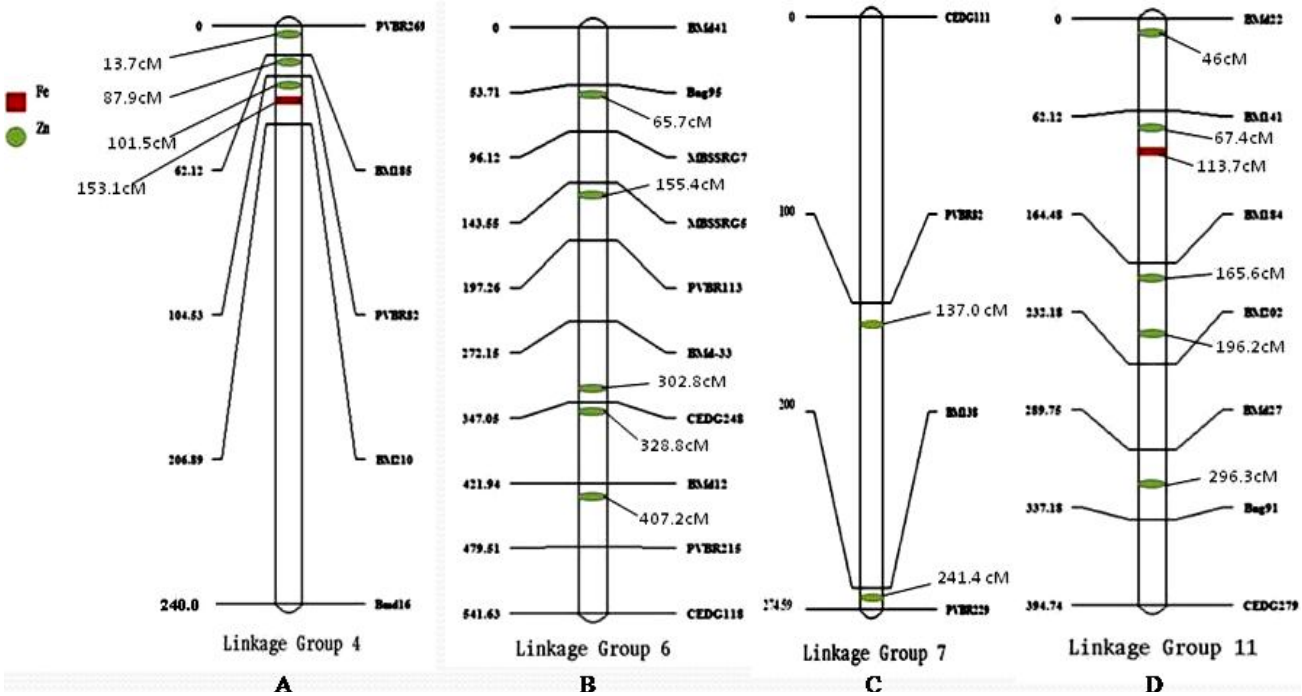

Fig-3: Chromosomal location of QTLs for Fe and Zn concentration identified by microsatellite markers (SSR) analysis: (A) three QTLs for Zn and one QTL for Fe on LG4; (B) five QTLs for Zn on LG6; (C) two QTLs for Zn on LG7; (D) five QTLs for Zn and one QTL for Fe on LG11

LG 11 of mungbean. Five SSR markers, PVBR269, BM185, PVBR82, BM210 and Bmd16 were located on LG 4; 10 SSR markers BMd41, Bng95, MBSSRG7, MBSSRG6, PVBR113, BMd33, CEDG248, BMd12, PVBR215 and CEDG118 were assigned on LG 6; while CEDG111, PVBR82, BM138 and PVBR 229 were located on LG 7; 7 SSR markers BMd22, BM141, BM184, BM202, BMd27, Bng91 and CEDG279 were found on LG 11 [Fig. 3 (A, B, C, D)].

QTL mapping for iron and zinc content in mungbean seeds: Quantitative trait loci analysis will allow gene discovery to move forward for genes underlying natural variation in seed Fe and Zn levels and can open the way for marker assisted selection to breed new varieties of mungbean with higher micronutrient concentration (Ghandilyan *et al.,* 2006). A total of 26 polymorphic SSR markers distributed on four mungbean chromosomes were used to map the QTLs associated with mineral content (iron and zinc) covering entire range of mineral concentration in 120 RILs. Composite Interval Mapping analysis by Win QTL Cartographer 2.5 revealed a total of 17 QTLs for mineral (iron and zinc) content on four linkage groups, LG 4, LG 6, LG 7 and LG 11 in RILs.

Composite interval mapping revealed two QTLs associated with seed iron content in mungbean mapped on chromosome 4 (qFe-4-1 at map position of 153.1 cM) [Fig. 3 (A)] and Chromosome11 (qFe-11-1 at map position of 113.7cM) [Fig.3 (D)]. QTL qFe-11-1 had the maximum additive effect of 7.17 (Table 1). The LOD score compares the likelihood of obtaining the test data if the two loci are indeed linked to the likelihood of observing the same data purely by chance. Positive LOD score favour the presence of linkage, whereas negative LOD scores indicate that linkage is less likely. The estimate with the highest LOD score considered the best estimate which revealed the presence of linkage. In this study, QTL qFe-4-1 (12.4) had maximum LOD values followed by QTL qFe-11-1(11.9).

The QTL qFe-11-1 found on chromosome 11 with an additive effect of 7.17 and accounted for 13% phenotypic variation contributed by ML776 and other QTL qFe-4-1 present on chromosome 4 with an additive effect -7.10 accounted for 21% phenotypic variation, was from Sattya because parent ML776 is identified as the source of the favorable allele *i.e.* higher iron content whereas parent Sattya identified for lower iron and zinc content.

Further, composite interval mapping revealed that three QTLs; qZn-4-1, qZn-4-2 and qZn-4-3 were associated with seed Zn content in mungbean at map distance of 13.7, 87.9 and 101.5 cM, respectively, on chromosome 4 [Fig. 3 (A)], five QTLs; qZn-6-1, qZn-6-2, qZn-6-3, qZn-6-4 and qZn-6-5 at map position of 65.7, 155.4, 302.8, 328.8 and 407.2 cM, respectively on chromosome 6 [Fig. 3 (B)], two QTLs; qZn-7-1 and qZn-7-2 at map position of 137.0 and 241.4cM, respectively on chromosome 7 [Fig. 3 (C)] and

five QTLs; qZn-11-1, qZn-11-2, qZn-11-3, qZn-11-4 and qZn-11-5 at map position of 46.0, 67.4, 165.6, 196.2 and 296.3cM, respectively on chromosome 11 [Fig. 3 (D)]. QTL qZn-7-1 had maximum LOD score of 8.0 showed the best linkage for zinc, followed by QTLs qZn-6-5, qZn-4-1 (7.6); qZn-11-4, qZn-11-5, qZn-7-2 (7.5); qZn-6-4, qZn-4-3 (7.4); qZn-6-3, qZn-11-1 (7.2); qZn-4-2 (7.1), qZn-6-2 (7.0) and qZn-11-2 (6.7) (Table 1).

15 QTLs were identified for mungbean seed zinc content, out of them three QTLs (qZn-4-1, qZn-4-2 and qZn-4-3) on chromosome 4 had additive effect of 2.58, 1.29 and 1.04, respectively accounted phenotypic variation range 11% to 22%; five QTLs (qZn-11-1, qZn-11-2, qZn-11-3, qZn-11-4 and qZn-11-5) on chromosome 11 with an additive effect of 1.64, 1.16, 2.26, 2.89 and 2.38, respectively accounted for 4% to 24% phenotypic variation, and one QTLs (qZn-6-1) on chromosome 6 with an additive effect of 2.14 accounted for 20% phenotypic variation, were from parent ML 776. On the other hand, two QTLs (qZn-7-1 and qZn-7-2) on chromosome 7 with an additive effect of -4.0 and -3.93, respectively, accounted for 14% to 38% of phenotypic variation and four QTLs (qZn-6-2, qZn-6-3, qZn-6-4 and qZn-6-5) on chromosome 6 with an additive effect of -2.66, -2.20, -2.85 and -2.28, respectively, accounted for 13% to 34% phenotypic variation, were contributed by parent Sattya (Table 1).

A notable aspect of this study was that out of two QTLs only one promising QTL (*qFe-11-1*) for iron was located on LG 11 is the most promising QTL for iron because of its high additive effect value (7.17). For zinc content, four promising QTLs (*qZn-11-4, qZn-11-5* on LG 11 and *qZn-4-1, qZn-4-2* on LG 4) were identified with additive effect of 2.89, 2.38, 2.58 and 1.28. These QTLs are placed to a linkage group for the first time on mungbean genetic map. Overlapping QTLs for Fe and Zn concentration were identified on two linkage groups. Earlier researchers also mapped QTLs for micronutrients in different crops; in common bean (Blair *et al.,* 2009; Blair *et al.,* 2010; Blair and Izquierdo, 2012), maize (Simic *et al.,* 2012), rice, (Anuradha *et al.,* 2012) and wheat (Tiwari *et al.,* 2009).

The SSR marker PVBR82 associated with QTLs for both high iron as well as high zinc content which could be an important marker for considering in MAS for biofortification, as being consistent in QTLs for both micronutrients. These values fall within the expected range of previously linkage map developed for iron and zinc content in different legumes using molecular markers by earlier researchers (Blair *et al.,* 2003; Blair *et al.,* 2006b; Blair *et al.,* 2008; Yu *et al.,* 2000).

The correlations and co-localizations for iron and zinc content: The correlation coefficient (r) between iron and zinc was highly consistent (0.16) but less than 0.50 (Fig. 4). Therefore, iron did not significantly correlate to zinc and was not significant from the biological point of view. In earlier studies on micronutrients in different crops, positive correlations between Zn and Fe concentration have been reported by Beebe *et al.,* (2000), Cakmak *et al.,* (2004), Gelin *et al.,* (2007), Stangoulis *et al.,* (2007) and Blair *et al.,* (2009). In the present study, positive correlations were observed between Zn and Fe concentration in mungbean seed with small correlation coefficient (less than 0.50). The weak relationship between iron and zinc concentration may be caused by some QTL co-localized (Stangoulis *et al.,* 2007; Tiwari *et al.,* 2009). There was also another possibility that

Table 1: Quantitative trait loci (QTL) associated with iron and zinc concentration

Seed trait	Linkage group	QTL	Position cM	LOD Score	Intervalmarkers	Additivity*	R^{2}** (%)
Fe	**LG11**	**qFe-11-1**	**113.7**	**11.9**	**BM141-BM184**	**7.17**	**13.0**
	LG4	**qFe-4-1**	**153.1**	**12.4**	**PVBR82-BM210**	**-7.10**	**21.0**
Zn	LG6	qZn-6-1	65.7	7.7	Bng95-MBSSRG7	2.14	20.0
		qZn-6-2	155.4	7.0	MBSSRG5-PVBR113	-2.66	23.0
		qZn-6-3	302.8	7.2	BMd33-CEDG248	-2.20	21.0
		qZn-6-4	328.8	7.4	CEDG248-BMd12	-2.85	34.0
		qZn-6-5	407.2	7.6	BMd12-PVBR215	-2.28	13.0
	LG11	qZn-11-1	46.0	7.2	BMd22-BM141	1.64	17.0
		qZn-11-2	**67.4**	**6.7**	**BM141-BM184**	**1.16**	**4.0**
		qZn-11-3	165.6	7.1	BM184-BM202	2.26	24.0
		qZn-11-4	196.2	7.5		2.89	22.0
		qZn-11-5	296.3	7.5	BMd27-Bng91	2.38	10.0
	LG7	qZn-7-1	137.0	8.0	PVBR82-BM138	-4.08	11.0
		qZn-7-2	241.4	7.5	BM138-PVBR229	-3.93	13.0
	LG4	qZn-4-1	13.7	7.6	PVBR269-BM185	2.58	11.0
		qZn-4-2	87.9	7.1	BM185-PVBR82	1.29	22.0
		qZn-4-3	**101.5**	**7.4**	**PVBR82-BM210**	**1.04**	**19.0**

**Effects of substituting a single allele from one parent to another. Positive values indicate that allelic contribution is from ML776 and negative from Sattya. **Percent Phenotypic variance explained by QTL.*

QTL were closely linked to each other. Although Zn or Fe concentration did not show biologically significant correlation. Total four QTLs for Zn or Fe concentration were located on the same chromosomal regions such as qZn-4-3 (101.5 cM) and qFe-4-1(153.1 cM) on chromosome 4 between PVBR82-BM210 markers; qZn-11-2 (67.4 cM) and qFe-11-1(113.7 cM) on chromosome 11 between BM141-BM184 markers. Most striking was the co-localization of QTL for Zn and Fe on chromosome 4 and 11. These co-localized QTLs probably, were closely linked to each other or were the same pleiotropic genomic region (Clemens, 2001). This inconsistency indicated that minerals accumulation in mungbean was very complex. Therefore, the correlations and their genetic mechanism between iron and zinc concentration need to be further studied.

Cross transferability of SSR markers from other related legumes into mungbean Genome: Limited availability of polymorphic DNA markers has been a major bottleneck in crop genomics. Therefore, developing and/or identifying polymorphic SSR motif of mungbean is the most important requirement for its improvement using genomic tools. The critical evaluation of sequence data from different crops indicates the existence of adequate homology between genomes in regions flanking the SSR loci. Hence, the primer pair designed for one species could be used to detect SSRs in related species. Weeden *et al.,* (1992) was the first researcher to report genetic relatedness in lentil and pea using RFLP markers. Their study revealed that 40% of the linked loci in the lentil linkage map were conserved in pea (*Pisum sativum*). Since then, many workers have reported transferability of sequence tagged microsatellite primers across major pulse crops. The transferability of SSRs due to homology of flanking regions between closely related species may reduce costs, avoiding the laborious cloning procedures involved in their development. Recent research suggests that successful cross-species amplification in plants is largely restricted to closely related genera. This also allows for the comparative map construction and molecular analysis of crop species which lack sufficient DNA markers.

In present study a total of 172 SSR markers from different legumes (17 SSR primers from mungbean, 35 SSR markers from common bean and 127 SSR primers from adzuki bean) were used. These SSR markers were analyzed for polymorphism in two parental genotypes (ML776 and Sattya). Based on polymorphism, the transferability percentage was 83.33% of adzuki bean SSRs and 77.14 % of common bean SSRs in mungbean. The interspecific cross transferability of *Phaseolus vulgaris, Pisum sativum, Vigna radiate, Medicago sativus, Medicago truncatula,* adzuki bean and common bean SSRs across the genotypes were successfully utilized in amplifying microsatellite sequences in *Vigna* species for inferring phylogenic relationships by earlier researchers (Aubert *et al.,* 2006; Phan *et al.,* 2007;

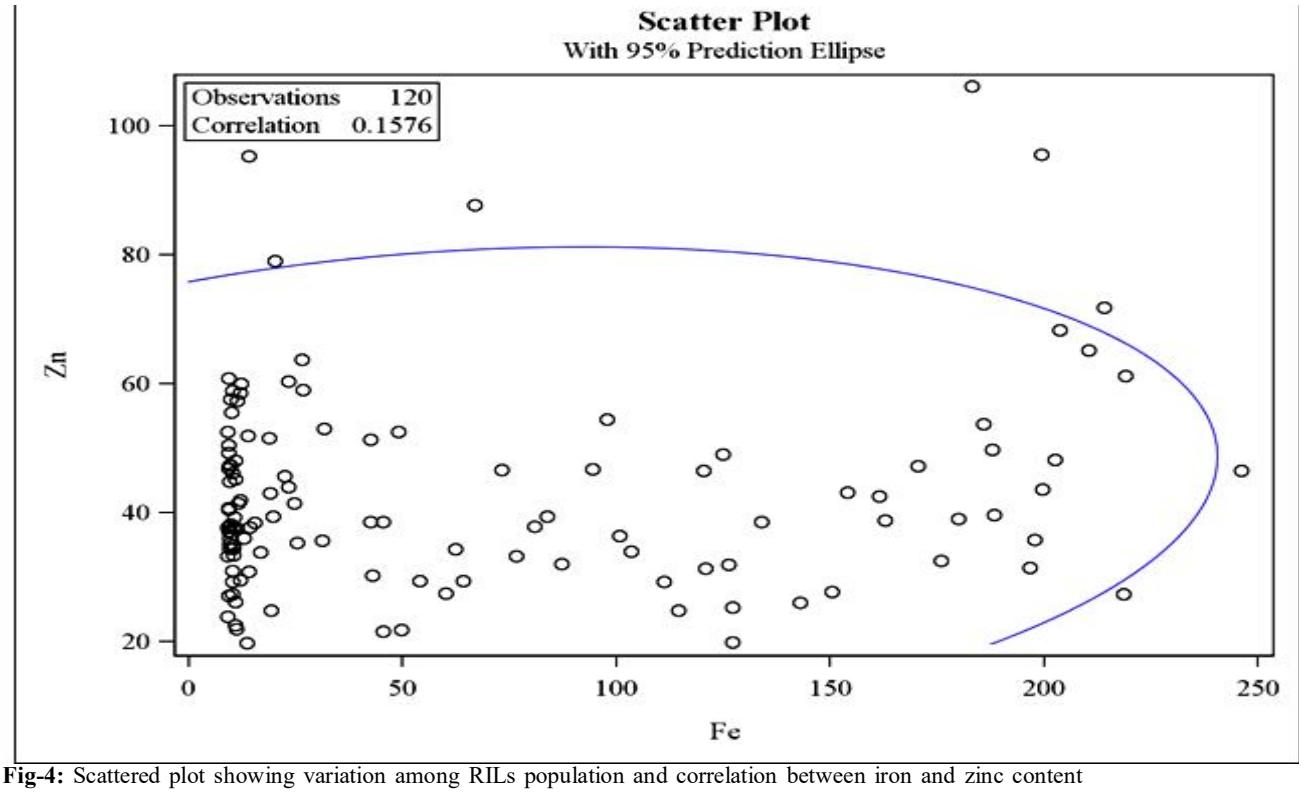

Fig-4: Scattered plot showing variation among RILs population and correlation between iron and zinc content

Choudhary *et al.,* 2008; Dikshit *et al.,* 2012; Wang *et al.,* 2009; Pandian *et al.,* 2000; Roorkiwal and Sharma, 2011).

CONCLUSION

The results from this research were interesting and novel. The positive correlation between Fe and Zn possibly, the genomic regions qZn-4-3 and qFe-4-1 on chromosome 4 between PVBR82-BM210 markers; qZn-11-2 and qFe-11-1 on chromosome11 between BM141-BM184 markers, were co-located on the same chromosomal regions for Zn or Fe concentration could be ideal candidates for further fine mapping and gene identification. The SSR markers associated with these QTLs for both high iron and zinc content could also be considered in marker assisted breeding for biofortification in mungbean. The genomic regions which were detected to be associated with high iron and zinc could be candidate for further fine mapping, gene identification and MAS in breeding. Thus, improved mungbean varieties developed with high iron and zinc content will also alleviate malnutrition and improve the nutritional status of Indian vegetarian population.

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