Research Article

Mapping Micronutrients using Recombinant Inbred Lines (Rils) in Mungbean (*Vigna Radiata* L.)

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Abstract

Malnutrition, in the developing world is an emergency that needs immediate attention. One of the methods to mitigate micro-nutrient deficiencies is to develop food crop cultivars with higher levels of essential micro-nutrients. As such, the food legumes are of major nutritional importance, especially in developing countries, because they have high protein contents with biological values. Mungbean is one of the major food legumes consumed by people in India sub-continent. As an initial step, available mungbean genotypes were assessed for genetic diversity for iron, zinc and protein contents. Marker analysis shows moderate genetic diversity ranging from 65 to 87 %. Chemical analysis showed a fair range of micro-nutrient and protein variation (Fe content ranging from 1.6 to 9.3 mg/100g; Zn content ranging from 1.5 to 3.9 mg/100g and total protein content ranging from21.1 to 30 %). Fe and Zn content showed a positive correlation (r= 0.469) along with fair heritability values (h^2 = 0.259 for Fe and 0.727 for (Zn). The existing genetic diversity provides an opportunity to make indirect selection for both the traits.

Keywords: Amplified fragment length polymorphism (AFLP), Iron, micronutrients, mungbean, recombinant inbred lines (RILs), Zinc

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Introduction

Mungbean (*Vigna radiata* L.) also called green gram is one of the principal legumes and is a very nutritive crop. The seeds are used for human consumption, the plant as fodder for livestock and green manure. The seeds contain 25 to 28% protein, 1.0 to 1.5 % fats, 3.5 to 4.5% fibre, 60 to 65% carbohydrates and are rich in lysine, ascorbic acid, potassium, iron, phosphorus and calcium. Moreover, its seeds are more palatable, nutritive and digestible and non-flatulent in comparison to other pulses [1]. Thus, mungbean is nature's gift to man in general and to children, pregnant or lactating women and especially to the elderly people. It thus has all the potential to be used as an economic food supplement to fight malnutrition. India is the largest producer and consumer of pulses in the world. In India, mungbean is grown on an area of 2.92 million ha, with a production of 1.42 million ton i.e. 486 kg/ha [2]. As it is one of the major sources of protein and mineral micronutrients it is often traded and consumed locally. Micronutrient malnutrition, and particularly Fe and Zn deficiencies (the so called 'hidden hunger'), affects over three billion people worldwide, mostly in developing [3]. Fortification of food is one step in combating these deficiencies but this is not always possible, for instance adding iron doesn't result in a stable product and makes the product unpalatable. To increase the concentration in the edible portions of crop plants it is necessary to incorporate micronutrient content in breeding programs.

Mungbean germplasm screening revealed genetic variation for the content of iron and zinc (ranging from 1.6–9,2 mg/100 g Fe and 1,5- 3,9 mg/100 mg Zn respectively. Iron and zinc concentrations in the seeds tend to be correlated with each other (r = 0.47) [4], making it possible to screen for high concentrations of both. The content is substantially influenced by genotype (G) x environment (E) interactions. Recombinant Inbred Lines (RILs) make it possible to screen for high iron and zinc concentrations under different conditions and make it possible to screen for associations between DNA markers and high concentrations (Quantitative Trait Loci). These markers later on can be used in marker assisted selection (MAS) and in this way make the breeding more efficient. Mineral accumulation in higher plants appears to be under control of many genes. In *Arabidopsis thaliana* seed mineral accumulation was found to be quantitative and associated with various candidate genes and 21 genes involved in ion homeostasis [5].

As mungbean is primarily used as a food, extensive research is available on seed quality traits such as size, shape, colour, hard-seededness, protein quality and quantity by Lambrides and Godwin [6] along with agronomic traits like drought resistance by Sholihin and Hautea [7], virus resistance (notably MYMV) by Anjum [8]. For micronutrient

content several studies were conducted in common bean, peas, chickpeas, lentils etc. but till this date, not much effort has been made to locate genes/QTLs responsible for micronutrients in mungbean [9- 12]. The present study was conducted (a) to choose the best parents to create mapping populations (recombinant inbred lines - RILs), (b) to determine the level of iron and zinc in the individuals of these RIL populations and (c) to analyse the segregation patterns of iron and zinc concentration in these RILs.

Experimental Results

The present studies were carried out at the Department of Biotechnology, CCS Haryana Agricultural University, India and Laboratory of Plant breeding, Wageningen University, Netherlands.

Plant materials

120 Recombinant inbred lines (F_6 RILs) obtained from a cross between BG 39 X 2KM 138 and 120 recombinant inbred lines from SMH 99-1 X BDYR1 were made through single seed descent (SSD) at the pulses research field, CCS HAU, Hisar (India). The parents used in both the crosses were contrasting in their micronutrient (Fe & Zn) content and agronomic characters [13].

Experimental design & mineral analysis

120 recombinant inbred lines (RILs) of both crosses were sown in a randomized block design (RBD). Each plant was threshed separately in a bag and used for chemical analysis. For both the crosses 210 RILs i.e. 85 (for cross 1) and 120 (cross 2) respectively were analyzed. Phenotyping for Fe and Zn was done using atomic absorption (AAS) analysis and was based on nitric/per-chloric acid digestion. With sample read on atomic absorption (Perkin Elmer Analysts 400 atomic absorption spectrophotometer) in the Shree Balaji Test Lab (SBTL).

Statistical analysis: Phenotypic/Chemical data

The phenotypic data obtained for iron and zinc content was analysed using t test ($t = \bar{X} - \mu / s/\sqrt{n}$). Where, s= standard deviation of the sample, n= number of observations, $\bar{X}$ = sample mean & μ = parent mean. Mean and range among the RILs in comparison with parents and correlation coefficients (r) among these traits were also estimated using the pooled data over environments (PE). The computation for the data was performed using the software package SAS.

Molecular marker analysis

Young leaves from 3 - 5 weeks-old seedlings were collected and immediately stored at -80^{o}C. The DNA isolation and molecular work was carried out at Wageningen-UR Plant Breeding, the Netherlands. Total genomic DNA was extracted using 96 well plate automated DNA isolation machine (according to Thermo Scientific™ KingFisher™ purification systems manual). The Li-Cor AFLP Kit was used according to the recommendations of the manufacturer (Invitrogen). 100 ng DNA was digested with restriction enzymes *Eco*RI and *Mse*I and enzyme adapters were ligated to the digested DNA. The selective amplification of restriction fragments was done with colour labelled primers with in total six selective nucleotides (**Table 1**). After selective amplification the total mixture was carefully mixed and heated for 5 minutes at 94^{o}C in a hot-block and then quickly cooled on ice. From the 10 µl, 8 µl was loaded on a 6% denaturing polyacrylamide gel 1X TBE buffer. Li-Cor 4300 S DNA analyser.

Marker-Trait associations

The software programs JoinMap® 4.1 were used to calculate linkage between markers [14].

Results and Discussions

Phenotypic data analysis

The values of the individual lines in the RIL population in cross 1 and cross 2 is provided in **Table 2**. A significant positive correlation was found between iron and zinc concentrations (r = 0.47). Frequency distribution of the pooled data for both the traits revealed in several cases transgressive segregation both on the negative and positive side (Table 2). A distribution in classes of the Fe and Zn content in both crosses are shown in **Figure 1**. The results showed a 2-3 fold difference in concentrations between individual lines and also some transgressive segregation

(values higher than the values of the parent with the highest value). In general the results showed that also in mungbean it is possible to breed for new cultivars with high micronutrient content. Studies in other pulses have shown similar results [15].

Table 1 Primer combinations used to screen for the highest level of polymorphisms

Eco RI		*MseI*	
Primers +0		*Primers +0*	
5' – GACTGCGTACCAATTCNNN-3'		*5'-GATGAGTCCTGAGTAANNN-3'*	
Primers +1	*A-3*	*Primers +1*	
E01	*A-3*	*M02*	*C-3*
Primers +3			
E31	*AAA-3*	*M47*	*CAA-3*
E32	*AAC-3*	*M48*	*CAC-3*
E35	*ACA-3*	*M49*	*CAG-3*
E36	*ACC-3*	*M50*	*CAT-3*
E37	*ACG-3*	*M51*	*CCA-3*
E40	*AGC-3*	*M52*	*CCC-3*
E45	*ATG-3*	*M53*	*CCG-3*
		M54	*CCT-3*
		M55	*CGA-3*
		M56	*CGC-3*
		M57	*CGG-3*
		M58	*CGT-3*
		M59	*CTA-3*
		M60	*CTC-3*
		M61	*CTG-3*
		M62	*CTT-3*

Table 2 Mean, range, standard deviation and correlation coefficients for iron and zinc in mungbean (*Vigna radiata* L).

Traits	**Parents Mean***	**Recombinant inbred lines**		
		Mean	**Range**	**St. Deviation**
Cross I- Fe	4.2 (6.0 and 2.4)	3.2	1.7 – 5.9	0.56
Cross I- Zn	2.3(2.6 and 2.0)	2.9	2.0 – 3.8	0.32
Cross II- Fe	4.3(3.9 and 4.5)	4.1	2.1 – 8.2	1.05
Cross II- Zn	2.8(3.1 and 2.5)	2.9	1.0 – 8.4	0.78
		Pearson's Correlation Coefficient**		
		Fe	**Zn**	
Fe		1.000	0.038	
		0.0	0.474	

* Between brackets the values of the two parents
**overall correlation between iron and zinc

Our results showed a positive correlation in mungbean between Fe and Zn content. This makes it possible to breed for new varieties high in both; similar observations have been reported in literature [16]. The uptake of both might be under control of similar mechanisms. To allow efficient breeding for good mungbean cultivars with high levels of Fe and Zn it is important that the tools such as developed molecular markers and contrasting parents are available that allow this.

DNA polymorphism

Level of polymorphism determined by AFLP analysis

In order to choose the enzyme primer combinations with the highest level of polymorphisms the four parents were subjected to in total 96 different enzyme primer combinations. **Table 3** shows the average number of polymorphisms for the parents of cross 1 was 4.4, this was somewhat lower between the parents of cross 2 (3.7 polymorphisms per combination).

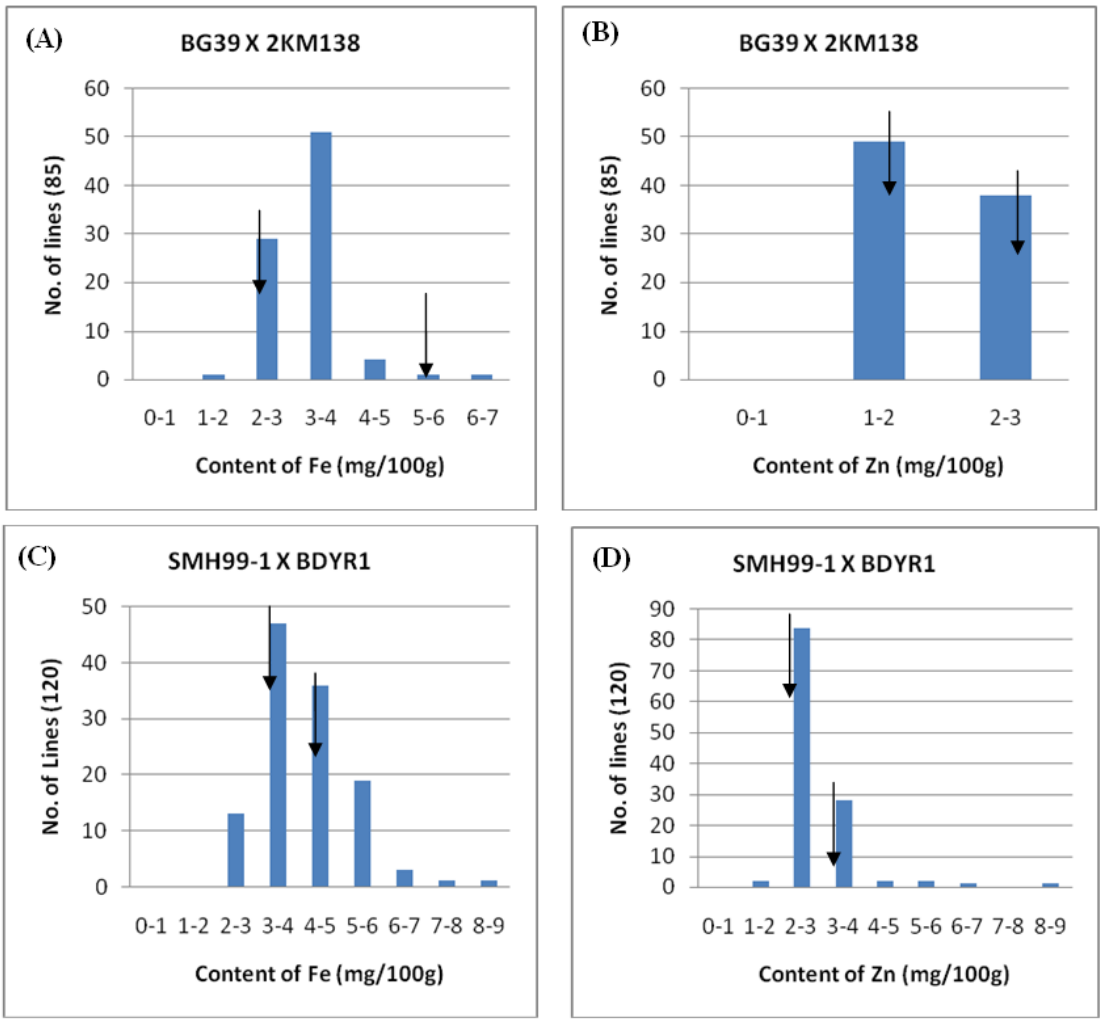

Figure 1 A. Distribution of Fe content in RIL population in cross 1; B. Distribution of Zn content in RIL population of cross 1; C. Distribution of Fe content in RIL population of cross 2; D. Distribution of Zn content in RIL population cross 2; Arrow represents the position of parental level in each cross.

Table 3 The number of polymorphisms between the parents of both crosses with different primer combinations

		M47	M48	M49	M50	M51	M52	M53	M54	M55	M56	M57	M58	M59	M60	M61	M62	
Pop 1	E32	8	6	1	8	6	3	7	6	1	4	1	2	3	4	x	x	60
Pop 1	E35	5	5	4	6	5	3	2	6	1	4	2	6	7	5	2	9	72
Pop 1	E36	4	7	4	9	8	5	8	3	4	4	3	5	4	6	4	7	85
Pop 1	E37	1	4	2	6	3	9	4	9	3	6	1	2	6	4	2	10	72
Pop 1	E40	7	7	1	7	7	3	5	4	1	2	2	4	6	0	6	8	70
Pop 1	E45	6	9	2	5	5	8	2	2	1	1	0	3	2	4	6	2	58
		31	38	14	29	34	31	28	30	11	21	9	22	28	23	20	36	417
Pop 2	E32	4	2	1	6	3	3	8	2	4	0	4	4	4	4	1	x	50
Pop 2	E35	6	5	0	5	2	2	4	8	3	2	4	1	4	2	3	8	59
Pop 2	E36	3	4	5	7	10	4	6	5	3	5	2	7	2	4	3	3	73
Pop 2	E37	4	2	5	7	4	7	3	8	3	3	3	2	3	4	3	8	69
Pop 2	E40	3	4	2	2	4	3	5	4	1	2	2	5	4	2	4	7	54
Pop 2	E45	3	7	4	9	4	5	3	1	1	2	2	0	1	1	3	4	50
		23	24	17	36	27	24	29	28	15	14	17	19	18	17	17	30	355

Screening RIL population of cross 2 for AFLPs

Analysing of the RIL population of cross 2 with the primer combinations E32M47 (8 polymorphisms), E32M48 (6 polymorphisms), E32M51 (6 polymorphisms), E32M53 (7 polymorphisms), E35M48 (5 polymorphisms), E35M51 (5 polymorphisms) and E40M47 (7 polymorphisms) was carried out. The choice of enzyme primer combinations was based on the level of polymorphisms and the quality of the overall AFLP pattern. Of the expected 44 polymorphisms

only 31 could be scored over the whole population. The other 13 were not clear enough for reliable scoring. This showed that only about four markers per combination were usable.

Mapping studies

The 31 segregating markers were used to calculate linkages between markers. Mungbean is diploid (2n=2x=22) and has a small genome size i.e. 0.60 pg/1C (579 Mbp) [17]. With the software package JoinMap® 4.1 seven linkage groups were found and six unlinked markers. Most linkage groups were small (2 markers in 2 cM, 3 markers in 3 cM, 3 markers in 17 cM, 2 markers in 16 cM and 2 markers in 10 cM) but a few were formed by more markers (9 markers in 61 cM and 4 markers in 25 cM). Fourteen markers had a significant skewed segregation, four towards parent 1 and ten towards parent 2 (**Figure 2**).

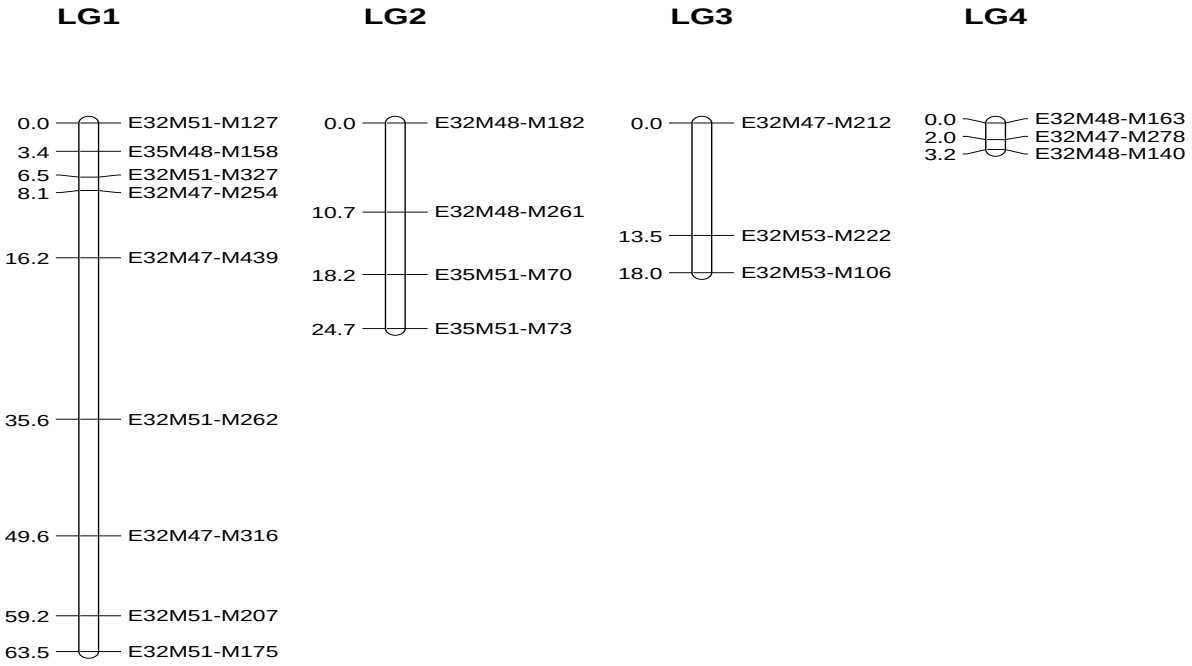

Figure 2 Four Linkage Groups with more than two markers based on the RIL population of cross 2

Conclusion

To allow efficient breeding for good mungbean cultivars with high levels of Fe and Zn it is important that the tools such as developed molecular markers and contrasting parents are available that allow this. Since over 20 years marker assisted selection has been such a tool in many crops [18]. Two steps are of importance: identification of marker trait associations and later using this information in molecular breeding programs. The molecular marker technology has been changing rapidly the last few years; due to rapid changes in efficiency of sequence technology it is possible to find enough polymorphisms. From the present study few scorable markers were generated. These markers could be use for generating high density linkage maps making it possible to look for marker trait associations. We hope that in the near future our mapping populations will be of use in combination with modern sequence based marker technology such as described in by Viquez Zamora [19]. Highly likely genotyping by sequencing will also be an affordable option in the near future. These methods will highly probable lead to markers linked to the genes playing a role in micronutrient levels in mungbean. This will give mungbean a role in solving the problem of the hidden hunger.

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