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Rapid generation advancement of RIL population and assessing the impact of *Rhizobium* nodulation on crop yields in Chickpea

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Chickpea, a widely cultivated legume, actively fix atmospheric nitrogen in root nodules through a symbiotic relationship with rhizobia bacteria. A recombinant inbred line (RIL) population, progressing from F_2 to F_7 generations, was developed in a short-period of 18 months using the Rapid Generation Advancement (RGA) protocol. The F_7 RILs were evaluated during the 2020-21 and 2021-22 crop seasons under typical field conditions to quantify the effects of nodulation on seed yield (SY) and its associated traits. The analysis of variance revealed a highly significant difference ($P < 0.01$) among genotypes for seed yield and other agronomic traits, with no significant seasonal effect. In the pooled analysis, nodulating genotypes (NG) exhibited a substantial increase ($P < 0.01$) in SY (62.55%), 100-seed weight (SW100; 12.21%), harvest index (HI; 6.40%), number of pods per plant (NPPP; 39.55%), and number of seeds per plant (NSPP; 44.37%) compared to non-nodulating genotypes (NNG). Both NG and NNG exhibited a significant ($P < 0.01$) positive correlation between SY and NPPP ($r = 0.64$ and 0.63), NSPP ($r = 0.66$ and 0.61), HI ($r = 0.27$), and number of primary branches per plant (PBr) ($r = 0.31$), respectively. The top-performing genotypes for yield and related traits were predominantly nodulating. Genotype-trait bi-plot analysis identified nine nodulating genotypes as the most adaptable across the two seasons—six for SY, plant height, SW100, and three for days to first flowering and maturity. These findings underscore the critical role of nodulation in maximizing chickpea yields and the significant yield penalties associated with non-nodulation. To boost chickpea production, future breeding efforts should focus on developing genotypes with high compatibility with rhizobium strains.

Keywords Chickpea, Rhizobium, Nodulation, Rapid generation advancement (RGA), Recombinant inbred line (RIL) population, Nodulating and Non-nodulating genotypes, Genetic variability

Abbreviations

RGA	Rapid Generation Advancement
RIL	Recombinant Inbred Line
SNF	Symbiotic nitrogen fixation
NG	Nodulating genotypes
NNG	Non-nodulating genotypes
DF	Days to first flowering
DFF	Days to 50% flowering
DM	Days to maturity
PH	Plant height
PBr	Number of primary branches per plant
SBr	Number of secondary branches per plant
NPPP	Number of pods per plant
NSPP	Number of seeds per plant

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SY	Seed yield
SW100	100-seed weight
HI	Harvest index
ANOVA	Analysis of variance
GCV	Genotypic coefficient of variation
PCV	Phenotypic coefficient of variation
GAM	Genetic advance of mean
GT biplot	Genotype-trait bi-plot analysis

Legumes have a specific mutualistic relationship with soil bacteria that fix the atmospheric nitrogen into plant-usable ammonical form in root nodules[1], in contrast to other plants. Development of nodules and nitrogen fixation depends majorly on the legume cultivar and its specific rhizobial strain[2–4] which will in turn improve the overall crop growth, productivity[5,6] and soil health. Around 20 to 22 million tonnes of nitrogen per annum is fixed in the agricultural systems by the Leguminosae family members[7] which contributes significantly towards reducing the global carbon footprint.

Chickpea (*Cicer arietinum* L.) is a highly nutritious, diploid ($2n = 2x = 16$) legume crop grown in an area of 14.2 million hectares across 56 countries globally[8], of which, India is the largest producer and consumer. It is considered a storehouse of proteins, complex carbohydrates, vitamins, and micronutrients that are required for human nutrition[9–13]. Being a leguminous crop, chickpea fixes the atmospheric nitrogen by association with *Rhizobium* species[14] which differentiates it from other cereal crops[15]. This ability to establish a symbiotic relationship is critical for legumes as it provides them with a readily available source of nitrogen, an essential macronutrient for plant growth and development. It stores the fixed nitrogen in the nodules present in its root system and converts it into ammonia that can be used by the plant[16,17]. It was estimated that about 70 kg of nitrogen per hectare is fixed annually by chickpea[18], which helps in providing nitrogen not only to the host but also to the subsequent crops grown[19] thereby, helping the farmers in reducing the cost of production.

Root nodulation is a complex process requiring the recognition of symbiotic bacteria, *Nod-factor* induced infection, and root growth[20–22]. Polyphenols named flavonoids released from the roots, stimulate *Nod-factor* production in rhizobia thereby initiating curling and colonization of root hair and the formation of root nodules[23]. Phytohormone signaling plays a major role during this process between the bacteria and the host[24]. The symbiotic nitrogen fixation (SNF) efficiency is dependent on the host, rhizobial strains, soil conditions[25], availability of phosphorous[26], and environmental conditions[27]. Under water stress, the biochemical activity in the nodules will get disrupted resulting in the senescence of nodules[28–30] along with downgraded leg-hemoglobin content and nitrogenase activity[31]. The ability of the nodules to supply energy, transport, regulate oxygen molecules, and assimilate ammonia to the plants will help in increasing plant growth and yield[32]. However, the consequences of the chickpea roots being unable to make a symbiotic relationship with rhizobium are not well understood and documented.

Nitrogen is a key component of proteins, nucleic acids, and chlorophyll, all vital for plant function. Hence, effective nodulation directly impacts legume productivity and nutritional value. Previous studies in legumes have identified numerous genes and signaling pathways involved in nodulation, shedding light on the molecular basis of signaling between the plant and the bacteria[33,34]. Conversely, the genetic basis of non-nodulation in certain legume species has been linked to mutations in key nodulation genes or disruptions within the signaling cascades vital for establishing this symbiosis[35]. For example, studies in *Medicago truncatula* have revealed that mutations in the NFP (Nod Factor Perception) gene result in nodulation deficiency[36]. Unraveling the genetic mechanisms underlying both successful nodulation and its absence is crucial not only for enhancing nitrogen fixation in crops but also for broadening our understanding of the intricate plant-microbe interactions that underpin sustainable agriculture. Several studies reported in chickpea[14,37–39] as well as in other leguminous crops viz., soybean[40], cowpea[30], lupin[41], groundnut[42], etc., were focused majorly on the external application of biochar, artificial fertilizers, growth hormones and the findings of strain-specific effect on nodulation and yield-related traits. However, the information on the impact of nodulation over non-nodulation on yield and yield-contributing traits was minimal. In this context, the current study aimed to (1) broaden the knowledge on the association between nodulation, yield, and its associated traits; and (2) quantify the value gain of the traits in a RIL population segregating for nodulation trait. In addition, we successfully showcased the utility of rapid generation advancement methods for developing mapping populations in a short period.

Results

Analysis of variance

The results of the combined analysis of variance (ANOVA) revealed significant differences (Prob < 0.01) among the genotypes for the traits under study while for the factors, block and replication, no significant difference was observed (Table 1). For nodulating genotypes (NG), mean performance of agro-morphological and yield-related traits over the seasons was in the range of 42.74–47.37 (Days to first flowering- DF), 47.57–51.76 (Days to 50% flowering- DFF), 94.24-100.92 (Days to maturity- DM), 38.74–44.36 (Plant height- PH), 1.94–2.17 (Number of primary branches per plant- PBr), 6.03–8.48 (Number of secondary branches per plant- SBr), 293.76-343.36 (Number of pods per plant- NPPP), 349.08-395.66 (Number of seeds per plant- NSPP), 17.12–17.26 (100 seed weight- SW100), 34.50-36.85 (Harvest index- HI), 2472.78-2689.62 (Seed yield- SY) (Table 2). Similarly, non-nodulating genotypes (NNG) exhibited comparable trends over the seasons ranging from 44.58 to 50.60 (DF), 49.27–54.87 (DFF), 96.20-103.46 (DM), 34.86–39.28 (PH), 1.98–2.10 (PBr), 6.97–8.42 (SBr), 194.53-265.36 (NPPP), 214.48–30.23 (NSPP), 14.29–16.27 (SW100), 33.19–33.87 (HI) and 1638.78-1665.05 (SY) (Table 2). In addition to this, checks exhibited a range of 42.61–43.38 (DF), 47.50-48.53 (DFF), 93.26–98.52 (DM), 54.02–

	Df	DF	DFF	DM	PH	PBr	SBr	NPPP	NSPP	SY	SW100	HI
Fixed Effects												
Season	1	5304.05**	3420.17**	5658.18**	1710.99**	144.85**	341.85**	1.8	15.35	178.75**	809.92**	87.48**
Rep (Season)	4	2.19	4.6	3.51	4.95	4.77	14.08**	0.3	1.45	3.81	0.4	5.49
Genotype	229	27.82**	23.31**	14.83**	36.13**	3.77**	1.53**	7.69**	10.26**	20.95**	46.96**	8.49**
Season×Genotype	229	12.58**	10.47**	9.14**	6.15**	3.63**	1.93**	6.04**	7.49**	8.17**	11.07**	7.81**
Random Effects												
Block (Rep*Season)		0.03	0.07	0.05	0.20	0	0.09	0.27	0.32	0	0	0.17
Residual-Season1		1.19	1.23	1.83	2.13	0.03	3.63	2.40	2.49	138,066	0.27	2.57
Residual-Season2		0.95	1.11	1.54	3.16	0.03	2.87	2.54	1.94	34,966	0.20	1.68
Comparison of Nodulation Vs Non-nodulation												
Nodulation	Yes	45.06	49.67	97.58	41.55	2.05	7.26	314.97	368.36	2685.23	17.26	35.67
	No	47.59	52.07	99.83	37.07	2.04	7.70	225.7	255.15	1651.91	15.38	33.53
Mean difference		-2.55**	-2.40**	-2.25**	4.48**	0.02	-0.44**	2.76**	3.24**	1033.32**	1.88**	2.15**
SE		0.07	0.07	0.09	0.10	0.01	0.10	0.11	0.10	19.79	0.03	0.10
%Change in nodulation		5.33(↓)	4.62(↓)	2.26(↓)	12.08(↑)	0.74(↑)	5.74(↓)	39.55 (↑)	44.37 (↑)	62.55(↑)	12.21 (↑)	6.40 (↑)

Table 1. Analysis of variance (ANOVA) for agro-morphological, yield, and yield component traits in Chickpea genotypes under pooled conditions. **Significance at 1% level, (↓)-% decrease, (↑)-% increase, DF-Days to first flowering, DFF-Days to 50% flowering, DM-Days to maturity, PH-Plant Height, PBr-Number of primary branches per plant, SBr-Number of secondary branches per plant, NPPP- Number of pods per plant, NSPP-Number of seeds per pod, SY-Seed yield, SW100-100 seed weight, HI-Harvest Index.

59.63 (PH), 1.99–2.07 (PBr), 5.06–7.81 (SBr), 296.37-321.63 (NPPP), 320.70–338.00 (NSPP), 21.55–21.67 (SW100), 31.68–34.34 (HI), 2644.09-2667.64 (SY) (Table 2) over the seasons.

The median values (black solid line) for agro-morphological and yield component traits across seasons were provided using Violin plot analysis (Fig. 1). All the traits exhibited almost consistent median values except for PH, PBr, and SW100 for both NG and NNG over the seasons, highlighting the importance of seasonal variations in the breeding selection process. Notably, the observed ranges of agro-morphological traits and yield components of NG, NNG, and checks, emphasize the inherent variability within the genotypes (nodulating and non-nodulating) across the seasons, which underscores the superiority of nodulating genotypes in terms of productivity.

Genetic parameters for the yield and agro-morphological traits

The genetic parameters for yield, yield components, and agro-morphological traits in NG and NNG chickpea are presented in Supplementary Tables 2 and 3, respectively. For NG (Supplementary Table 2), low Genotypic coefficient of variation (GCV) and Phenotypic coefficient of variation (PCV) were observed for DF, DFF, and DM; moderate to low for PH; low to moderate for PBr; and high to low for SBr. The genetic advance of mean (GAM) estimates was low for DM; low to moderate for DF and DFF; low to high for SBr; and moderate to high for PH and PBr in pooled data. Yield and yield component traits showed low PCV and GCV for HI, moderate to low for SW100, and high to moderate for NPPP, NSPP, and SY. GAM estimates were high for NPPP, NSPP, and SY, while SW100 and HI had moderate to low estimates in pooled data. For NNG (Supplementary Table 3), PCV and GCV estimates were low for DF, DFF, and DM; moderate to low for PH and PBr; and high to low for SBr. GAM estimates were low to moderate for DF and DFF; low for DM; moderate to high for PH; and low to high for PBr and SBr.

High estimates of PCV, GCV, heritability, and GAM for yield and yield components were observed for NPPP, NSPP, and SY. PCV and GCV were low to moderate for SW100 and HI, and high for SY. Promising NG and NNG genotypes were identified for yield and yield components (Table 3). Of the NG, NG-172 recorded the highest yield (3966.7 kg/ha) followed by NG-88 (3730.7 kg/ha), NG-188 (3584.7 kg/ha), NG-159 (3563.8 kg/ha), and NG-13 (3561.6 kg/ha). Despite the non-significant increase in SY, it was higher than the best checks, RVG 204 and Phule Vikram (Table 3). Among NNG, NNG-206 recorded the highest yield (3153.7 kg/ha) followed by NNG-182 (2881.3 kg/ha), NNG-163 (2676.4 kg/ha), NNG-80 (2661.9 kg/ha), and NNG-152 (2648.5 kg/ha), demonstrating comparable seed yield with the best checks.

Correlation of agro-morphological, yield, and yield component traits

Karl Pearson's correlation coefficients were calculated for the agro-morphological, yield, and yield component traits of NG and NNG (Supplementary Tables 4 and 5). In NG under both seasons, SY showed a significant positive correlation with PH, NPPP (except 2020-21), NSPP, and SW100. Similarly, in pooled, SY was positively associated with PH, PBr, NPPP, NSPP, and HI. DF and DFF traits showed a significant negative correlation with DM and SW100 in both the crop seasons and pooled data. Whereas PH showed a significant positive correlation with SW100 in both the seasons and pooled. For NPPP, a positive significant correlation was recorded with NSPP and HI whereas NSPP recorded a significant positive correlation with HI alone (Fig. 2).

In NNG, the SY showed a significant negative correlation with NPPP in *Rabi* 2020-21; PH, NPPP, and NSPP in *Rabi* 2021-22 whereas it was significantly positively correlated with NPPP and NSPP in pooled. In both the

Character	Season	Nodulating genotypes	Non-nodulating genotypes	Checks
Agro-morphological traits				
Days to first flowering	*Rabi* 2020-21	47.37 ± 0.75	50.60 ± 0.75	43.38 ± 0.78
	Rabi 2021-22	42.74 ± 0.68	44.58 ± 0.68	42.61 ± 0.70
	Pooled	45.12 ± 0.50	47.60 ± 0.51	42.99 ± 0.52
Days to 50% flowering	*Rabi* 2020-21	51.76 ± 0.77	54.87 ± 0.77	48.53 ± 0.80
	Rabi 2021-22	47.57 ± 0.72	49.27 ± 0.73	47.50 ± 0.73
	Pooled	49.72 ± 0.53	52.08 ± 0.53	48.01 ± 0.54
Days to maturity	*Rabi* 2020-21	94.24 ± 0.93	96.20 ± 0.93	93.26 ± 0.97
	Rabi 2021-22	100.92 ± 0.86	103.46 ± 0.87	98.52 ± 0.81
	Pooled	97.63 ± 0.63	99.85 ± 0.64	95.89 ± 0.63
Plant Height	*Rabi* 2020-21	38.74 ± 0.95	34.86 ± 0.93	54.02 ± 1.07
	Rabi 2021-22	44.36 ± 1.19	39.28 ± 1.19	59.63 ± 1.29
	Pooled	41.07 ± 0.76	37.05 ± 0.76	56.82 ± 0.84
Number of primary branches per plant	*Rabi* 2020-21	2.17 ± 0.13	2.10 ± 0.12	1.99 ± 0.14
	Rabi 2021-22	1.94 ± 0.12	1.98 ± 0.12	2.07 ± 0.12
	Pooled	2.05 ± 0.09	2.04 ± 0.09	2.03 ± 0.09
Number of secondary branches per plant	*Rabi* 2020-21	8.48 ± 1.12	8.42 ± 1.11	7.81 ± 1.11
	Rabi 2021-22	6.03 ± 0.99	6.97 ± 0.99	5.06 ± 0.99
	Pooled	7.29 ± 0.75	7.70 ± 0.75	6.44 ± 0.75
Yield component traits				
Number of pods per plant	*Rabi* 2020-21	343.36 ± 1.13	194.53 ± 1.12	321.63 ± 1.15
	Rabi 2021-22	293.76 ± 1.12	265.36 ± 1.13	296.37 ± 1.01
	Pooled	316.20 ± 0.80	225.45 ± 0.80	304.34 ± 0.77
Number of seeds per pod	*Rabi* 2020-21	395.66 ± 1.16	214.48 ± 1.15	338.00 ± 1.17
	Rabi 2021-22	349.08 ± 1.04	307.23 ± 1.04	320.70 ± 1.00
	Pooled	370.74 ± 0.78	254.91 ± 0.78	324.48 ± 0.77
100 seed weight	*Rabi* 2020-21	17.25 ± 0.36	14.29 ± 0.36	21.55 ± 0.37
	Rabi 2021-22	17.26 ± 0.31	16.47 ± 0.32	21.67 ± 0.32
	Pooled	17.12 ± 0.24	15.38 ± 0.24	21.61 ± 0.24
Harvest index	*Rabi* 2020-21	36.85 ± 1.15	33.19 ± 1.16	31.68 ± 1.11
	Rabi 2021-22	34.50 ± 0.92	33.87 ± 0.93	34.34 ± 0.91
	Pooled	35.75 ± 0.74	33.53 ± 0.75	33.01 ± 0.72
Seed yield	*Rabi* 2020-21	2472.8 ± 132.1	1665.1 ± 263.8	2667.6 ± 263.1
	Rabi 2021-22	2689.6 ± 147.0	1638.8 ± 132.7	2644.1 ± 132.4
	Pooled	2685.2 ± 263.8	1652.3 ± 147.7	2655.9 ± 147.3

Table 2. Mean performance (± SE) of nodulating and non-nodulating genotypes of RIL population agro-morphological, yield and yield components traits in Chickpea.

cropping seasons and pooled season, a significant positive correlation was recorded between DF and DFF, PH and SW100, and NPPP and NSPP (Fig. 2).

Scatter plot for yield and yield component traits in Chickpea RILs

The relationships between two numeric variables in the data set for the target traits and the performance of the genotypes in two environments were analyzed using a scatter plot (Fig. 3). The RILs that were in the 2nd and 4th coordinates are adaptable when considering the environment as the main factor while, those in the 1st and 3rd coordinates are stable across the environments. Specifically, genotypes situated in the 1st and 3rd coordinates were identified as NG and NNG, respectively.

The scatter plot analysis revealed distinct performance patterns for yield and yield component traits across different seasons in the NG and NNG (Fig. 3). In pooled (2nd and 3rd coordinate), RILs 172, 88, 188, 159, and 13 for seed yield; RILs 206, 156, 13, 172 and 17 for NPPP; the RILs 172, 40, 206, 13, 56 for NSPP; RILs 92, 199, 194, 17 and 63 for HI; RILs 163, 157, 194, 22, 68 for SW100 exhibited better performance, emphasizing the trait-specific adaptability of genotypes (Fig. 3). For *Rabi* 2020-21 (4th coordinate), the RILs 172, 88, 188, 178, 159 for SY; the RILs 172, 188, 178, 40, 159 NPPP; the RILs 172, 98, 188, 221, 13 for NSPP; the RILs 92, 199, 194, 17 and 63 for HI; RILs 163, 22, 207, 68, 157 for SW100 whereas for *Rabi* 2021-22 (2nd coordinate), the RILs 209, 101, 110, 151, 136 for SY; the RILs 156, 94, 4, 199, 196 for NPPP; RILs 156, 4, 94, 102, 175 for NSPP; the RILs 114, 22, 67, 199, 194 for HI; the RILs 163, 157, 169, 185, 208 for SW100 (Fig. 3) were scattered in their respective coordinates

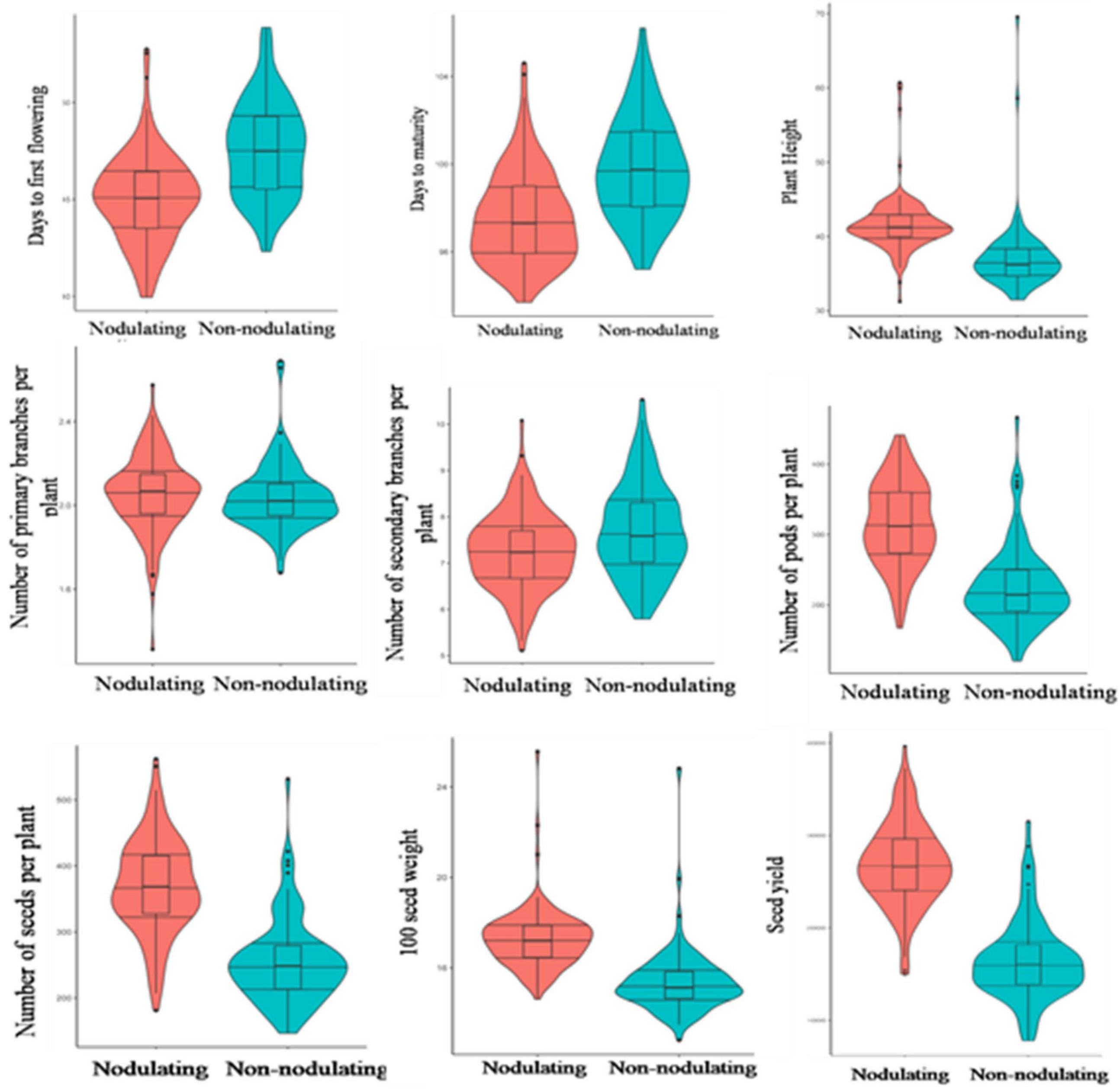


Fig. 1. Variation in agro-morphological and yield component traits under nodulating and non-nodulating genotypes (pooled analysis).

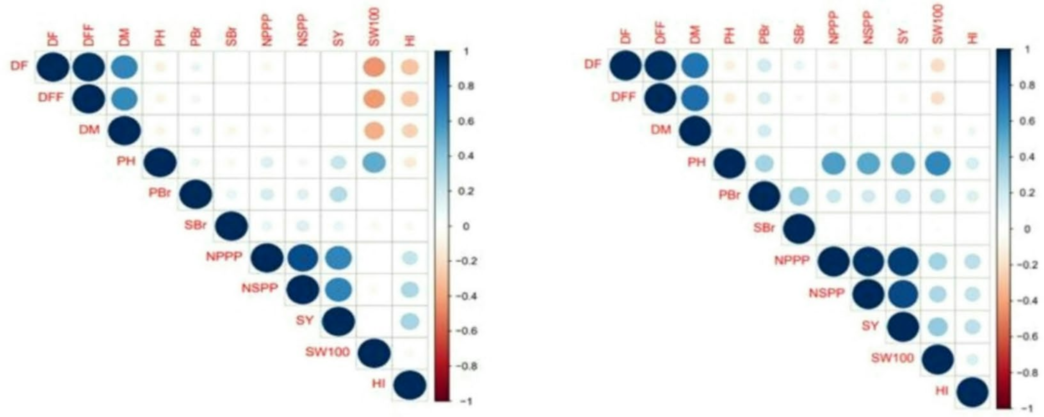
from other RILs depicting their better performance in their respective seasons, highlighting their potential for adaptability and yield enhancement.

Genotype × trait (GT) bi-plot

The GT bi-plot analysis revealed that 53.92% of the trait variation could be explained, with PC1 and PC2 accounting for 40.72% and 13.20% of the total variance, respectively (Fig. 4). Apart from the checks, genotypes 68, 159, 13, 17, 187, and 123 demonstrated superior adaptability for the traits SY, PH, and SW100 across the seasons. Conversely, genotypes 213, 128, 106, 109, and 46 exhibited the highest adaptability for traits DFF and DM across the seasons (Fig. 4). Of the checks, PhuleVikram and RVG 204 were best adaptable for the traits SY, PH, and SW100 in both the seasons (Fig. 4). Notably, there were differences in genotype rankings for HI between seasons, as indicated by angles greater than 90° between vectors. Based on the vectors, the ranking of the genotypes for SY, PH, and SW100 can be observed as, PhuleVikram = RVG 204 > RILs 68 > 159 > 13 > 17 > 187 > 123. The polygon view depicted in Fig. 4 illustrated the distribution of genotypes in the trait bi-plot, highlighting important yield, yield components, and agro-morphological traits in chickpea.

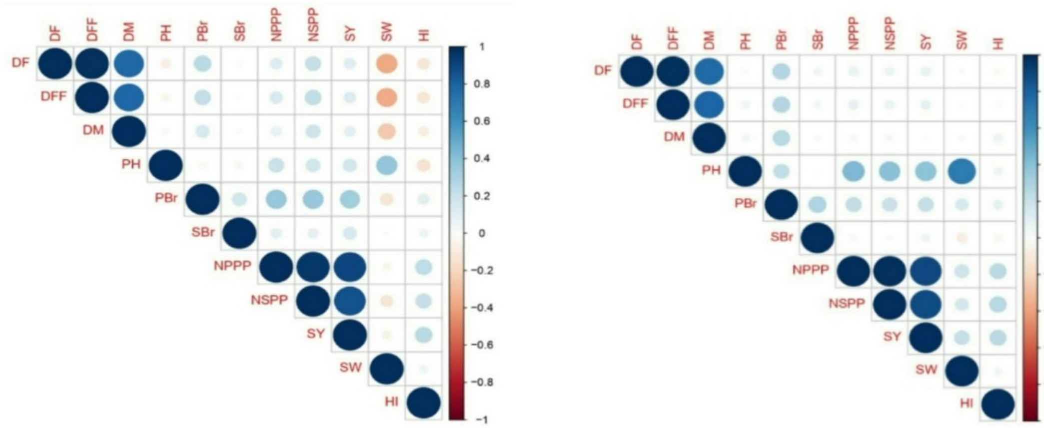
S.No	Genotype	Days to first flowering	Days to 50% flowering	Days to maturity	Plant Height (cm)	Number of primary branches per plant	Number of secondary branches per plant	Number of pods per plant	Number of seeds per plant	100 seed weight (g)	Harvest Index (%)	Seed yield (kg/ha)
Nodulating genotypes												
1	NG-172	51.28	55.85	104.09	44.32	2.11	8.11	425.19	562.11	15.71	35.14	3966.7
2	NG-88	46.74	51.39	99.15	41.09	2.35	5.86	294.62	335.90	17.49	34.98	3730.7
3	NG-188	47.29	51.52	100.34	40.14	2.43	7.99	408.33	502.47	15.91	33.64	3584.7
4	NG-159	42.52	47.13	96.21	44.65	1.87	7.99	350.80	446.68	16.80	36.86	3563.8
5	NG-13	46.20	50.52	97.71	43.22	2.34	7.00	430.07	514.43	18.17	36.60	3561.6
Non-nodulating genotypes												
6	NNG-206	48.74	53.41	99.14	42.12	2.06*	8.26	466.59	531.88	15.48	35.05	3153.7
7	NNG-182	49.40	54.25	97.31	38.43	2.04*	6.50	232.36	243.31	16.40	35.24	2881.3
8	NNG-163	53.32	58.08	101.52	69.55	2.29	6.87	242.60	248.73	24.83	30.26	2676.4
9	NNG-80	49.44	52.70	99.93	38.71	2.04	8.33	375.88	401.02	15.57	34.67	2661.9
10	NNG-152	46.80	51.35	97.54	40.07	1.82	8.21	383.81	407.67	14.76	34.09	2648.5
Checks												
12	Phule Vikram	43.47	47.93	96.63	57.14	2.05	5.68	363.23	375.45	17.57	35.13	3201.6
13	RVG 204	43.54	48.96	96.40	59.97	2.26	6.98	381.76	409.69	21.01	32.76	3478.4
14	NBeG 47	45.02	49.72	95.71	60.69	1.73	6.47	167.34	180.91	25.57	28.92	1501.8
15	JG 14	39.95	45.44	94.82	49.50	2.09	6.61	305.02	331.87	22.31	35.23	2441.8
16	ICC 4918NN	51.26	46.77	97.53	39.13	1.88	7.05	248.72	278.34	14.87	33.35	1614.4
17	ICC 4918	49.75	45.47	97.20	42.99	2.12	6.50	198.44	234.43	17.69	35.88	2231.4
	CD (0.05)	3.71	3.57	3.3	4.88	0.1	0.1	82.72	107.91	1.94	1.54	912

Table 3. Details of promising nodulating and non-nodulating genotypes identified for yield and yield related traits.



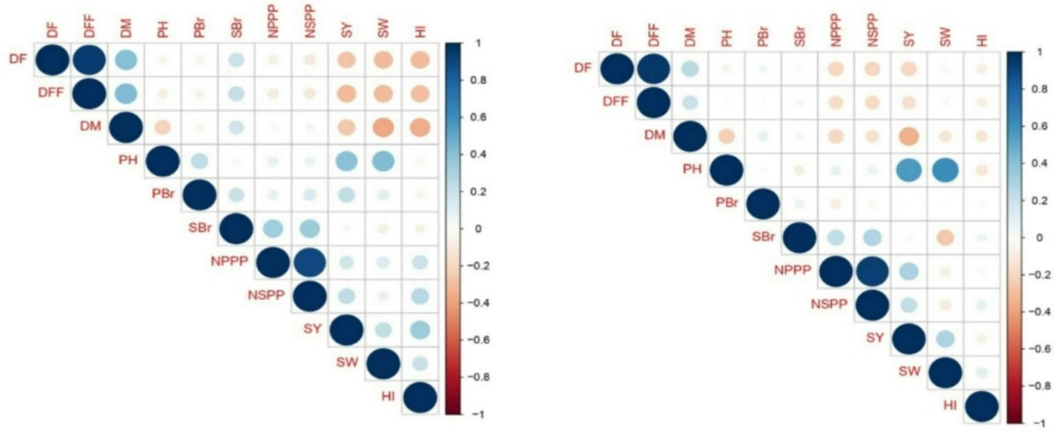
Nodulating genotypes - pooled

Non-nodulating genotypes - pooled



Nodulating genotypes – *Rabi* 2020-21

Non-nodulating genotypes – *Rabi* 2020-21



Nodulating genotypes – *Rabi* 2021-22

Non-nodulating genotypes – *Rabi* 2021-22

Fig. 2. Correlation of yield and yield components in nodulating and non-nodulating chickpea genotypes (DF-Days to first flowering, DFF-Days to 50% flowering, DM- Days to maturity, PH-Plant height, PBr- Number of primary branches per plant, SBR- Number of secondary branches per plant, NPPP- Number of pods per plant, NSPP- Number of seeds per plant, SW100-100 seed weight, HI-Harvest index, SY-Seed yield).

Discussion

The formation of rhizobium nodulation is a key symbiotic mechanism in legume crops for their adaptation to marginal environments. The quantitative assessment of their impact on plant growth and economic yields is crucial for cultivar improvement and optimizing agricultural productivity. In this study, the selected parents are

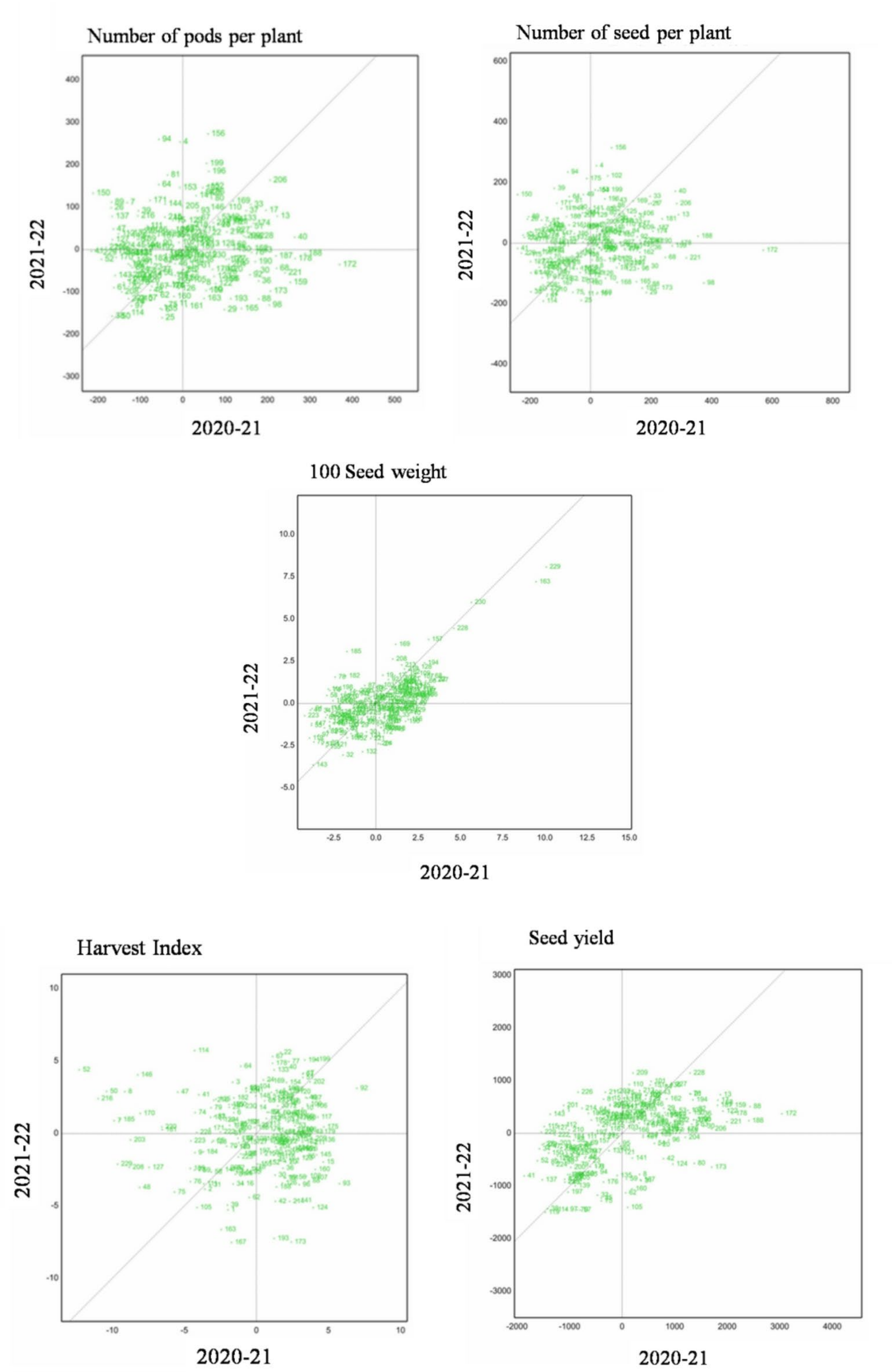


Fig. 3. Scatter Plot for yield and yield component traits in the RILs of ICC 4918NN x ICC 4918 of chickpea.

landraces, distinguishing one as a non-nodulating mutant (ICC 4918NN) derived from the other germplasm (ICC 4918). By employing the RGA approach, we successfully generated a RIL population within a mere 18 months, a testament to the efficiency and robustness of the methodology utilized[43]. While the potential of RGA has been acknowledged in other crops such as pea[44], pigeon pea[45], barley and wheat[46], and canola[46], its application in practical breeding programs remains largely unexplored. The variance analysis revealed a significant difference ($P < 0.01$) among the RILs for all the traits under the study. This indicates the presence of ample variability for the

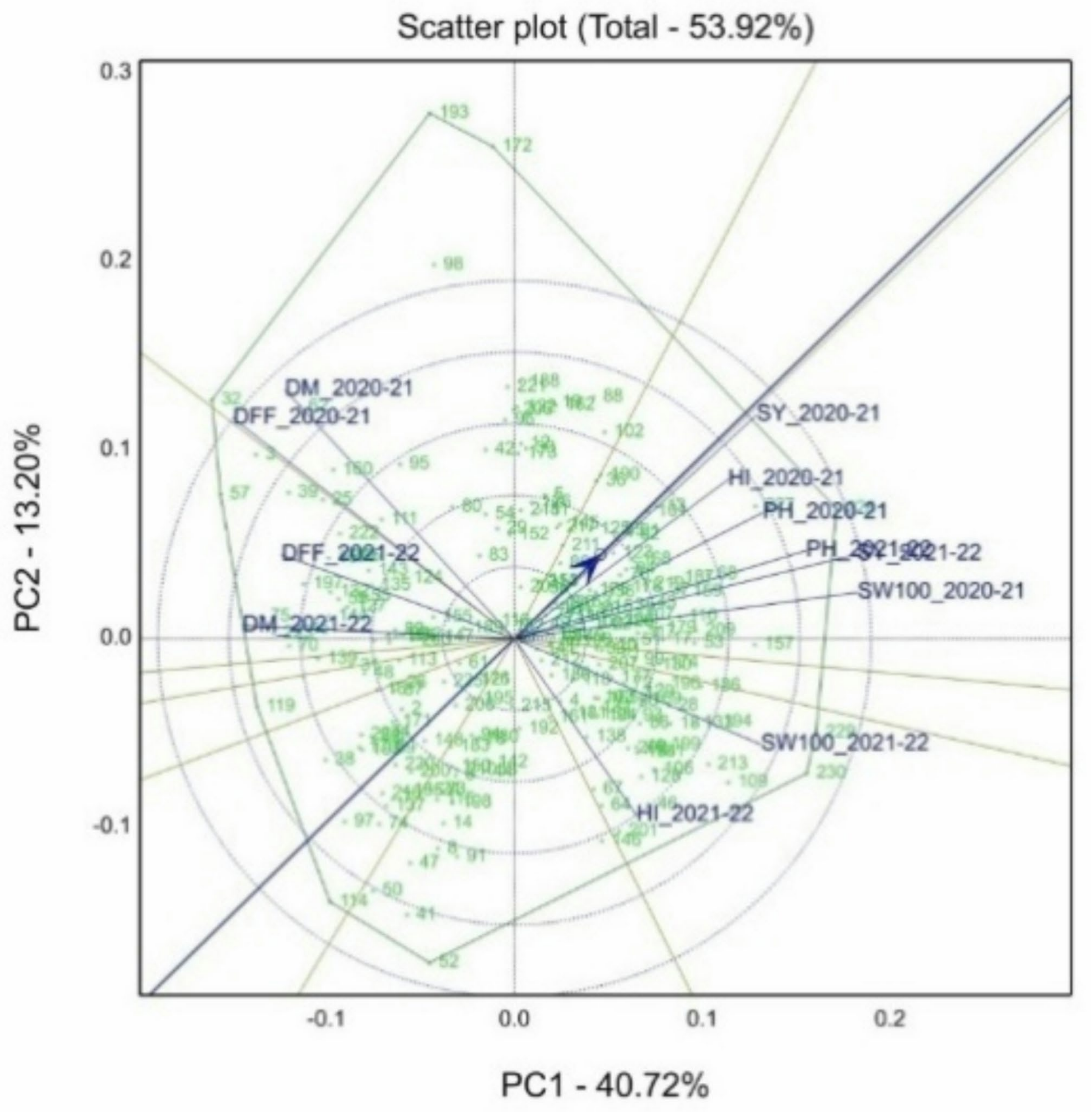


Fig. 4. Polygon view of genotypes in trait bi-plot for important yield, yield components, and agro-morphological traits in chickpea (DF-Days to first flowering, DFF-Days to 50% flowering, PH-Plant height, SY-Seed yield, SW100-100 seed weight, HI-Harvest index).

traits in the population (Table 1). Significant genetic variability among genotypes was observed in earlier studies evaluated for nodulation-related traits in chickpea[47,48].

A notable finding in our study is the substantial increase in yield (62.55%) in NG compared to NNG. The improvement was majorly contributed by the increase in the NPPP (39.5%) and NSPP (44.4%). On a moderate level, nodulation has reduced the flowering time and maturity and enhanced the PH, SBr, SW100 and HI (Table 1). These results indicate that NGs were more efficient in synthesizing photosynthates, which led to produce more number of flowers for generating a large number of pods and seeds[49,50] than NNG. In addition, the yield advantage reflects not only the inherent genetic potential of the legume plant but also the synergistic effects of the established symbiosis between roots and rhizobial bacteria. The result agrees with an earlier study in chickpea, which reported a 31% higher yield in NG compared to NNG counterparts[51,52]. The current study, with a more pronounced yield advantage, emphasizes the significance of rhizobial nodulation in optimizing chickpea productivity.

The poorer performance of NNG may be attributed to the deficiency of nitrogen fixation through nodulation, emphasizing its crucial role in chickpea productivity. The reliance on alternative nitrogen sources becomes crucial for NNG genotypes, and supplementing nitrogen in the form of fertilizers may be necessary to enhance yield[53]. This aligns with previous findings in chickpea and groundnut, where NNGs in nitrogen-rich soils could attain yields on par with NG's[54,55]. Though the degree of reliance on nodulation for nitrogen fixation varies throughout legumes, it is perpetually present. For instance, in soybean, nodulation can contribute to a significant proportion of the plant's nitrogen needs, with non-nodulating variants often displaying stunted growth and reduced yields[25]. Similarly, nodulation is critical for optimal growth in common beans, especially under nitrogen-deficient conditions[56]. Further investigations into the performance of NNG and NG under varying nitrogen availability can offer insights into their adaptability and yield potential across different soil conditions.

The present investigation aimed to bridge a crucial gap in the existing knowledge base by assessing the impact of nodulation on agro-morphological and yield traits in chickpea. Our results align with existing literature on the positive effects of rhizobial nodulation on various growth characteristics, emphasizing the importance of this symbiotic association. The observed higher values in PH, PBr, biomass, and yield traits in NG compared to NNG underscore the pivotal role of rhizobial nodulation in enhancing plant growth and productivity in chickpea (Table 2) and mobilization of insoluble nutrients in the soil, leading to improved nutrient uptake in other legumes[57,58]. In addition, the absence of rhizobium nodulation resulted in a significant reduction in various growth parameters which might be due to a deficit in (a) host-dependent strain fitness[59], (b) up-regulated expression of *nif* genes related to flavonoid synthesis[60], and (c) maintenance of plant Pi (Inorganic phosphate) levels[61].

The estimation of genetic variability and inheritance through GCV, PCV and heritability allows the breeders to identify the traits with substantial genetic control and potential for selection in crop improvement programs. For phenological traits, the small difference between GCV and PCV values suggests a predominant influence of genetic factors on their variance. HI exhibited low estimates of both PCV and GCV, indicating a more substantial influence of environment (Supplementary Tables 2 and 3). This aligns with previous studies highlighting the major role of genetic components in the inheritance of flowering, maturity, and the environmental factors in determining HI[62–65]. For the traits NPPP, NSPP, HI, PBr, and SBr, a magnitude of low to high heritability was observed, suggesting several genetic factors controlling the inheritance of these traits. In particular, SY demonstrated a high magnitude of GCV, PCV, and heritability emphasizing its potential for genetic improvement through simple selection even in early generations efficiently[63,65,66,70,71]. This diverse heritability pattern underscores the importance of trait-specific breeding strategies to achieve improvements in chickpea agronomics[64,65,72,73]. High heritability coupled with high GAM was recorded for SY and SW100 across seasons (Supplementary Tables 2 and 3), indicating the predominance of additive gene action for these traits. Similar findings were reported under diverse genetic backgrounds in chickpea[71,74,75], blackgram[76] as well as in cowpea[77,78].

Interestingly, the association among SY, NPPP, and NSPP was significantly positive in both NG and NNG (Fig. 2). This indicates the possibility of simultaneous improvement of multiple traits in chickpea genotypes and cowpea advanced breeding lines[39,62,78,77]. The ability to enhance multiple traits concurrently is crucial for developing improved chickpea varieties with enhanced agronomic performance. The scatter plot analysis (Fig. 3) demonstrated distinct performance patterns for yield and yield component traits across various seasons (Pooled, *Rabi* 2020-21 & 2021-22) in the NG and NNG indicating their superior performance in their respective seasons (Fig. 3). The use of scatter plots to differentiate genotypes based on their mean performance and its strategic application enhances the accuracy of genotype selection based on mean performance which is consistent with previous research conducted in chickpea[66,67] and mungbean[68,69]. Further analysis of the genotypes for their yield and trait interactions using GT bi-plot identified the checks, Phule Vikram and RVG 204 on its vertex with high values for all the traits under the study which can be considered the best adaptable genotypes (Fig. 4). For SY, PH, and SW100, the NG #68, 159, 13, 17, 187, and 123 while 106, 109, and 46 for DFF and DM were best adaptable over the seasons. Comparable research of this kind in various crops[79–81], provide a valuable insight for the selection of superior genotypes with enhanced adaptability and nodulating nature in the chickpea breeding programs.

The impact of rhizobium nodulation on grain yield and its association with yield-related traits was prominent in different legume crops. Earlier studies in chickpea emphasized the role of nodulation in enhancing seed yield and pod development[62,71]. Similarly, the seed yield was significantly improved by 40% in cowpea[82], 33% in common bean[56], and 45.6–50% in faba bean[83,84] compared to control or non-inoculated treatments, indicating the critical role of symbiotic nitrogen fixation in achieving higher seed yields in legumes. Moreover, the crop-specific strains, soils, and weather factors are crucial in achieving yields. These shared trends highlight the conserved nature of the nodulation mechanism inherited historically for thriving legume crops under diverse agro-ecologies.

The comprehensive assessment of genetic variability, heritability, and genetic advance in this study provides a robust foundation for future chickpea breeding programs. The identification of traits with high heritability and substantial genetic advance, coupled with positive correlations among yield-related parameters, highlights avenues for effective genetic improvement. However, it's crucial to recognize the crop-specific nature of these findings and to tailor breeding strategies accordingly. Future research should delve deeper into the molecular mechanisms underpinning nodulation in chickpea and explore comparative studies across leguminous crops to enhance our understanding of these complex interactions.

Materials and methods

Description of the environment

The present investigation was carried out at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India located at 17°30′ N latitude and 78°16′ E longitude with an altitude of 549 m above sea level during the normal crop season i.e., *Rabi* 2020-21 and 2021-22. The average rainfall, humidity, minimum, and maximum temperatures were recorded as 3.1 mm, 73.8%, 14.7 °C and 29.7 °C respectively during *Rabi* 2020-21 whereas in *Rabi* 2021-22 they were recorded as 8.0 mm, 63.3%, 15.7 °C and 30.0 °C respectively (http://icrisatintranet/nrmp/agroclimatology/weather.asp).

Development of the RIL population through rapid generation advancement (RGA)

The RIL population was developed using the Rapid Generation Advancement (RGA) method[43] by crossing between two parents ICC 4918NN (female, non-nodulating type) and ICC 4918 (male, nodulating type). The hybridity confirmed F_1 seeds were advanced to F_2 in a glasshouse. From F_2 the generations were advanced by selfing and single seed decent method. In 2019-20, 307 F_2 seeds were harvested in the glasshouse and advanced to F_3 through RGA where the immature pods were harvested 50 days after sowing (DAS) and sown on the same day for generation advancement. Four generations starting from F_2 to F_6 advanced in one year (Table 4). A maximum temperature of 25 ± 1 °C, 70–80% humidity, and artificial light with the help of incandescent bulbs was provided for a period of 12 h (from 18:00–6:00) during the RGA experiment. The F_{6-7} and F_{7-8} seeds were sown in the field in *Rabi* 2020-21 and 2021-22 for agronomic characterization (Fig. 5). A total of 230 RILs (130 nodulating genotypes, 94 non-nodulating genotypes), four checks (Phule Vikram, RVG 204, NBeG 47, and JG 14), and two parents (ICC 4918NN and ICC 4918) were used in our experiment. The parental genotypes were obtained from the gene bank of ICRISAT, India. The genotypes (RIL population) were categorized based on the presence or absence of nodules in Supplementary Table 1.

Field experiment

The experimental material was sown on Vertisols in Alpha Lattice Design with three replications. Each genotype was sown with a spacing of 60 × 15 cm (inter and intra) covering an area of 1.2 m^2. Planting of the population was completed during the first week of November in *Rabi*, 2020, and in the second week of November in *Rabi*, 2021. All the standard agronomic practices were followed for better crop establishment. Two irrigations were provided, one at the initial stage of planting and the other at the podding stage to maintain sufficient moisture regime for better crop establishment. Data related to the agronomic traits such as DF, DFF, DM, PH, PBr, SBr were recorded in each plot. The genotypes were harvested when all the plants matured completely. The post-harvest data related to the NPPP, NSPP, SW100, HI and SY were recorded from their respective plots.

Screening for nodulation

To distinguish the nature of the genotypes in terms of nodulation, the RIL population was grown in semi-controlled glasshouse condition as per the protocol outlined in the previous studies of chickpea[14]. The experimental material was surface sterilized with sodium hypochlorite and treated individually with the rhizobial strains IC59 and IC76A, the predominant nodulating bacteria in south-central India[54,85]. The seeds of each genotype were sown in 9” pots filled with potting mixture composed of sterile soil, sand, and vermicompost (3:2:2). After germination, the seedlings were thinned to three and uprooted at 35 days after sowing[86], to determine the presence or absence of nodules in the planting material. Each genotype was designated as NG and NNG based on the presence or absence of root nodules (Fig. 5).

Statistical analysis

A combined analysis of variance was carried out using the SAS MIXED procedure[87], to test the significance of the main and interaction effects of seasons and genotypes, considering the season, genotype, replication as fixed, and block as random. Individual season variance was modeled into combined analysis with repeated statements using the REML (Restricted Maximum Likelihood) method. To analyze the fixed effects, BLUEs (Best Linear Unbiased Estimates) are estimated for both main and interaction effects from the pooled analysis. The significance of NG and NNG means were compared using t-statistics from the combined analysis. Karl Pearson’s correlation was performed among the agronomic traits by using the SAS CORR procedure[87].

S.No	Filial generations	Year	Growth condition	No. of plants advanced	Plant Type	
					Nodulating genotypes	Non-nodulating genotypes
1	Crossing	2018-19	Field	-	*	*
2	F_{1-2}	2018-19	Field	1	*	*
3	F_{2-3}	2019-20	Glass House	307	*	*
4	F_{3-4}	2019-20	Glass House	302	*	*
5	F_{4-5}	2019-20	Glass House	281	*	*
6	F_{5-6}	2019-20	Glass House	270	*	*
7	F_{6-7}	2020-21	Field evaluation	224	130	94
8	F_{7-8}	2021-22	Field evaluation	224	130	94

Table 4. Development of RIL mapping population of nodulating and non-nodulating genotypes.

Nodulating genotypes - RIL#108, 109, 117, 118

Non-nodulating genotypes – RIL#121, 122, 132, 135

Fig. 5. Nodulating and non-nodulating genotypes uprooted at 35 days after sowing (DAS) with intact root system [Nodulating genotypes #108,109, 117, and 118) (Top), Non-nodulating genotypes #121, 122, 132, and 135) (Bottom)].

The magnitude of variation and its utility can be explained by genetic parameters like GCV, PCV, broad sense heritability, and GAM. These parameters were also estimated for the target traits using the SAS CORR procedure[87]. The estimates of PCV and GCV were classified as high (> 20%), moderate (10–20%) and low (< 10%)[88]; heritability as high (> 60%), moderate (30–60%), low (< 30%)[89]; GAM as high (> 20%), moderate (10–20%) and low (< 10%)[89].

To visualize the associations among the traits and the trait profiles of the genotypes a Genotype × Trait (GT) bi-plot was generated. It is based on Single Value Decomposition (SVD) of trait-wise standardized data ($Z \sim N(0,1)$). The correlation between the traits was interpreted by using cosine angles of the vectors in between the traits. The angle < 90°, > 90°, and equal to 90° states the positive, negative, and no correlation between the traits, respectively. As the vector length of a particular trait explains the variation in genotypes, the longest vector explains more variation among the genotypes and the shortest vector depicts a very low level of variation among genotypes for a given trait.

Conclusion

The RIL mapping population was developed in less than two years, the first report in chickpea to show the practical application of the RGA protocol in developing breeding material. The influence of nodulation on yield was distinctly favorable in nodulating genotypes, characterized by increased SY, biomass, and PH compared to NNG. The stable performing genotypes with high yield and early flowering nature were mostly nodulating in nature depicting the beneficial effect of nodulation on the crop growth under moisture stress conditions. The promising genotypes identified can serve as donors for use in chickpea breeding programs. Furthermore, the results emphasize the critical role of compatible *Rhizobium* strain/s in achieving optimal yields. The study highlights that the absence of nodulation can lead to substantially lower yields in chickpea. Therefore, the distribution and presence of *Rhizobium* strains in cultivated fields emerge as influential factors affecting final yield levels. To enhance the understanding of nodulation in chickpea, further research on identifying the genomic regions/QTLs/markers associated with nodulation is in progress to enhance our knowledge and provide valuable insights into the molecular mechanisms controlling nodulation in chickpea, ultimately contributing to improved crop productivity and promoting sustainable agricultural practices.

Data availability

The data of this article supporting the conclusions will be made available by the corresponding author upon request.

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S.R.N: Data collection and curation, Trial establishment, Software, Visualization, Writing-original draft preparation. M.D.M: Data curation, formal analysis, validation, writing the manuscript. U.C: Field layout, Intercultural operations, Data curation. S.S.G: Conceptualization of rhizobium inoculation, Methodology, Review, and editing the manuscript. S. R.V: Methodology, Review, and editing the manuscript. S.V: Rhizobium culture preparation and application, glasshouse evaluations. S.T: Conceptualization, Review, and editing of the manuscript. R.S: Data curation, Formal analysis, Editing of the manuscript. A.V: Statistical Analysis, Validation of Results, Visualization. H.K: Methodology and editing of the manuscript. S.S: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

International guidelines and legislation

This experimental research on chickpea (*Cicer arietinum* L.) crop adheres to international guidelines and legislation to ensure ethical, sustainable, and scientifically robust practices. The study complies with institutional, national, and international standards, including the Convention on Biological Diversity (CBD) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), ensuring the sustainable use and conservation of biodiversity.

Additional information

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