INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0637/2018/20–3–683–688
DOI: 10.17957/IJAB/15.0549
http://www.fspublishers.org

Full Length Article

Characterization for Nodulation and Detection of Duplicate Gene Action of Dominant Epistasis Controlling Root Nodulation in Chickpea (*Cicer arietinum*)

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Abstract

Diverse germplasm of chickpea (*Cicer arietinum* L.), procured from national and international organizations was categorized for nodulation as well as assessed for nodules per plant and yield per plant in The University of Agriculture, Peshawar. Forty three genotypes exhibited nodulation (Nod+) whereas four genotypes failed to produce any nodule (Nod-). ANOVA showed highly significant variances for both the studied characters between genotypes, in addition these traits showed high heritability estimate. Out of four non-nodulated genotypes, one new mutant strain was used to understand the mode of inheritance of non-nodulation. A spontaneous non-nodulating mutant ICC19181 was crossed with two different highly nodulated strains i.e., NDC 5-S-10 and NDC 4-20-4 and differential results were obtained in the F2 generation and backcross progenies. The former (ICC19181 X NDC 5-S-10) showed 3:1 and the latter (ICC19181 X NDC 4-20-4) showed 15 : 1 segregation for Nod+ : Nod-. The distribution of nodulated and non-nodulated progenies i.e., 1 Nod+: 1 Nod- and 3 Nod+: 1 Nod- in the backcross progenies of hybrid A and hybrid B, with ICC19181, respectively authenticate the results of segregating populations. The latter suggests that two independent loci were involved in forming non-nodulation phenotype. These are in complete dominance but the effect is epistatic to the others. NDC 5-S-10 is expected to be homozygous dominant in one gene but homozygous recessive in the other gene.

Keywords: Chickpea; Nodulation; Inheritance; Mutant
Abbreviations: ICARDA International Center for Agricultural Research in Areas, Syria; ICRISAT, International Crops Research Institute for the Semi-Arid Tropics, India; NIFA, Nuclear Institute for Food and Agriculture, Pakistan.; Gram Research Station (GRS) Karak; N+: Nodulated; N-: Non-nodulated

Introduction

Many legume species usually form a complex symbiotic association with specific soil bacteria known as rhizobia (*Rhizobium leguminoserum*). This association constructs highly specialized organ, called the root nodule. Rhizobia live in these nodules and fix atmospheric nitrogen into a usable form for plants. Legume crops like chickpeas do not respond to high doses of nitrogen fertilizer as they mainly depend upon atmospheric nitrogen. Improving symbiotic nitrogen fixation ability in legume crops could be the most efficient way of improving yield (Nutman, 1969; Caldwell and Vest, 1977). This is also one of the main goals of recent agronomical studies to reduce consumption of artificial nitrogen fertilizer (Devine and Weber, 1977), as synthetic nitrogen based fertilizers produced every year met only 50% of the world's requirement (WIPO, 2007).

Symbiotic nitrogen fixation ability has been known to be influenced by three important factors i.e., host genotypes, bacterial strains and environmental conditions. Genotypic variation for nodule formation ability (nodule number and nodule dry weight) and N_2 fixation ability was found in clover (Nutman, 1967), common bean (*Phaseolus vulgaris* L.) (Chavera and Graham, 1992; Montealegre *et al.,* 1994), soybean (Videira *et al.,* 2001) chickpea (Romdhane *et al.,* 2007). Using diallel cross populations, it also has been shown that nodule formation ability is under polygenic control, in which non-additive gene action as well as substantial additive effect was presented (Bhapkar and Deshmukh, 1982). Cultivars response to inoculation with diverse rhizobial strains also has been studied in several leguminous plants. Hungría and Neves (1987) indicated that there is an interaction of strains of *Rhizobium* with plant genotypes for growth and nitrogen content in the common bean. Interactions between *Rhizobium* strains and *Phaseolus* cultivars on N_2 fixation were further investigated by Rodríguez-Navarro *et al.* (1999). Differential response of chickpeas in nodulation to environmental conditions was

To cite this paper: Gul, R., H. Khan, N.U. Khan, A. Latif and K. Harada, 2018. Characterization for nodulation and detection of duplicate gene action of dominant epistasis controlling root nodulation in chickpea (*Cicer arietinum*). *Int. J. Agric. Biol*., 20: 683–688

also reported in high soil salinity (Saxena and Rewari, 1992) and drought conditions (Romdhane *et al*., 2008). Considering these facts it should be proposed that breeding of host plants in combination with rhizobia strains might be requested for improving nitrogen fixation. In order to improve the nitrogen-fixing ability of legumes through breeding, understanding the genetic control that underlines nodulation is required (Wyne *et al.,* 1981). Mutants showing aberrant responses to inoculation with Rhizobium in chickpea were first isolated by selection following γ-irradiation mutagenesis (Davis *et al.,* 1985). Some of these mutants showed temperature sensitive expression of nodulation. When root temperature was controlled under greenhouse conditions, some mutants produced effective nodules at 24ºC, but nodulation was strongly suppressed or eliminated at 29ºC. Devis (1988) revealed that these aberrant phenotypes or "ineffective" nodulation are under the control of single recessive genes (rn1, rn2 and rn3). Additional recessive mutant genes, which are non-allelic to the previously reported genes, were identified for ineffective nodulation mutants and assigned as rn4 and rn5 (Davis *et al.,* 1986). However, recessive epsitatic relationship was observed between these two genes. Beside these studies, Rupela and Sudarshana (1986) identified spontaneous non-nodulating mutants in chickpea.

Singh *et al.* (1992) evaluated the mode of inheritance of these strains and identified a single recessive gene controlling non-nodulating trait. Because this gene is not allelic to the other genes identified in the induced mutation studies, they assigned the symbol *rn6* to this mutant. Singh and Rupela (1998) reported three additional non-nodulating genotypes from three different morphs originated in India i.e., Annigeri, (ICC4918), and Rabat (ICC 4993) through pure line selection. In these mutants, one new recessive mutant gene (*rn8*) was identified, which might be the first mutant gene in Kabuli genotypes.

This study was conducted to get a clearer understanding of the genetics of nodulation in chickpea. The specific objective was to determine the inheritance of nodulation for two chickpea crosses using ICC9181 (non-nodulated) as female parent in both the crosses and applying *Rhizobium leguminoserum* strain Cl2 as seed inoculant.

Materials and Methods

Classification of chickpea germplasm for nodulation and its evaluation for number of nodules per plant and seed yield per plant was accomplished in pots in the net house facility of The University of Agriculture Peshawar (UAP), during 2006–2007. The experimental material consisted of 47 genotypes (Table 1), procured from national (NIFA, Peshawar and GRS, KARAK) and international (ICARDA Syria and ICRISAT India) organizations, were studied. Completely Randomized Design (CRD) with three replications was used to carry out the experiment. Diameter of each pot was 22 cm containing 4.5 kg soil (50% clay and 50% sand). Six seeds were sown per pot.

Statistical software, SAS (statistical analysis system) version 9 was used to analyze the data. Out of 47 genotypes, 43 exhibited nodules while only four genotypes remained completely non-nodulated. Thus, data of only 43 genotypes was analyzed for number of nodules per plant and seed yield per plant. Genotypic means were compared, by LSD test at 5% probability level by the formula of Steel and Torrie (1980). While for heritability (broad-sense), coefficient of variation, genotypic and phenotypic variances formula of Hanson *et al.* (1956) and Burton (1952) was used respectively.

Table 1: Pedigree and origin of genotypes/accessions used in the study

Genotype Name	Parentage	Origin	Genotype name	Parentage	Origin
NDC-122	C-44 x ILC-195	NIFA, Pakistan	NKC-10-99	Flip98-138c x Sel99th15039	ICARDA/,Syria
NDC-727	C-44/M	NIFA, Pakistan	NKC-5-S12	BAHODIR x SEL99TER85530	ICARDA, Syria
NDC-728-5	C-44/M	NIFA, Pakistan	NKC-5-S13	SEL99TH15039 x S98008	ICARDA, Syria
NDC-730-2	C-44/M	NIFA, Pakistan	NKC-5-S14	SEL99TH15039 x S98008	ICARDA, Syria
NDC-15-1	Pb-91/M	NIFA, Pakistan	NKC-5-S15	FLIP98-15C x S98033	ICARDA, Syria
NDC-15-2	Pb-91/M	NIFA, Pakistan	NKC-5-S16	S99456 x SEL99TER85314	ICARDA, Syria
NDC-15-3	Pb-91/M	NIFA, Pakistan	NKC-5-S17	S99456 x SEL99TER85314	ICARDA, Syria
NDC-15-4	Pb-91/M	NIFA, Pakistan	NKC-5-S18	(ILC4291xFLIP98-129C) x S98008	ICARDA, Syria
NDC-4-15-1	C-44/M	NIFA, Pakistan	NKC-5-S19	(ILC4291xFLIP98-129C) x S98008	ICARDA, Syria
NDC-4-15-2	C-44/M	NIFA, Pakistan	NKC-5-S20	(FLIP98-138C x SEL99TH15039)	ICARDA, Syria
NDC-4-15-3	C-44/M	NIFA, Pakistan	NKC-5-S21	GLK95069 x SEL99TER85530	ICARDA, Syria
NDC-4-20-1	C-44/M	NIFA, Pakistan	NKC-5-S22	CA9783007 x SEL99TER85534	ICARDA, Syria
NDC-4-20-2	C-44/M	NIFA, Pakistan	NKC-5-S23	CA9783007 x SEL99TER85534	ICARDA, Syria
NDC-4-20-3	C-44/M	NIFA, Pakistan	NKC-5-S24	CA9783007 x SEL99TER85534	ICARDA, Syria
NDC-4-20-4	C-44/M	NIFA, Pakistan	HASSAN-2K	ILC-195/M	NIFA, Pakistan
NDC-4-20-5	C-44/M	NIFA, Pakistan	Karak 1	Local selection	Karak, Pakistan
NDC-4-20-6	C-44/M	NIFA, Pakistan	Karak 2	Local selection	Karak, Pakistan
NDC-4-20-7	C-44/M	NIFA, Pakistan	Karak 3	Local selection	Karak, Pakistan
NDC-5-S10	JG74xICC12071	ICRISAT, India	Sheenghar	Local selection	Karak, Pakistan
NDC-5-S11	JG74xICC12071	ICRISAT, India	Lawaaghar	Local selection	Karak, Pakistan
NIFA-88	6153/M	NIFA, Pakistan	ICC 4993	Rabat	Karnataka, India
NIFA-95	6153/M	NIFA, Pakistan	ICC 19183	ICC 4993	ICRISAT
NIFA-2005	PB-91/M	NIFA Pakistan	ICC 4918	Annigeri	Morocco
			ICC 19181	ICC 435	ICRISAT

For inheritance study ICC19181 (one of the four spontaneous non-nodulating mutants of Desi chickpea genotype sent from ICRISAT) and two highly nodulated chickpea genotypes i.e., NDC 5-S-10 and NDC 4-20-4 were selected among the bulk of 47 chickpea genotypes (Table 1). The selected genotypes were raised and crossed for F1 during growing season of 2007–2008 at the University of Agriculture, Peshawar, Pakistan. F2 populations and backcrosses were developed at NIFA during 2008–2009. The mating scheme is shown in Table 2.

The parental genotypes, F1, F2 and backcross populations were raised in pots during 2009 in a net house at the UAP, Pakistan. To prepare inoculation, slurry 40 g of *Rhizobium leguminosarum* culture of CPL2 was added in 300 mL of 5% sugar solution. The whole inoculation procedure was completed in the shade. Three seeds were sown per pot, without fertilizer addition and irrigated when required. Data for nodulation was recorded after six weeks of planting. For examining nodulation, plants were gently uprooted from pots and then the soil was removed by dipping the plant roots three times with tap water to completely clean roots from soil particles. All the plants of different genotypes were carefully scored for presence/absence of nodules on roots by evaluating the roots carefully in trays filled with water. Data for the presence/absence of nodules were obtained from 120 plants of parental genotypes (40 plant for each parent), 57 plants of F1 (27 of hybrid A and 30 of hybrid B), 226 F2 (109 of hybrid A and 117 of hybrid B), and 263 backcrossed (84 of hybrid A and 79 of hybrid B) plants. To test the goodness of fit for appropriate genetic hypotheses the Chi-square ($\chi 2$) test using Yates correction for adjustment of small population size was used (Yates, 1934).

Results

Genotypes Evaluation for the Nodules

All collected genotypes of chickpea were characterized for the occurrence or non-occurrence of nodules (Table 2) in UAP. As a result of characterization 43 genotypes i.e., from NDC 122 to Lawaghar (Table 2) showed nodule including genotypes from ICARDA, Syria; ICRISAT, India; NIFA, Peshawar and GRS, Karakhence, these genotypes were reported as nodulated ones. Whereas, only four genotypes (ICC4993, ICC19183, ICC4918 and ICC19181) procured from ICRISAT, India did not produce any nodule and are characterized as non-nodulated genotypes.

Evaluation of the Germplasm for Nodules and Seed Yield per Plant

Genotypes exhibited highly significant variances for nodules per plant as well as for seed yield per plant (Table 3). Mean values for genotypes showed a wide range of variation for nodules plant^{-1} in addition with seed yield per plant and displayed highly significant variation between genotypes (Table 2 and Fig. 1). Genotype NDC-5-S10 exhibited highest (14.5) number of nodules per plant followed by genotype NDC 4-20-4 which produced 12.5 nodules per plant, whereas lowest (2.4) number of nodules plant^{-1} were shown by genotype Lawaghar. Maximum (28.9 g) seed yield per plant was recorded for genotype Karak 3, whilst genotype NDC-15-2 showed minimum (4.1 g) seed yield per plant (Table 2).

For number of nodules per plant moderate phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) i.e., 32.85 and 17.48 respectively, were revealed by the Components of variance (Table 4).

Table 2: Mean values of nodules per plant and seed yield per plant for chickpea genotypes

Genotype name	Nodules Present/absent	No. of Nodules per plant	Seed yield per plant (g)
NDC-122	Present	8.0	9.4
NDC-727	Present	7.8	6.4
NDC-728-5	Present	7.2	6.8
NDC-730-2	Present	7.9	6.8
NDC-15-1	Present	8.1	14.8
NDC-15-2	Present	7.3	3.6
NDC-15-3	Present	9.3	4.7
NDC-15-4	Present	9.9	8.8
NDC-4-15-1	Present	8.1	4.9
NDC-4-15-2	Present	8.8	6.8
NDC-4-15-3	Present	8.7	4.5
NDC-4-20-1	Present	11.0	17.9
NDC-4-20-2	Present	12.0	5.6
NDC-4-20-3	Present	10.4	4.5
NDC-4-20-4	Present	12.5	4.3
NDC-4-20-5	Present	9.2	5.3
NDC-4-20-6	Present	8.8	14.8
NDC-4-20-7	Present	9.0	4.4
NDC-5-S10	Present	14.5	8.3
NDC-5-S11	Present	9.6	8.6
NIFA-88	Present	6.4	6.7
NIFA-95	Present	6.7	6.0
NIFA-2005	Present	9.1	13.8
NKC-10-99	Present	7.2	6.8
NKC-5-S12	Present	6.6	12.7
NKC-5-S13	Present	6.9	6.5
NKC-5-S14	Present	6.9	13.7
NKC-5-S15	Present	7.7	12.3
NKC-5-S16	Present	4.7	11.4
NKC-5-S17	Present	9.6	12.5
NKC-5-S18	Present	3.1	12.6
NKC-5-S19	Present	6.8	9.3
NKC-5-S20	Present	4.0	12.4
NKC-5-S21	Present	5.1	11.1
NKC-5-S22	Present	7.4	9.5
NKC-5-S23	Present	4.9	11.3
NKC-5-S24	Present	4.9	14.1
HASSAN-2K	Present	7.8	12.1
Karak 1	Present	8.1	17.1
Karak 2	Present	7.9	15.5
Karak 3	Present	6.7	27.8
Sheenghar	Present	5.8	13.4
Lawaghar	Present	2.4	12.6
ICC 4993 (Rabat)	Absent	0.0	7.0
ICC 19183	Absent	0.0	6.0
ICC 4918 (Annigeri)	Absent	0.0	8.4
ICC19181	Absent	0.0	5.9

Table 3: Mean square for inoculation effect on nodules per plant and seed yield per plant

Source of Variation		Mean Squares	
		Seed yield per plant	Nodules per plant
	Degrees of Freedom		
Treatment	1	648.3**	1104.01**
Genotype	42	28.6**	201.59**
Treatment X Genotype	42	8.0**	28.72**
Error	172	2.19	11.86

Table 4: Estimates of variability parameters of number of nodules and seed yield per plant

Variability parameters	Number of nodules per plant	Seed yield per plant
Genotypic variance	3.51	21.3
Phenotypic variance	6.62	27.9
Genotypic coefficient of variation	17.48	43.6
Phenotypic coefficient of variation	32.85	49.9
Heritability (broad sense)%	53.2	77

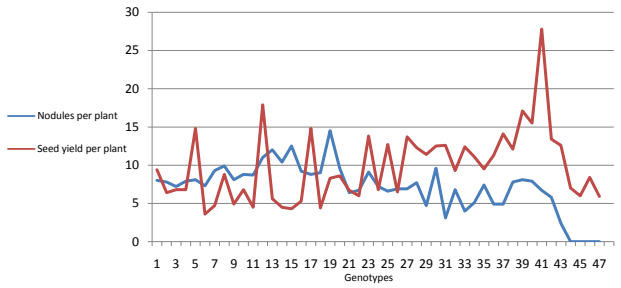

Fig. 1: Mean values of number of nodules per plant and seed yield per plant of diverse chickpea genotypes

Whereas it's broad sense heritability was a bit high with value of 53.2%. However, high heritability i.e., 77%, with a greater extent of PCV (49.9) as well as GCV (43.6) was reported for seed yield plant^{-1}.

Hybridization of Selected Parents and Evaluation of Inheritance Pattern of Nodulation

After characterization of genotypes for nodulation and their evaluation for nodules per plant (Table highly nodulated genotypes NDC 5-S-10 and NDC 4-20-4 were hybridized with non-nodulated genotype ICC19181 to study the inheritance pattern of nodulation (Table 5).

Results revealed that all the F1 plants of hybrid A i.e., NDC 5-S-10 x ICC 19181 (Table 6) as well as hybrid B i.e., NDC 4-20-4 x ICC19181 (Table 7) showed normal nodulation indicating dominant effect for nodulation in these genotypes. In NDC 5-S-10 x ICC 19181, segregation in F2 population showed a good fit to the 3:1 ratio for nodulated and non-nodulated phenotypes. This is the expected ratio when there is single recessive gene behind a non-nodulated phenotype. In backcross populations, the

Table 5: Hybrid and backcross populations used in study

No.	Cross combination	Cross designation
1	ICC19181 ×NDC 5-S-10	Hybrid A (F11)
2	ICC19181 × NDC 4-20-4	Hybrid B (F12)
3	(ICC19181 × NDC 5-S-10) × ICC19181	BC11(P1)
4	(ICC19181 × NDC 5-S-10) × NDC 5-S-10	BC12(P2)
5	(ICC19181 × NDC 4-20-4) × ICC19181	BC13 (P1)
6	(ICC19181 × NDC 4-20-4) × NDC 4-20-4	BC14 (P2)

P1 and P2: Parents. F1: first filial generation. F2: second filial generation. BC1: first backcross generation. ‡The parents in right side serve as female and that in left side as male

Table 6: Segregation of nodulation in parents, F_1, F_2 and back cross generations in hybrid ICC19181 X NDC 5-S-10

Generation†	Parent or cross‡	Number of Plants		Expected ratio	χ^2	P
		Nod^+	Nod^-			
Hybrid A						
P_1	ICC19181	0	40	-		
P2	NDC 5-S-10	40	0	-		
F_1	P_2 x P_1	27	0	-		
F_2	F_1	84	25	3 : 1	0.14	0.68 – 0.70
$BC_1(P_1)$	P_1 x F_1	45	39	1 : 1	0.29	0.50 – 0.70
$BC_1(P_2)$	P_2 x F_1	48	0	-		

Table 7: Segregation of nodulation in parents, F_1, F_2 and back cross generations in hybrid ICC19181 X NDC 4-20-4

Generation†	Parent or cross‡	Number of Plants		Expected ratio	χ^2	P
		Nod^+	Nod^-			
P_1	ICC19181	0	40	-		
P2	NDC 4-20-4	40	0	-		
F1	P2 x P_1	30	0	-		
F_2	F_1	105	12	15 : 1	2.55	0.15 – 0.20
$BC_1(P_1)$	P_1 x F_1	53	26	3 : 1	2.22	0.15 – 0.20
$BC_1(P_2)$	P_2 x F_1	59	0	-		

progenies of BC1 (P1) showed a good fit to the expected 1 Nod^+: 1 Nod^-, however, all progenies were Nod^+ when F1 populations of hybrid-A were crossed with the nodulated parent (BC1 (P2). These ratios confirmed the single mutant gene effects nodulation in the recessive homozygotes (Table 6). In the hybrid-B, F_2 populations were most appropriately segregated in 15:1 ratio for Nod^+ and Nod^- phenotypes ($P > 0.15$), respectively. In backcross populations, the (BC1(P1)) progenies were segregated in 3:1 ratio $P > 0.15$ for N^+ and N^-, respectively, however, all the progenies were nodulated when F1 population of hybrid-B were crossed with P2 (NDC 4-20-4) (Table 7).

Discussion

Nodules per plant and seed yield per plant are two very important traits of chickpea, as one is vital for nitrogen supply to the plant and the soil, while the other trait is the basic characteristic for improvement in every breeding program of crop plants specifically grain legumes in order to nourish the increasing population effectively. In our study significant differences for these traits showed that the

germplasm under study is expressively variable for these traits and is very important from breeding point of view to be utilized in future yield improvement and nitrogen fixation upgradation programs of chickpea. Tellawi *et al.* (2007) and Gallani *et al*. (2005) reported significant variation in nodulation among chickpea genotypes. Likewise, significant difference in the seed yield of soybean genotypes was recorded by Muhammad and Khalil (2013). Moderate amount of PCV and GCV revealed that there is sufficient quantity of dissimilarity among genotypes for the studied characters of chickpea. Our results showed that it is easy to achieve adequate genetic improvement in nodules per plant and seed yield per plant because their heritability is moderate to high. The role of genotype is projecting in the expression of phenotype when heritability is moderate to high. This shows that environmental variance is less than the genotypic variance in the phenotype, so selection for the studied traits is dependable and perpetual improvement could be achieved in the genotypes for these traits.

Results indicated that Nod^- phenotype in the parent strain ICC19181 was controlled by duplicated mutant genes and segregate independently. These results further revealed that operation of dominant epistasis occurred between duplicated loci. This is explained as follows: suppose these loci to be M and N, the genotypes of ICC19181 and NDC 4-20-4 must be *mmnn* and *MMNN*, respectively. Segregation in F2 in hybrid B, are then, 9 *M-N-* : 3 *M-nn*: 3 *mmN-* : 1 *mmnn* if there are no linkages. This will show 15 Nod^+: 1 Nod^- ratio if either of the genes when dominant, epistatic to the other. The 3:1 segregating ratio in the F2s in cross A is understood if the genotype of NDC 5-S-10 is *MMnn* (or *mmNN*). Thus, far all the reported mutants for non-nodulation are recessive. Furthermore, in all the duplicated gene actions up to now reported, either recessive homozygotes is epistatic to the effect of the other gene (i.e. *M-nn* and *mmN-* genotypes are both non-nodulated and segregate in 9 Nod^+: 7 Nod^- in F2). This is the first case of dominant epistasis in chickpea for non-nodulating phenotypes. The probable underlying mechanism controlling the phenotype might be due to two mutations occurred independently in the duplicated pathway to produce necessary products for nodulation.

During the development of root nodules, legumes express a set of nodule-specific host polypeptides referred to as "nodulins" (Spaink *et al.*, 1998). More than 20 such polypeptides other than leghemoglobin were expected to be expressed in nodules (Legocki and Verma, 1980). These may include proteins responsible for the maintenance of nodule structure, and enzymes necessary for the specific assimilation of reduced nitrogen (Fuller *et al.*, 1983). The mutations in present materials might have occurred in some of the pathways to produce nodulins. Our mutations could be provided for the molecular studies of the symbiotic interaction of rhizobium and legumes. Reports of some other scientist's research studies in legumes, also authenticate our findings like Gallo-Meager *et al.* (2001) who proposed a three gene model for nodulation and suggest nodulation is controlled by tree genes present on separate loci, with nodulation to be the result of two genes and stopped by the dominant form of a third gene when the other are homozygous recessive. In soybean two dominant alleles were observed to control promiscuous nodulation (Gwata *et al.*, 2005). Two duplicate recessive genes were also reported by Nigam *et al.* (1982) that control non-nodulation in groundnut. Dutta and Reddy (1988) also reported duplicate gene action as well as single recessive gene controlled non-nodulation in peanut. It was stated that duplicate gene action as well as single recessive gene controlled non-nodulation in peanuts.

Conclusion

The present study revealed highly significant differences among genotypes for nodules per plant and seed yield per plant. In the current study, for the first time duplicate gene action with dominant epistasis has been reported in chickpea. High heritability estimates revealed that the studied traits could be used as selection criteria. Moreover the studied germplasm could be utilized successfully for the development of chickpea varieties with increased nodulation and improved seed production.

Acknowledgments

Authors are thankful to ICRISAT and NIFA for providing germplasm for this study

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(Received 10 June 2017; Accepted 11 December 2017)