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GENETIC LINKAGE EFFECT ON INHERITANCE OF NODULATION AND LEAF COLOR IN CHICKPEA (*Cicer arietinum* L.)

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SUMMARY

Mode of inheritance of nodulation and leaf color are the two important morphological markers, thus in this study their relationship was investigated in chickpea (*Cicer arietinum* L) at The University of Agriculture, Peshawar. The study was carried out via parents, F_1, F_2 and backcross populations of two cross combinations i.e. ICC 19181 (Nod^- and dark green leaves) × NDC 5-S10 (Nod^+ with light green leaves) and ICC 19181 × NDC 4-20-4 (Nod^+ and light green leaves) designated as "Hybrid A" and "Hybrid B", respectively. Hybrid A showed dominant monogenic (F_2 = 3:1, $x^2 = 0.45$) inheritance while hybrid B revealed duplicate gene action (F_2 = 15:1, $x^2 = 2.55$) for nodulation. Both hybrids, revealed single gene dominant inheritance (F_2 = 3:1) for light green leaf color. In F_1 population, the nodulation (Nod^+) and light green leaf color were dominant over non-nodulation (Nod^-) and dark green leaf color. In F_2 populations, the segregation diverted from the normal ratio (9:3:3:1), and revealed an indication of linkage between these loci. Recombination frequency between loci RN (root nodule) and L (leaf color) was recorded as 0.1575 $\pm$0.0928 and 0.264 $\pm$0.084 in genotype NDC 5-S10 and NDC 4-20-4, respectively. Results revealed that the loci of nodulation and leaf color are residing on the same chromosome in these selected genotypes (NDC 5-S10 and NDC 4-20-4) of chickpea.

Keywords: Chickpea, inheritance, linkage, nodulation, leaf color

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INTRODUCTION

Although chickpea requires less quantity of nitrogenous fertilizer however, low availability of nitrogen is responsible for limited growth of agricultural crops. Synthetic fertilizers like ammonia, nitrate, or urea met at least 50% of the global requirement. Among plant nutrients nitrogen plays an important role in crop productivity and is regarded as growth and yield determinant. With continuous cropping system the nitrogen supplied from decomposition of organic matter must be supplemented from other sources. In developing countries like Pakistan, it is not possible due to high cost of chemical fertilizers. Therefore we have to rely on exploitation of biological nitrogen fixation (BNF) in legumes, which is one of the most important sources of nitrogen for agriculture (Kumar *et al.*, 2007).

Chickpea is one of the world's most important but less-studied leguminous food crop. It has great importance as food, feed and fodder. It provides a protein-rich supplement to cereal-based diets (Talebi *et al.*, 2008). Apart from providing dietary benefits to human beings, chickpea is very useful in the management of soil fertility due to its nitrogen fixation ability (Erler *et al.*, 2009). Chickpea forms symbiotic relationship with rhizobia that results in root nodules where atmospheric nitrogen is fixed and used by the plant. For breeding high yielding genotypes in legume crops, proper consideration must be given to genotypes showing high nitrogen fixation ability.

Selection of plants with desirable characters is a basic criterion in plant breeding. However, problems arose during selection of traits (like nodulation) through evaluation of genotypes and should be selected indirectly. The difficulty might be overcome by identification of markers closely linked to the desired traits, then indirect selection becomes possible, if location of the desired gene in relation to other gene is known. Major component of characterization of crops is the evaluation of gene linkage relationship among agronomically important genes. Morphological markers play a pivotal role in selection of desirable traits in plant breeding programs. Monogenic markers are useful in estimating the rate of out-crossing in predominantly self-pollinating crops. They also help in identification of F_1 hybrids in the breeding programs (Muhammad *et al*., 2010).

Due to importance of biological nitrogen fixation, breeding for an efficient nodulation in legume crops is important.

Information on the inheritance of nodulation would facilitate to incorporate this important character in a high yielding and well adapted genotype. Moreover, nodulation can't be observed or evaluated under nitrogen rich field conditions, until the plants are uprooted, this problem can be solved by identification of marker traits linked to nodulation. The present study was thus designed to study the mode of inheritance of nodulation and leaf color in chickpea, and to identify linkage among loci controlling these two important morphological markers.

MATERIALS AND METHODS

The study was performed by using chickpea genotypes ICC19181 (non-nodulating with dark green leaf color), NDC 5-S10 (nodulating with light green leaf color) and NDC 4-20-4 (nodulating with light green leaf color) which were hybridized during the crop season 2007-2008 at The University of Agriculture, Peshawar, Pakistan. The crosses were grown during 2008-2009 in the field of Nuclear Institute for Food and Agriculture (NIFA), Peshawar to get F_1 and backcross seed as per Table 1.

Seed from the F_1 plants (including backcross seeds as well as F_2 seeds) were collected on individual plant basis.

Table 1. Hybrid and backcross populations used in study

No.	Cross combination	Cross designation
1	ICC19181 ×NDC 5-S-10	Hybrid A (F_{11})
2	ICC19181 × NDC 4-20-4	Hybrid B (F_{12})
3	(ICC19181 × NDC 5-S-10) × ICC19181	$BC_{11}(P_1)$
4	(ICC19181 × NDC 5-S-10) × NDC 5-S-10	$BC_{12}(P_2)$
5	(ICC19181 × NDC 4-20-4) × ICC19181	BC_{13} (P_1)
6	(ICC19181 × NDC 4-20-4) × NDC 4-20-4	BC_{14} (P_2)

During 2009, the parents, F_1, F_2 and backcross populations were evaluated for presence/absence of nodules and leaf color in a green house. Data were recorded on 120 plants of parents (40 plants parent^{-1}) in combination with 27 plants of F_1, 109 F_2 and 132 backcross plants 84 plants of F_1 BC_1 (P_1), 48 of F_1 BC_1 (P_2) of cross ICC19181 × NDC 5-S10 while for second cross (ICC 19181 × NDC 4-20-4) 30 F_1 plants, 117 F_2 plants and 138 plants of backcross population (79 plants of F_1 BC_1 (P_1), 59 of F_1 BC_1 (P_2). Three seeds were sown in each pot [22-cm-diameter pots filled with 4.5 KG soil (50% sand + 50% clay)]. Seed were inoculated at the time of sowing with *Rhizobium leguminosarum* strain. Plants were assessed after eight weeks of planting for presence or absence of nodules and leaf color (light green/dark green).

In order to collect data on nodules, plants along with soil were removed from pots and washed with tap water three times.

The Chi-square (χ^2) test using Yates correction factor to adjust small population size was used to test the goodness of fit for appropriate genetic hypotheses (Steel *et al.*, 1980). Data on leaf color and presence / absence of nodules in F_2 and backcross populations were tested based on the hypothesis that it will fit to the expected ratios of 3:1, 1:1, respectively. The test statistics was calculated as described by Yates (1934). The leaf color and nodulation data in F_1 and F_2 generations were analyzed through Linkage 1 computer program (Sulter *et al.*, 1983).

RESULTS

Inheritance of nodulation

Results revealed that F_1 hybrid A (ICC 19181 × NDC 5-S10) and F_1 hybrid B (ICC19181 × NDC 4-20-4) produced normal nodules (Tables 2 & 3) indicating dominant gene effect for nodulation in chickpea. After segregation in F_2 population the nodulated and non-nodulated phenotypic ratio was 3:1 in hybrid A indicated monogenic inheritance for nodulation. Distribution of nodulated and non-nodulated phenotypes in the backcross progenies further confirmed the results which showed a good fit as 1 Nod^+: 1 Nod^- the same as expected for single gene inheritance. The chi-square values for F_2 data of cross ICC 19181 × NDC 5-S10 has probability between 68 to 70%, which authenticate the presence of single dominant gene for nodulation or monogenic inheritance in genotype NDC 5-S10.

After F_2 segregation in hybrid B, the nodulation fit well to the ratio of 15 nodulated: 1 non-nodulated (Table 4). The said segregation pattern was supported by backcross progenies in which the ratio for nodulated and non-nodulated phenotypes also showed a good fit to the expected ratio of 3 Nod^+: 1 Nod^- (Table 3). Thus, hybrid B showed that nodulation in genotype NDC 4-20-4 was controlled by two alleles at two independent loci with dominant gene action. Normal nodules production in F_1s, and segregation of backcross and F_2 progenies for nodulated and non-nodulated phenotypes, confirmed that nodulation in genotype NDC 4-20-4 was controlled by duplicate dominant genes. The probability of chi-square values for F_2 population of cross ICC19181 × NDC 4-20-4 was between 15 to 20% which also supports the duplicate gene action of nodulation in genotype NDC 4-20-4.

Inheritance of leaf color

F_1 population of both the crosses (ICC19181 × NDC 5-S10 and ICC19181 × NDC 4-20-4) showed light green color (Table 1). The F_2 populations segregated for leaf color as light green and dark green. Segregating ratios of both F_2 populations were in good agreement with a Mendelian dominant genetic model and fit to 3:1 ratio (Tables 5, 6). The backcross progenies of both crosses also verified the result of F_2 segregating population by showing a good fit to the ratio of 1 light green: 1 dark green. Thus, it may be concluded that leaf color in chickpea is controlled by a single dominant gene. F_2 data of both crosses showed high level of probability (50%) for Chi-square values which confirmed the monogenic inheritance of leaf color in chickpea.

Table 2. Morphological markers of parents and their F_1s used in linkage studies of chickpea.

Parents/Hybrids	Leaf Color	Nodulation
ICC 19181 (P_1)	L	rn
NDC 5-S10 (P_2)	L	RN
NDC 4-20-4 (P_3)	L	RN
ICC 19181 × NDC 5-S10 (F_1)	L	RN
ICC 19181 × NDC 4-20-4 (F_1)	L	RN

L = Light green; l = Dark green; RN + Nodulated; rn = Non-nodulated

Table 3. Nodulation response of non-nodulated (Nod-) and nodulated (Nod+) parents, $F_{1,}$ F_2, and backcross progenies of ICC 19181 × NDC 5-S10 cross to rhizobial inoculation.

Populations	Parent / Hybrid	Plants		Expected ratio	χ^2	<P <
		Nod^+	Nod^-			
Parent (P_1)	(ICC 19181)	0	40	-		
Parent (P_2)	(NDC 5-S10)	40	0	-		
F_1	($P_1 \times P_2$)	27	0	-		
F_2	($P_1 \times P_2$)	84	25	3:1	0.14	0.70-0.68
BC_1 (P_1)	($F_1 \times P_1$)	45	39	1:1	0.29	0.70-0.50
BC_1 (P_2)	($F_1 \times P_2$)	48	0	-		

Table 4. Nodulation response of non-nodulated (Nod-) and nodulated (Nod+) parents, F_1, F_2, and backcross progenies of ICC19181 × NDC 4-20-4 cross to rhizobial inoculation.

Populations	Parent / Hybrids	Plants		Expected ratio	χ^2	<P <
		Nod^+	Nod^-			
Parent (P_1)	(ICC 19181)	0	40	-		
Parent (P_3)	(NDC 4-20-4)	40	0	-		
F_1	($P_1 \times P_3$)	30	0	-		
F_2	($P_1 \times P_3$)	105	12	15:1	2.55	0.20-0.15
BC_1 (P_1)	($F_1 \times P_1$)	53	26	3:1	2.22	0.20-0.15
BC_1 (P_3)	($F_1 \times P_3$)	59	0	-		

Table 5. Inheritance of leaf color in light green and dark green leaf colored parents, $F_{1,}$ F_2, and backcross progenies of cross ICC 19181 × NDC 5-S10.

Populations	Parent/Hybrids	Plants		Expected ratio	χ^2	<P <
		Light green	Dark green			
Parent (P_1)	(ICC 19181)	0	40	-		
Parent (P_2)	(NDC 5-S10)	40	0	-		
F_1	($P_1 \times P_2$)	27	0	-		
F_2	($P_1 \times P_2$)	78	31	3:1	0.49	0.50
BC_1 (P_1)	($F_1 \times P_1$)	47	37	1:1	0.96	0.50 – 0.30
BC_1 (P_2)	($F_1 \times P_2$)	48	0	-		

Table 6. Inheritance of leaf color in light green and dark green leaf colored parents, F_1 F_2, F_2, and backcross progenies of ICC19181 × NDC 4-20-4 cross.

Populations	Parent / Hybrid	Plants		Expected ratio	χ^2	<P <
		Light green	Dark green			
Parent (P_1)	(ICC 19181)	0	40	-		
Parent (P_3)	(NDC 4-20-4)	40	0	-		
F_1	($P_1 \times P_3$)	30	0	-		
F_2	($P_1 \times P_3$)	88	29	3:1	0.45	0.50
BC_1 (P_1)	($F_1 \times P_1$)	46	33	1:1	1.82	0.30
BC_1 (P_3)	($F_1 \times P_3$)	59	0	-		

Table 7: Joint segregation for markers of chickpea F_2 population for Leaf color and nodulation

Crosses	Gene	A- B-	A- bb	aa B-	aa bb	Sum	χ^2	P	R $\pm$ SE
ICC 19181 × NDC 5-S10	L: N	73	5	11	20	109	42.37	0.00	0.1575 $\pm$ 0.092
ICC 19181× NDC 4-20-4	L: N	85	3	20	9	117	18.084	0.00	0.264 $\pm$ 0.084

Leaf color vs. nodulation

Results of both the crosses (ICC19181 × NDC 5-S10 and ICC19181 × NDC 4-20-4) are presented in Table 7. Linkage analysis of F_2 population of ICC19181 × NDC 5-S10 manifested that out of 109 plants, 73 plants were recorded with root nodules (RN) and light green leaf color (L), 5 plants were non-nodulated (rn) with light green leaf color (L), 11 plants showed root nodules (RN) and dark green (l) leaf color, while 20 plants were observed as non-nodulated (rn) with dark green leaf color (l). The recombination value between genes for leaf color and root nodulation was observed to be 0.1575 $\pm$0.0928 (15%).

In F_2 population of second cross (ICC19181 × NDC 4-20-4) there were total 117 plants out of which 85 plants showed root nodules (RN) with light green leaf color (L), 3 plants were non-nodulated (rn) with light green leaf color (L), 20 plants showed root nodules (RN) and dark green (l) leaf color, while 9 plants were observed as non-nodulated (rn) with dark green leaf color (l). The recombination frequency between genes for leaf color and root nodulation was observed to be 0.264 $\pm$ 0.084 i.e. 26% (Table 7). Results of F_1 and F_2 generations of both crosses revealed that L (leaf color) and RN (root nodulation) loci are linked (Table 7).

DISCUSSION

Results obtained from hybrid A in this study fit to the monohybrid Mendelian ratio i.e. 3 (nodulated): 1 (non-nodulated), which indicated that nodulation in genotype NDC 5-S10 was controlled by a single dominant gene. In chickpea, similar results of monogenic inheritance of nodulation have been reported by Singh and Rupela (1998); Devis (1988) and Singh *et al.* (1992). In addition to chickpeas, it was found that super nodulation in Pea (Sidorova*et al.*, 2003) and hyper nodulation in *Lotus Japonicus* (Oka-Kera *et al.*, 2005) are also governed by Mendelian segregation. The F_1 BC_1 data (1:1) of hybrid A and the highest probability level (68-70%) of Chi-square values also provided strong evidence to support the monogenic inheritance of nodulation in the said genotype NDC 5-S10.

The second cross (hybrid B) revealed normal nodulation (all plants produced nodules) in the $F_{1,}$ while in the F_2 segregating population, the ratio of 15 (Nod^+): 1 (Nod^-) was observed. Whereas, the backcross progenies revealed a ratio of 3 (Nod^+): 1(Nod^-). Result of F_2 population showed that nodulation in genotype NDC 4-20-4 was controlled by two independent dominant genes i.e. duplicate gene action. Furthermore, this result strengthened by the ratio of backcross progenies (1Nod^+: 1Nod^-), and the probability levels of chi-square of F_2 and backcross data. In chickpea this will be the first report about duplicate gene action controlling nodulation, however, the presence of two genes for certain other traits like resistance to wilt, *Ascochyta blight* and leaf type have also been reported (Danehloueipour *et al.*, 2008). Furthermore, in some other pulses there are evidences of duplicate gene action behind nodulation e.g. in soybean, two dominant alleles were observed to control promiscuous nodulation (Gwata *et al.*, 2005). Similarly, Gallo-Meagher *et al.* (2001) reported a 3-gene model for nodulation in pigeon peas and suggested that there are three genes which control nodulation and these genes are located at three independent loci (with nodulation being a product of two genes and inhibited by a third gene when it is dominant and the others are homozygous recessive). Moreover, Nigam *et al.* (1982) also observed two duplicate recessive genes that control non-nodulation in groundnut. Dutta and Reddy (1988) also proposed a genetic model in peanut (*Arachis hypogaea* L.), involving three genes in the inheritance of non-nodulation. They further mentioned that two genes produced the nodulation while the third gene inhibits nodulation only when it is dominant and the former two genes were in recessive homozygous condition.

In our previous study (Khan *et al.*, 2010) performed by using simple sequence repeats (micro satellite) markers, it was concluded that the two genotypes (NDC 5-S10 and NDC 4-20-4) fall into distinct groups in the cluster analysis/ phylogenetic tree, constructed by UPGMA (unweighted paired-group method) and NJ

(Neighbor Joining) methods even, the principal component analysis (PCA) based on morphological data also exhibited that these two genotypes belong to separate classes. High level genetic diversity both at morphological and molecular levels also supported the possibility of different genetic background for nodulation in these two genotypes.

Results revealed that leaf color in chickpea was controlled by single gene in both cross combinations. Previous studies also reported monogenic inheritance for morphological markers including leaf characteristics (other than color) in chickpea. Davis (1991) also reported monogenic inheritance for simple leaf, filform leaves, ineffective nodulation and white color flower traits in chickpea. Similarly, Pundir and Reddy (1998) observed single gene inheritance for chickpea small leaf size and open flower characteristics.

Monogenic markers are useful in estimating the rate of out-crossing in predominantly self-pollinating crops and also help in identification of F_1 hybrids in the breeding programs. In case of complete dominance, the detection of heterozygotes for morphological markers is not possible (Arshad *et al.*, 2005). Gene linkage study showed that both qualitative characters (nodulation and leaf color) deviated from the expected Mendelian segregating ratio (9:3:3:1). This shows the presence of linkage between the studied traits i.e. genes for both the parameters are present on the same chromosome.

Rare linkage study has been reported among genes controlling nodulation with other morphological markers in chickpea. Gwata *et al.* (2005) studied the inheritance of nodulation with the help of leaf color in soybean, and found that yellow leaf color with non-promiscous nodulation (which forms no or nonfunctional nodules) was dominant over green leaf color with promiscuous nodulation (which form functional nodules). From plant breeding point of view, such method of identifying nodulated genotypes is most rapid, less expensive and effective. Likewise, Oka-Kera *et al.* (2005) reported that hyper nodulation, aberrant leaf vein formation and significantly delayed flowering in *Lotus Japonicus,* are linked in a monogenic and recessive manner, indicating that these phenotypes are caused by either a single mutation, or tightly linked mutations. The present study may be the first effort to report linkage between genes for nodulation and leaf color in chickpea. Our results corroborate the findings of other researchers, who reported linkage of nodulation with various morphological traits e.g. Davis (1991) observed strong linkage between genes for simple leaf (*slv*) and root nodulation (*rn3*) and among genes controlling filform leaf and open flower (*fil* and *w2*). Yasuhero *et al*. (2006) also suggested that LjNSP2 gene (encodes a putative transcription factor) acts as a transcription factor to directly or indirectly switch on the NF-induced genes required for nodule initiation. Sinjushin *et al.* (2008) reported linkage between genes for stem architecture and nodules number in garden pea (*Pisum sativum* L.).

It may be concluded that nodulation in chickpea is controlled by a single or two dominant genes, depending on the nature of germplasm. Leaf color in chickpea also has monogenic inheritance. Presence of linkage between genes for nodulation and leaf color is of great importance and could be used efficiently in future breeding programs for rapid screening method in nodulation.

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