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ORIGINAL ARTICLE

Genotyping-by-sequencing based genetic mapping reveals large number of epistatic interactions for stem rot resistance in groundnut

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Abstract

Key message **Genetic mapping identified large number of epistatic interactions indicating the complex genetic architecture for stem rot disease resistance.**

Abstract Groundnut (*Arachis hypogaea*) is an important global crop commodity and serves as a major source of cooking oil, diverse confectionery preparations and livestock feed. Stem rot disease caused by *Sclerotium rolfsii* is the most devastating disease of groundnut and can cause up to 100% yield loss. Genomic-assisted breeding (GAB) has potential for accelerated development of stem rot resistance varieties in short period with more precision. In this context, linkage analysis and quantitative trait locus (QTL) mapping for resistance to stem rot disease was performed in a bi-parental recombinant inbred line population developed from TG37A (susceptible) × NRCG-CS85 (resistant) comprising of 270 individuals. Genotyping-by-sequencing approach was deployed to generate single nucleotide polymorphism (SNP) genotyping data leading to development of a genetic map with 585 SNP loci spanning map distance of 2430 cM. QTL analysis using multi-season phenotyping and genotyping data could not detect any major main-effect QTL but identified 44 major epistatic QTLs with phenotypic variation explained ranging from 14.32 to 67.95%. Large number interactions indicate the complexity of genetic architecture of resistance to stem rot disease. A QTL of physical map length 5.2 Mb identified on B04 comprising 170 different genes especially leucine reach repeats, zinc finger motifs and ethyleneresponsivefactors, etc., was identified. The identified genomic regions and candidate genes will further validate and facilitate marker development to deploy GAB for developing stem rot disease resistance groundnut varieties.

Introduction

Groundnut, also known as peanut (*Arachis hypogaea* L.), is an important global crop commodity and serves as a major source of cooking oil, diverse confectionery preparations and livestock feed. Stem rot caused by *Sclerotium rolfsii*, a necrotrophic soilborne fungus, is most serious among biotic stress of groundnut, which commonly occurs in the tropics, subtropics and other warm temperate regions of the world (Deepthi and Reddy 2013). The disease occurs in almost all the groundnut-producing areas with yield loss commonly ranging from 10 to 40% but can reach over 80% in heavily infested fields (Akgul et al. 2011; Bera et al. 2014). The pathogen (*S. rolfsii*) mainly infects the stems, but it also attacks leaves, pods and all parts of the plant, therefore causing severe damage during all stages of crop growth (Cilliers et al. 2000; Ganesan et al. 2007). Although several

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strategies, such as cultural methods and use of chemicals and fungicides, can be employed to control this disease, these methods cannot be used effectively due to late diagnosis of disease. Further, the above strategies bring extra financial burden on farmers in addition to damaging the ecosystem. Such situation can be dealt by developing stem rot resistant varieties using reliable host–plant resistance and cultivation in farmers' field. In order to ensure timely replacement of improved varieties with enhanced resistance to stem rot, GAB has the potential to facilitate accelerated development of improved varieties with more precision (Varshney et al. 2009; Pandey et al. 2012; Varshney et al. 2013).

In order to deploy GAB, tightly linked markers are required for precise selection of breeding lines. Among all the trait mapping approaches, sequencing-based genetic mapping would be the most suitable method for performing high-resolution mapping for candidate gene discovery and marker development (Pandey et al. 2016). We can generate thousands of data points for conducting high-resolution trait mapping using next-generation sequencing technologies such as genotyping-by-sequencing (GBS), whole-genome resequencing (WGRS) or SNP array-based genotyping (Pandey et al. 2017). Most recently, a sequencing-based trait mapping approach, QTL-Seq, has been successfully deployed for detection of genomic regions, candidate gene discovery and marker development for foliar disease resistance (Pandey et al. 2017). Considering the cost-effectiveness, GBS seems to have comparative advantages over other sequencing-based methodologies. Nevertheless, several mapping efforts were successfully deployed in past using different genotyping methods including GBS leading to identification of genomic regions and linked markers for several agronomic, quality and disease resistance traits (Vishwakarma et al. 2017).

Molecular breeding has already demonstrated its strength in accelerated improvement of target traits in groundnut including disease resistance traits (Varshney et al. 2014). Now, reference genome for cultivated tetraploid (https://peanutbase.org/node/587309) has also become available in addition to earlier available genome assemblies of diploid progenitors (Bertioli et al. 2016; Chen et al. 2016). The availability of tetraploid genome assembly will further enhance the precision of sequence analysis, annotation, candidate gene discovery and marker development. Further, recombinant inbred line (RIL) population provides better option for generating replicated and multi-season phenotyping data in different locations to decipher the environmental effect on target traits. Such phenotyping data are must for detection of stable and consistent QTLs followed by detection of linked markers for use in breeding. Considering above facts, this study reports development and use of a RIL population for generating GBS-based genotyping data and multi-environment phenotyping data for resistance to stem rot disease. The results of this study helped in developing better trait understanding and mapping leading to successful identification of genomic regions controlling stem rot resistance.

Materials and methods

Plant materials and development of RIL population for stem rot disease

A recombinant inbred line (RIL) population was developed using TG37A (stem rot susceptible) × NRCG CS85 (stem rot resistant) comprising of 270 individuals (RILs) at ICAR-Directorate of Groundnut Research (DGR), Junagadh, India (Fig. 1). The resistant parent, NRCG CS85, had pedigree of (CT 7-1 × SB11) × *A. kretschmeri* with growth habit type—Virginia bunch and resistant to multiple diseases, viz. PBND, stem rot, late leaf spot, rust and *Alternaria* leaf blight (Bera et al. 2011). This parent was selected and used as the resistant donor based on its stable resistance for stem rot disease over the years and locations (Bera et al. 2011). On the other hand, the female parent, TG37A (TG 25 × TG 26), with botanical type Vulgaris and growth habit type Spanish bunch, is high yielding, moderately tolerant to collar rot, rust and LLS, and drought tolerant and possess fresh seed dormancy up to 15 days (Kale et al. 2004).

The hybridization was performed in net house at ICAR-Directorate of Groundnut Research (DGR), Junagadh, India, in the rainy season of 2013. The pots (30 cm diameter and 35 cm height) containing mixture of dry soil, fine sand and well-decomposed farmyard manure (3:1:1 proportion) were kept on raised platforms. One seed per pot was sown, and a total of 15 plants for female parent while 10 plants for male parent were raised. Emasculation and pollination were performed as described by Nigam et al. (1990) with minor modifications. The single seed of putative hybrids (F_1s) obtained through crossing were grown in cups filled with fine sand and screened with polymorphic SSR primers detected between parents. The identified hybrid plants were transferred to earthen pots, and the confirmed F_1s were advanced to F_2s by selfing. The single seed descent (SSD) method under field conditions was used till F_6 generation for developing RIL population (Fig. 1).

Phenotyping for stem rot resistance and yield traits

For phenotyping, a total of 270 RILs (F_6 generation) were planted in 3 replications along with their parents in earthen pots under controlled P-II glasshouse conditions in three seasons [*rainy* 2016 (S1), *post-rainy* 2017(S2) and *rainy* 2017(S3)] (Fig. 1). The plant height was measured in centimetres (cm) from the ground level to the tip of the main

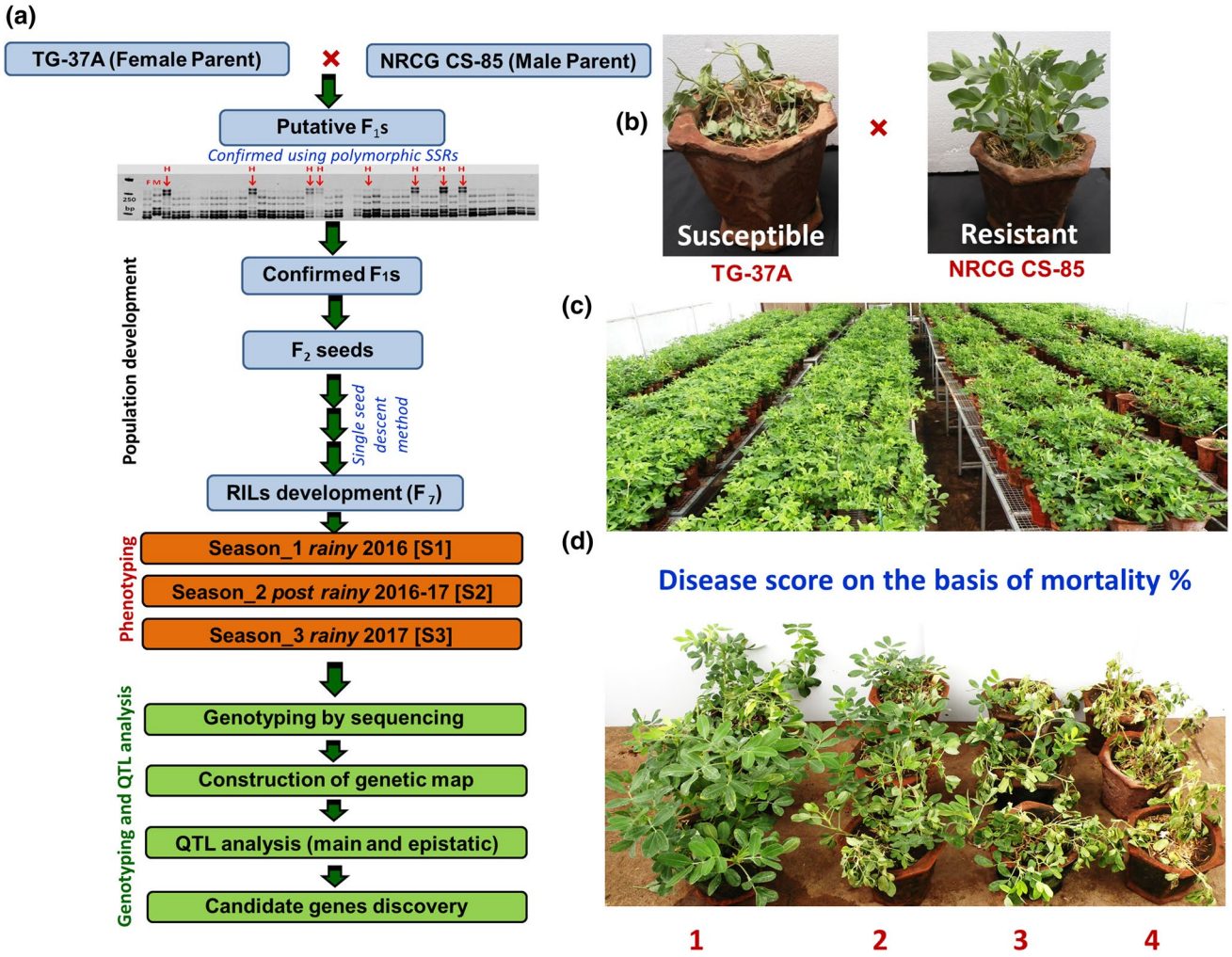


Fig. 1 Flow chart for population development, high-density genotyping and multi-season phenotyping. **a** Development of RIL population following SSD method followed by phenotyping and genotyping; **b** Susceptible (TG 37-A) and resistant (NRCG CS-85) parents used for developing RIL population; **c** 70-days-old plants used for the infection with *Sclerotium rolfsii* spores; and **d** Mortality percentage recorded at 6DAI, 9DAI and 12DAI after infection

stem after 60 days of sowing. Number of primary branches and total number of secondary branches were recorded after 60 days of sowing. Stem surface of each RIL was observed after 60 days of sowing for presence of hairs on the stems lines and was scored as 0: glabrous; 1: pubescent (sparse); 2: pubescent (dense). Shape of fully expanded apical leaflet of the third leaf of the main stem was observed and scored as 1: ovate and 2: lanceolate. Length of ten mature pods and seeds from each RIL was measured in mm after harvesting. Shelling percentage was calculated for each RIL as kernel weight/pod weight and expressed in percentage.

For disease screening, 70-days-old plants were infected with *S. rolfsii*. To do so, wheat straw was spread to the soil surface of the pots and each plant was infected by applying the *S. rolfsii* which was multiplied on autoclaved sorghum grains for 15 days at 2 g per plant around the base of the main stem. Wilting (%) of plants was recorded at regular interval of 3 days. First observation was recorded at 6 days after inoculation (6 DAI), second at 9 days after inoculation (9 DAI) and third at 12 days after inoculation (12 DAI) in season S1, S2 and S3. Disease intensity was measured using 1–4 scale (modified scale of Shokes et al. 1998) as described in Table 1.

DNA extraction and sequencing

DNA from 270 RILs and the two parents was extracted using NucleoSpin Plant II kit (Macherey-Nagel, Duren, Germany). The DNA quality and quantity were checked on 0.8% agarose and then Qubit 2.0 fluorometer (Thermo Fisher Scientific Inc., USA). Genotyping-by-sequencing (GBS) (Elshire et al. 2011) was performed for entire RIL

Table 1 Scoring of disease reaction, disease intensity and rating scale used (modified from the 1 to 5 scale of Shokes et al. 1998)

S. No.	Disease reaction	Disease intensity	Rating scale
1	R: Resistant	< 10% drying of plant	1
2	MR: Moderately resistant	10 to 25% drying of plant	2
3	MS: Moderately susceptible	> 25 to 50% drying of plant	3
4	S: Susceptible	> 50% drying of plant	4

population for simultaneous SNP discovery and genotyping of mapping population. To do so, 10 ng DNA from each RIL was digested using the restriction enzyme *ApeKI* endonuclease which recognizes the site: G/CWCG. The ligation enzyme, T4 ligase, was used to ligate the digested products with uniquely barcoded adapters. Such digestion and ligation were performed for each RIL, and then, equal proportion from each sample was mixed to construct the libraries. These libraries were then amplified and purified to remove excess adapters. These DNA libraries were then sequenced on HiSeq 2500 platform (Illumina Inc, San Diego, CA, USA) to generate genome-wide sequence reads.

Sequence analysis and SNP calling

The sequence raw reads in the form of FASTQ files generated for RIL population and parental genotypes were used for SNP discovery using TASSEL v4.0 (Bradbury et al. 2007). The draft genome sequences of groundnut progenitors, namely *A. ipaensis* and *A. duranensis*, were used as reference assembly for SNP calling (Bertioli et al. 2016; Chen et al. 2016). Initially, the in-house script was used to detect perfectly matched barcode with four base remnant of the digestion site of the restriction enzyme in sequencing reads generated for RIL and parental genotypes. The above information on barcoding was then used for sorting and demultiplexing the sequence reads. These reads were then used for trimming up to first 64 bases starting from the cut site of the restriction enzyme. In order to filter quality sequencing data, the reads containing '*N*' within first 64 bases were identified and discarded from further analysis. Finally, those reads containing '*N*' within first 64 bases were rejected. The sequence reads which passed above quality filtering criteria (called as tags) were aligned against draft genome sequence of groundnut progenitors using Burrows-Wheeler Alignment (BWA) tool (Li and Durbin 2009). The alignment file was then processed for SNP calling and genotyping. The individuals with less than 80 Mb data were not selected for further analysis to avoid false-positive detection. Lines with more than 50% missing data and minor allele frequency (MAF) of ≤ 0.3 were filtered out. Further, imputation of missing data was carried out using FSFHap algorithm implemented in TASSEL v4.0 in the mapping populations. The imputed SNPs were again filtered with MAF cut-off of 0.2 to remove missing data, and such filtered SNPs were used for genetic mapping and QTL studies.

Phenotypic data analysis

Phenotyping data were analysed using software Genstat 15th edition. Association among different stem rot disease-related traits and yield-related traits was established using Pearson correlation and calculating a two-tailed p value with 95% confidence intervals. Skewness, kurtosis, standard deviation, p(W) p values corresponding to Shapiro–Wilk test for normality and coefficient of variation were also calculated using ICIM mapping software version 4.0 (Wang et al. 2012).

Construction of genetic linkage map

The chi-square (χ^2) values calculated for each SNP marker were used to determine the goodness of fit to the expected 1:1 segregation ratio; highly distorted and unlinked markers were filtered out and not considered for the linkage map construction. Allelic calls in the population based on the parents were assigned the numeric values of − 1, 0 or 2 as specified in the manual. The genetic map was constructed using JoinMap version 4 (Van Ooijen 2006). The grouping and ordering of markers were carried out using regression mapping algorithm. Kosambi's mapping function was used for converting the recombination frequency into map distance in centiMorgan (cM). Markers at the same locus (with 0 cM interval) were counted with zero recombination frequency. The markers were ordered in 20 linkage groups by applying the LOD score (logarithm of the odds) with LOD threshold ranging from 3 to 10 with minimum recombination frequency threshold (∂) of 50%. For better visualization and clarity, software called MapChart was used to draw final genetic map (Voorrips 2002).

Quantitative trait loci (QTLs) analysis

Composite interval mapping (CIM) using the software QTL cartographer (Wang et al. 2011) was performed using the genotyping and phenotyping data together with genetic map information. In addition, the above information was also used to perform inclusive composite interval mapping-additive (ICIM-ADD) analysis using the ICIM software (Meng et al. 2015). In ICIM, the p values for entering variables

(PIN) and removing variables (POUT) were set at 0.001 and 0.002, and the scanning step was 1.0 cM. LOD threshold value of 3.0 was used to declare the presence of a QTL. A QTL was considered to be major only if had a LOD of ≥ 3 and PVE explained > 10%; the rest were considered as minor QTLs. The QTLs were designated with initial letter 'q' followed by the trait name and chromosome number. If there were more than one QTL for a trait, then it was suffixed by the numeric values as _1, _2 and so on.

Identification of epistatic (*Q* × *Q*) and environment effect (*Q* × *E*) QTLs

Realizing the complexity of stem rot resistance, we identified $Q \times Q$ interactions (epistatic QTLs) for disease resistance to detect combined effect of two or more genomic regions (QTLs) on the stem rot disease. The genetic map information together with phenotyping data was used for identification of epistatic QTLs for stem rot using ICIM mapping software version 4.0 (Wang et al. 2012). Inclusive composite interval mapping (ICIM) for epistatic QTLs with additive (two-dimensional scanning, ICIM-EPI) method with 5 cM step and 0.001 probability mapping parameters in stepwise regression were employed in QTL analysis. The LOD threshold score of 3.0 was used as minimum significance level for epistatic QTLs.

Results

Phenotypic variation for disease resistance and yield-related traits in RIL population

Replicated and multi-season phenotyping data generated in *rainy* 2016 (S1), *post-rainy* 2017 (S2) and *rainy* 2017 (S3) for stem rot disease in RIL population showed good variation with highest PCV % of 74.0 (6 DAI) followed by 36.0% (9 DAI), and 22.3% (12 DAI) while highest GCV % of 42.1% (6 DAI) followed by 14.9% (9 DAI), 10.7% (12 DAI). The level of resistance was ranging from 0 to 100% damage across infection stages (6 DAI, 9 DAI and 12 DAI) and seasons. The parental genotypes 'NRCG CS85' showed resistance to stem rot disease with just 0–10% percentage of infection while 'TG37A' showed susceptibility with 50–100% percentage of infection. Disease scoring of stem rot disease followed normal distribution at 6 DAI. But due to high disease pressure during 9 DAI and 12 DAI, the curve was quite skewed due to high disease pressure across three seasons (S1, S2 and S3) and three treatments (6 DAI, 9 DAI and 12 DAI) (Fig. 2). All the three observations provided a clear understanding about the infection percentage, which increased with increasing time; i.e., curve is getting skewed with increasing time of infection. At primary stage of infection (6 DAI, 9 DAI), the disease did not show significant correlation with yield-related traits. However, stem rot disease showed significant negative correlation with pod length and seed length at advanced stage of infection (12 DAI) (Table 2).

Genotyping-by-sequencing, SNP discovery and genetic mapping

GBS approach was used to generate a total of 30.02 Gb (297.77 million reads) clean reads using HiSeq 2500 platform for 270 RILs segregating for stem rot disease and the parental genotypes. The reads from individual progenies ranged from 0.80 to 4.79 million reads across RIL population. A total of 1.14 million SNPs were detected across RIL population, and 36,429 SNPs were found polymorphic between the parents. Out of these polymorphic SNPs, 9115 SNPs were filtered out having MAF) of 0.3 and less than 30% missing data. These polymorphic SNPs were non-uniformly distributed across different pseudomolecules. Of the 9115 SNPs (average read depth of 70.7), further filtering based on per cent heterozygosity and polymorphic information identified 735 high-quality polymorphic SNPs for further genetic analysis. Stringent SNP selection criteria reduced number of SNPs from several millions to few hundreds.

The imputed 735 polymorphic SNPs from RIL population were used for construction of genetic map. Finally, a genetic map with 585 SNP loci was developed with a total map distance of 2430 cM. The remaining unmapped 250 loci either were highly distorted or had more missing data. The length of individual linkage groups varied from 4.9 cM (A02) to 262.5 cM (A03). Total 266 loci were mapped in A sub-genome with total map distance 1092 cM, whereas 319 loci were mapped in B sub-genome with total map distance 1337.8 cM. A maximum number of loci mapped in individual linkage group varied from 3 (A02) to 62 (B10). The average inter-marker distance per linkage group ranged from 1.5 cM (A10) to 16.1 cM (B01). The average map density was high (0.66 loci/cM) for the linkage group A10 and minimum (0.061 loci/cM) for B01 (Table 3).

Genomic regions controlling plant architecture and stem rot resistance identified using QTL Cartographer and ICIM

QTL analysis was performed using two different methods composite interval mapping (CIM) using software QTL cartographer and inclusive composite interval mapping additive (ICIM-ADD) using the software ICIM. In total, 20 main-effect QTLs (M-QTLs) were identified for various plant architecture- and disease-related traits using QTL cartographer and ICIM with LOD score ranging

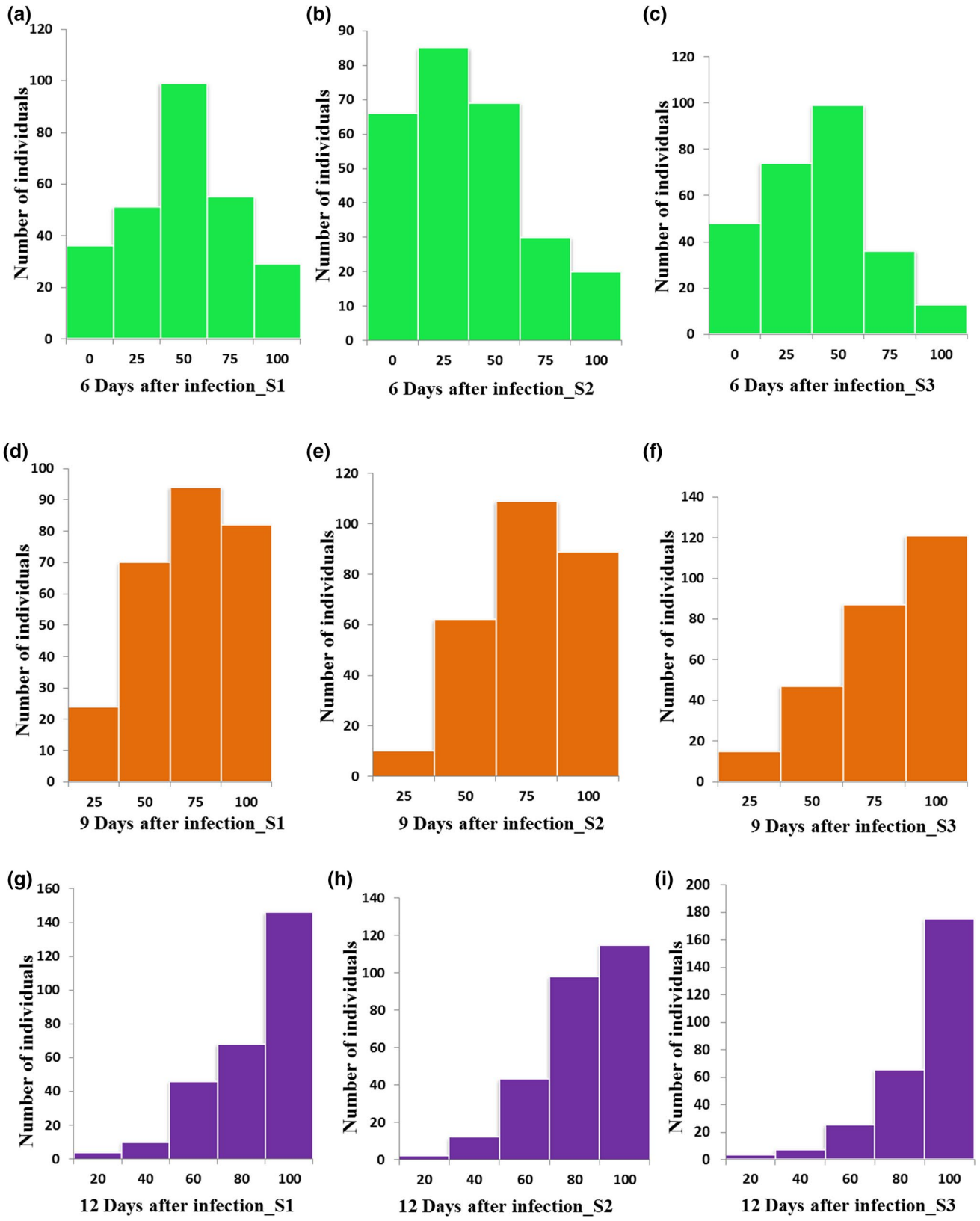


Fig. 2 Frequency distribution for stem rot disease resistance at different infection stages. The *x*-axis shows the percentage of disease, and the *y*-axis represents the number of individuals in the F_8 population. Seasons are denoted as (S1)-*rainy 2016*, (S2)-*post-rainy 2017* and (S3)-*rainy 2017*

Table 2 Pearson correlation between disease and yield-related traits

Variables	PH	LL	LW	SP	PL	PW	SL	SW
6 DAI	0.11	−0.06	−0.03	0.02	−0.05	−0.06	−0.06	−0.04
9 DAI	0.09	−0.03	−0.07	0.06	0.02	0.01	0.03	0.05
12 DAI	0.03	−0.04	−0.02	−0.09	−0.16**	0.00	−0.18**	−0.01

PH plant height, *LL* leaf length, *LW* leaf width, *SP* shelling percentage, *PL* pod length, *PW* pod weight, *SL* seed length, *SW* seed weight, *6 DAI* 6 days after infection, *9 DAI* 9 days after infection, 12 DAI 12 days after infection

*, **Statistically significant at 1% and 5% level of significance respectively

Table 3 Features of the saturated genetic map with 585 mapped loci for TG37-A x NRCG CS-85

Linkage group	Mapped loci	Length of LG (cM)	Map density (cM/loci)	Linkage group	Mapped loci	Length of LG (cM)	Map density (cM/loci)
A sub-genome				*B sub-genome*			
A01	52	221.8	4.3	B01	10	161.4	16.1
A02	3	4.9	1.6	B02	34	90.9	2.7
A03	35	262.5	7.5	B03	34	200.8	5.9
A04	19	46.8	2.5	B04	38	106.3	2.8
A05	30	97.0	3.2	B05	15	135.9	9.1
A06	17	67.2	4.0	B06	43	92.3	2.1
A07	12	94.8	7.9	B07	59	230.7	3.9
A08	7	33.0	4.7	B08	13	43.0	3.3
A09	50	202.0	3.4	B09	11	173.4	15.8
A10	41	62.5	1.5	B10	62	103.0	1.7
Total	266	1092.5	4.11		319	1337.8	4.2
Grand total	585	2430	4.15				

from 3.2 to 12.2 and phenotypic variance explained (PVE %) ranging from 4.91 to 10.34% (Table 4; Fig. 3).

QTLs for stem rot resistance

Seven main-effect QTLs were identified for stem rot resistance with LOD score ranging from 3.1 to 5.4 and PVE ranging from 5.0 to 8.5% (Fig. 3). Three QTLs for stem rot resistance were identified at 12 DAI, namely *q12DAI_S1B06.1* on B06 with 3.5 LOD and 8.4% PVE, *q12AI_S3B04.2* on B04 with 3.6 LOD and 5.5% PVE and *q12DAI_S3B10.3* on B10 with 3.2 LOD and 6.3% PVE. Two QTLs at 9 DAI were identified namely *q9DAI_S1B08.1* on B08 (4.3 LOD and 7.0% PVE), *q9DAI_S3B10.2* on chromosome B10 (5.4 LOD and 8.5% PVE). Two QTLs at 6 DAI were identified namely *q6DAI_S2B03.1* on B03 (4.2 LOD and 6.7% PVE) and *q6DAI_S3B04.2* on B04 (3.1 LOD and 5.0% PVE). A QTL on chromosome B04 at 28 cM found to be expressed at both infection stages, 6DAI and 12DAI.

QTLs for plant architecture-related traits

In total, 11 main-effect QTLs were identified for plant architecture-related traits such as root hairiness (HRN), leaf shape (LS), number of primary branches (NPB), number of secondary branches (NSB), plant height (PH), pod length (PL), shelling percentage (SP) and seed weight (SW) (Fig. 3).

Two main-effect QTLs, each on chromosome A01 (*qHRN_S1A01.1* with 3.3 LOD and 6.5% PVE) and on chromosome B09 (*qHRN_S1B09.3* with LOD 6.0 and PVE 54.4%), were identified for root hairiness, interestingly a common QTL on chromosome A06 for number of primary branches (*qNPB_S2A06.1* with LOD 3.6 and PVE 6.0%) and number of secondary branches (*qNSB_S2A06.1* with LOD 3.3 and PVE 10.3%). A single main-effect QTL was identified for leaf shape (*qLS_S1A01.1* with LOD 3.2 and PVE 4.9%) on A01, for plant height (*qPH_S1A10.1* with LOD 3.4 and PVE 6.5) on chromosome A10, for pod length (*qPL_S1B07.1* with LOD 4.2 and PVE 7.3%) on chromosome B07 and for seed weight (*qSWS1B09.1* with LOD 4.0 and PVE 6.6%) on B09. Three QTLs were

Table 4 Summary of main-effect QTLs identified for stem rot resistance and plant architecture

Trait	Season	Method (CIM/ ICIM-ADD)	QTL	Chr	Position (cM)	Left flanking marker	Right flanking marker	LOD	PVE%	Additive effect
QTLs for stem rot resistance										
12 DAI	S1	*CIM*	*q12DAI_S1B06.1*	B06	65.7	S16_126812379	S16_134339084	3.5	8.4	−0.14
	S3	*ICIM-ADD*	*q12DAI_S3B04.2*	B04	28.0	S14_103838285	S14_109137568	3.6	5.5	−3.80
	S3	*ICIM-ADD*	*q12DAI_S3B10.3*	B10	32.0	S20_127503069	S20_14425894	3.2	6.3	4.09
6 DAI	S2	*CIM*	*q6DAI_S2B03.1*	B03	100.2	S13_8258337	S13_11316796	4.2	6.7	0.17
	S3	*CIM*	*q6DAI_S3B04.2*	B04	28.0	S14_103838285	S14_109137568	3.1	5.0	0.10
9 DAI	S1	*CIM*	*q9DAI_S1B08.1*	B08	38.0	S18_113539069	S18_49660038	4.3	7.0	3.65
	S3	*CIM*	*q9DAI_S3B10.2*	B10	0.0	S20_62052887	S20_95957766	5.4	8.5	−0.16
QTLs for plant architecture										
HRN	S1	*CIM*	*qHRN_S1A01.1*	A01	123.2	S1_9530252	S1_6936082	3.3	6.5	−4.67
	S1	*ICIM-ADD*	*qHRN_S1B09.3*	B09	148.0	S19_141233870	S19_4467306	6.0	54.4	0.59
	S2	*ICIM-ADD*	*qHRN_S2B09.4*	B09	145.0	S19_141233870	S19_4467306	5.1	54.1	0.58
LS	S1	*CIM*	*qLS_S1A01.1*	A01	102.5	S1_82874814	S1_89256537	3.2	4.9	−4.95
	S1	*ICIM-ADD*	*qLS_S1A01.2*	A01	104.0	S1_82874814	S1_89256537	12.2	20.6	0.20
NPB	S2	*CIM*	*qNPB_S2A06.1*	A06	49.7	S6_83876842	S6_2770531	3.6	6.01	3.74
NSB	S2	*CIM*	*qNSB_S2A06.1*	A06	52.0	S6_83876842	S6_2770531	3.3	10.3	33.55
PH	S1	*CIM*	*qPH_S1A10.1*	A10	47.5	S10_18160081	S10_16903283	3.4	6.5	0.21
PL	S1	*CIM*	*qPL_S1B07.1*	B07	65.0	S17_68870700	S17_65719160	4.2	7.3	−0.31
SP	S1	*ICIM-ADD*	*qSP_S1A09.1*	A09	154.0	S9_90565863	S9_108801949	3.8	6.3	2.69
	S1	*CIM*	*qSP_S1B07.2*	B07	75.5	S17_17834940	S17_26195616	3.7	9.2	−0.56
	S1	*ICIM-ADD*	*qSP_S1B06.3*	B06	34.0	S16_14446457	S16_14373891	3.4	5.7	−2.56
SW	S1	*CIM*	*qSW_S1B09.1*	B09	104.1	S19_141991194	S19_141233870	4.0	6.6	−1.82

CIM composite interval mapping was performed using QTL cartographer; *ICIM-ADD* inclusive composite interval mapping additive was performed using ICIM software

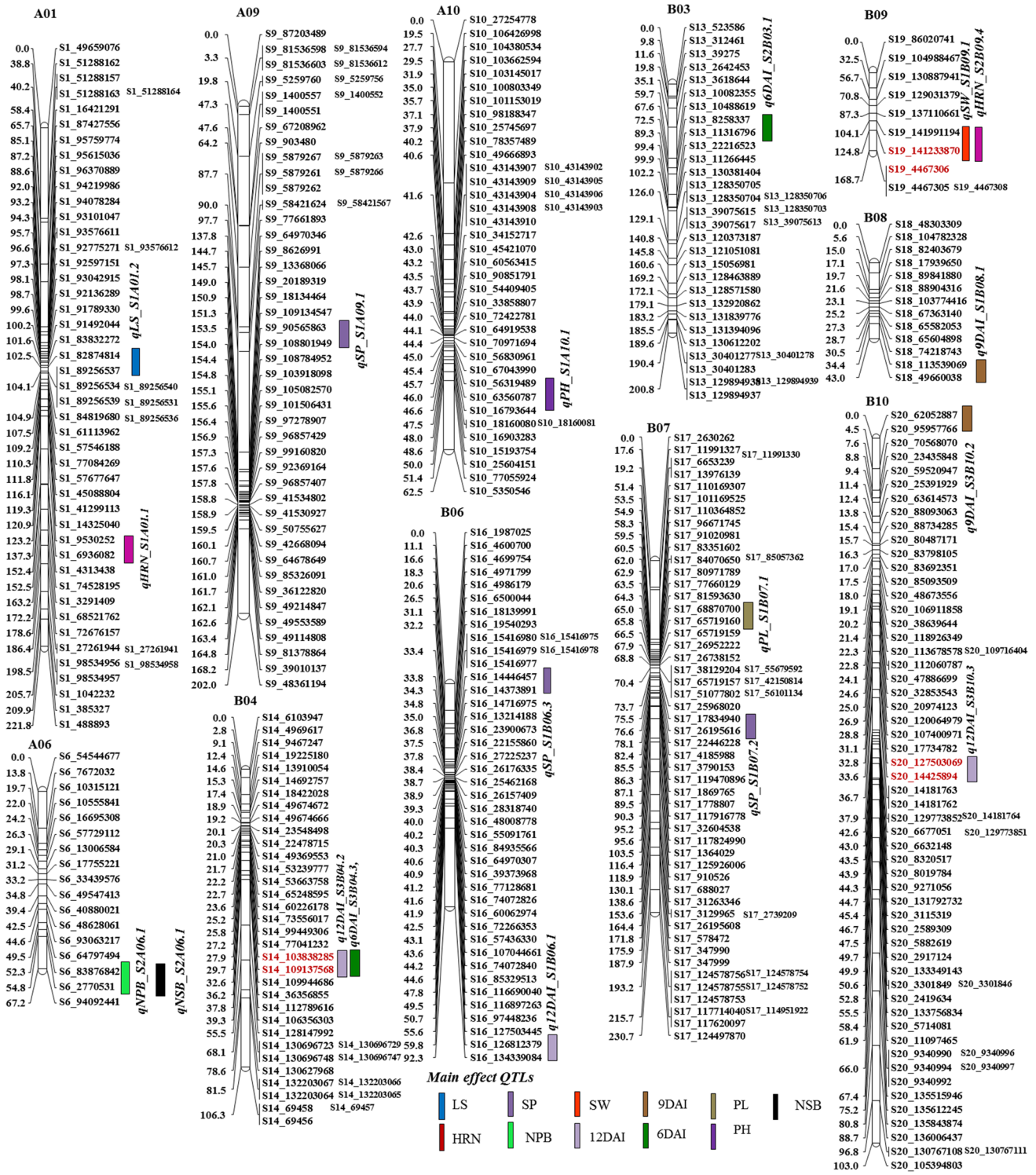


Fig. 3 Selected linkage groups (LGs) of dense genetic map of the RIL population showing main-effect QTLs associated with resistance to stem rot resistance and plant architecture

identified for shelling percentage namely *qSP_S1A09.1* on A09 (LOD 3.8 and PVE 6.3%), *qSP_S1B07.2* on B07 (LOD 3.7 and PVE 9.2%) and *qSP_S1B06.3* on B06 (LOD 3.4 and PVE 5.7%).

Epistatic (QTL × QTL) QTLs for stem rot disease resistance

Epistatic interaction ($Q \times Q$) analysis identified a large

number of epistatic QTLs (total 514) with the large range of PVE % (5.8–59.0%). Across three (S1, S2 and S3) seasons, 169 epistatic QTLs were identified for 12 DAI, 196 epistatic QTLs for 9 DAI and 149 epistatic QTLs for 6 DAI (Table 5, Fig. 4). Among epistatic QTLs, 8 consistent epistatic QTLs with high PVE were identified across the infection stages and seasons (Table 6). Three epistatic QTLs namely *qtlA01.1* (PVE 43%), *qtlB01.1* (PVE 43%) and *qtlA03.1* (PVE 61%) were identified in season S1 which interacts with various regions during resistance to stem rot disease. Similarly, two epistatic QTLs namely *qtlB04.1* (PVE 46%) and *qtlA03.2* (PVE 61%) were detected in S2 on chromosome B04 and A03, respectively. Three epistatic QTLs namely *qtlA03.3* (PVE 55%), *qtlB01.2* (PVE 43.7%) and *qtlA09.1* (PVE 53.3%) were mapped on chromosome A03, B01 and A09, respectively. The QTL on chromosome A03 expressed in two seasons (S1 and S3).

Discussion

Stem rot resistance is very important for sustainability of groundnut production in stem rot affected areas of India and in other countries. This disease is most devastating and incurs huge yield loss including fodder. GAB can facilitate faster development of stem rot resistance varieties for timely replacement in farmers' field. However, no dedicated trait mapping and marker discovery study for stem rot resistance has been conducted so far in groundnut due to which currently GAB cannot be deployed. In this context, this comprehensive study has been conducted which resulted in availability of multi-season phenotyping data, high-quality genetic map, QTLs and linked markers for stem rot resistance. The information generated through this study also provides further opportunity for fine mapping, gene discovery and marker development for stem rot disease in groundnut.

Stem rot disease: the most devastating disease in groundnut

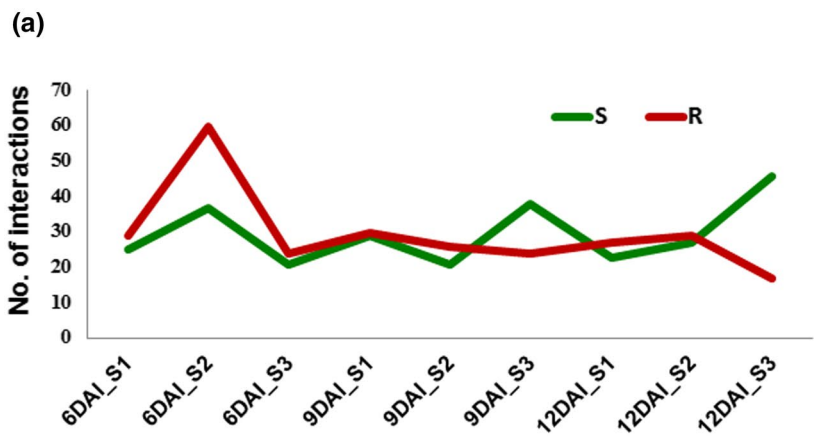
This disease has always been well known as the most devastating disease of the groundnut. The present experiment also confirms the same assumption and observed 50%, 75% and 100% loss of plant population at 6 DAI, 9 DAI and 12 DAI, respectively. The above results clearly indicated major crop damage (mostly yield and yield-related traits), highest at very advanced stage of the infection. Therefore, the advanced stage should be the perfect stage for phenotyping for conducting genetic mapping and also making reliable selection in the breeding programme for developing resistant varieties.

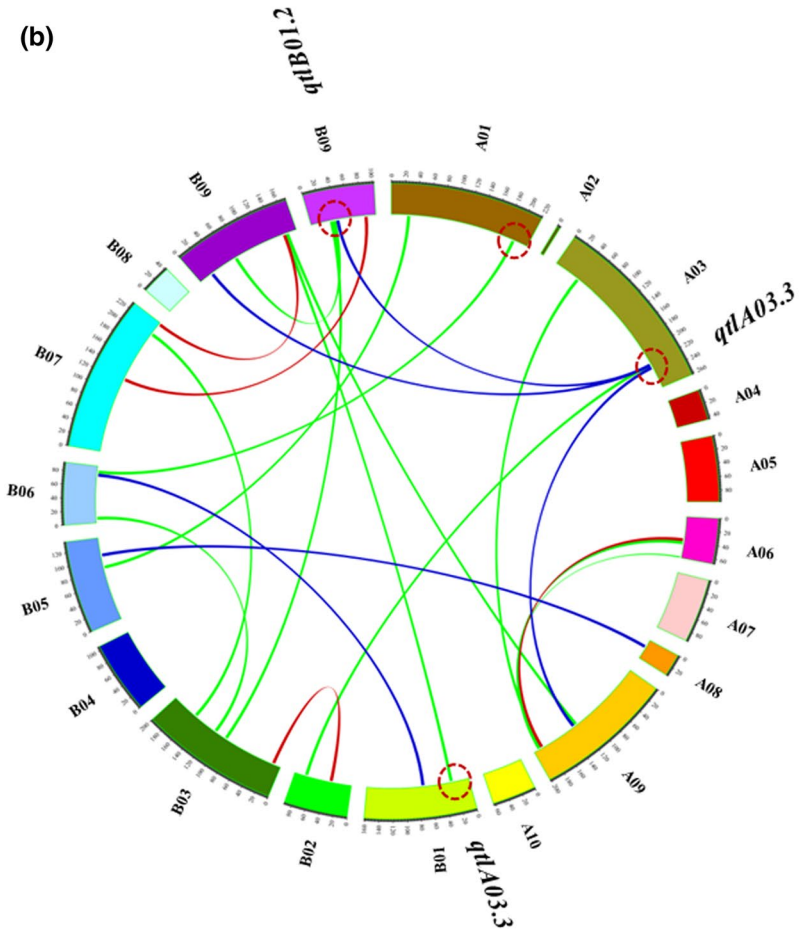
Sequencing-based genotyping facilitated development of high-quality genetic map

The large tetraploid genome (2.7 GB) of cultivated groundnut with narrow genetic base always posed challenge in achieving optimum genetic density. High marker density genetic maps are essential for conducting high-resolution genetic mapping and marker discovery. For example, the SSR-based genetic maps could achieve 135–191 mapped loci (Varshney et al. 2009; Ravi et al. 2011; Sujay et al. 2012; Gautami et al. 2012). Realizing this problem, high-density mapping has been achieved through sequencing technologies such as GBS, DArT/DArTseq and whole-genome resequencing (WGRS). The sequencing/SNP genotyping-based genetic maps were prepared with 1685 loci (Zhou et al. 2014) and DArT/DArTseq-based genetic maps with 854–1435 loci in F_2 populations (Vishwakarma et al. 2016; Shasidhar et al. 2017). The present study reports development of high-quality genetic map with 585 SNP loci with spanning the distance of 2430 cM with an average inter-marker distance of 4.1 cM. Earlier, Bera et al. (2017) tried to develop SSR-based genetic map for conducting mapping for the same

Table 5 Summary of Epistatic QTLs detected in RIL population (TG37-A x NRCG CS85)

S. No.	Trait name	Total number of epistatic ($Q \times Q$) interactions	Epistatic ($Q \times Q$) interactions contributing towards susceptibility	PVE range	Epistatic ($Q \times Q$) interactions contributing towards resistance	PVE (%) range
1	12DAI_S1	50	23	6.2–60.0	27	4.8–61.7
2	12DAI_S2	56	27	5.2–20.6	29	4.4–44.1
3	12DAI_S3	63	46	5.0–38.9	17	4.5–41.3
4	9DAI_S1	54	29	6.0–59.1	30	5.2–58.1
5	9DAI_S2	97	21	6.1–42.9	26	5.5–32.4
6	9DAI_S3	45	38	6.8–61.5	24	7.0–53.7
7	6DAI_S1	59	25	5.5–50.0	29	5.0–44.0
8	6DAI_S2	47	37	5.6–59.5	60	6.1–68.0
9	6DAI_S3	43	21	5.4–33.5	24	6.2–46.0





(c)

Selected candidates from epistatic QTLs					Disease Score of selected RILs (Mortality %)	
S1_49659076	S11_1411092	S11_124094853	S3_33444558	S3_123995085		
A	A	A	A	B	100	Susceptible RILs
A	A	A	A	B	100	
A	A	A	A	B	100	
A	A	A	A	B	100	
A	A	A	A	B	100	
A	A	A	A	B	100	
A	A	A	A	B	100	
A	A	A	A	B	100	
A	A	A	A	B	100	
A	A	A	A	B	100	
A	A	A	A	B	100	
B	B	B	B	A	29	Resistant RILs
B	B	B	B	A	33	
B	B	B	B	A	35	
B	B	B	B	A	34	
B	B	B	B	A	36	
B	B	B	B	A	35	
B	B	B	B	A	29	
B	B	B	B	A	25	
B	B	B	B	A	26	
B	B	B	B	A	31	
B	B	B	B	B	22	
B	B	B	B	A	25	

Fig. 4 Number of epistatic interactions contributing towards resistance and susceptibility against time of infection. **a** Major epistatic interactions (PVR% > 30%) for stem rot resistance; **b** Circos plot represents 20 linkage groups which are named as A01 to A10 and B01 to B10. Links inside the circle indicate the interactions between various genomic regions (epistatic loci) for stem rot resistance at different time of infections (12 DAI, 9 DAI and 6 DAI); **c** Selected five loci from the regions of epistatic QTLs showing association with the phenotype

trait, however, could only identified 12 SSR polymorphic marker loci for stem rot disease resistance. Nevertheless, the availability of reference genomes of diploid progenitors (Bertioli et al. 2016; Chen et al. 2016) and now cultivated tetraploid genomes have enhanced the precision in SNP and candidate gene discovery in groundnut.

Table 6 Summary of consistent epistatic QTLs identified across the infection stages and seasons

QTL_Name	Trait name	Chr	Position	Left marker	Right marker	Chr	Position	Left Marker	Right Marker	LOD	PVE (%)
Epistatic interactions during season 1 (S1)											
qtlA01.1	6DAI_S1	A01	15	S1_49659076	S1_51288162	A04	0	S4_115184231	S4_59704073	3.1	14.1
	9DAI_S1	A01	20	S1_49659076	S1_51288162	B07	10	S17_2630262	S17_11991327	4.6	53.5
	12DAI_S1	A01	25	S1_49659076	S1_51288162	B07	10	S17_2630262	S17_11991327	3.4	43.4
qtlB01.1	12DAI_S1	B01	115	S11_1411092	S11_124094853	B05	65	S15_137667262	S15_125202697	3.1	30.8
	9DAI_S1	B01	110	S11_1411092	S11_124094853	B07	10	S17_2630262	S17_11991327	4.7	43.8
qtlA03.1	9DAI_S1	A03	220	S3_3769933	S3_33444558	B09	20	S19_86020741	S19_104988467	3.2	58.2
	12DAI_S1	A03	220	S3_3769933	S3_33444558	A09	120	S9_77661893	S9_64970346	3.9	61.4
Epistatic interactions during season 2 (S2)											
qtlB04.1	9DAI_S2	B04	95	S14_132203065	S14_69458	B07	220	S17_117620097	S17_124497870	3.5	24.1
qtlA03.2	12DAI_S2	A03	35	S3_127066093	S3_123995085	B09	15	S19_86020741	S19_104988467	3.0	44.1
Epistatic interactions during season 3 (S3)											
qtlA03.3	12DAI_S3	A03	240	S3_3769933	S3_33444558	A03	250	S3_33444558	S3_37240291	6.4	39.3
	6DAI_S3	A03	225	S3_3769933	S3_33444558	B06	80	S16_126812379	S16_134339084	2.5	37.6
qtlB01.2	12DAI_S3	B01	25	S11_130102229	S11_11800657	B09	150	S19_141233870	S19_4467306	3.6	43.7
	12DAI_S3	B01	30	S11_130102229	S11_11800657	B03	50	S13_3618644	S13_10082355	2.6	31.5
	6DAI_S3	B01	30	S11_130102229	S11_11800657	B07	110	S17_1364029	S17_125926006	3.4	20.2
qtlA09.1	6DAI_S3	A09	170	S9_39010137	S9_48361194	B09	45	S19_104988467	S19_130887941	3.1	13.4

Genetic dissection and QTL discovery analysis indicated complex genetic nature for stem rot resistance

Use of two different genetic softwares showed difference in results where QTL cartographer showed relatively higher PVE than ICIM. A lone study conducted with just 12 mapped loci for stem rot resistance in groundnut reported one major QTL *qstga01.1* with flanking markers GM2350 and TC4H02 (Bera et al. 2017). The high-quality SNP-based genetic map developed in present study allowed to conduct high-resolution genetic mapping leading to detection of 7 main-effect QTLs for stem rot resistance at different infection periods (6 DAI, 9 DAI, 12 DAI) with cumulative 47.4% PVE. This study clearly identified 'B' genome as the source of resistance which hosted all the 7 main-effect QTLs for stem rot resistance. The analysis also identified a potential genomic region for further investigation which harbours two main-effect QTLs for stem rot resistance.

In case of complex trait, the phenotype is a cumulative effect of few or many genes which is a barrier to recognize exact region controlling the effect of phenotype. The epistasis analysis conducted in present study identified large number of epistatic QTLs explaining PVE ranging from 4.4 to 68%. It gives a clear understanding about genetic nature of stem rot disease which is a complex trait influenced by the cumulative effect of various genes across the genome. Similar results were also reported earlier for drought tolerance-related traits in groundnut (Ravi et al. 2011). In present study, the results showed an interesting trend among epistatic interactions against different time periods of infections. During the initial stage of infection (6 DAI), very few epistatic interactions were functioning in favour of susceptibility; in contrast, more interactions were contributed towards resistance. But, with increasing time after infection, the number of interactions was increased for susceptibility and decreased for resistance. This is may be due to the systemic acquired resistance (SAR) activated by (JA) and (SA) by activating effector triggered immunity (ETI) in response to infection of disease (Table 6).

Candidate genes for stem rot resistance

Based on the results obtained from QTL analysis, a genomic region of 5.2 Mb was identified with two main-effect QTLs for stem rot disease resistance. This genomic region was identified on B04 with flanking markers (S14_103838285–S14_109137568 (Fig. 5). The 5.2-Mb QTL region on chromosome B04 (1.8 cM on genetic map) harbours two main-effect QTLs for stem rot resistance explains up to ~ 11% PVE. Evaluation of this region with the peanut genome assembly followed by genome annotations identified a total of 170 genes (Table 7). This region is rich in NBS-LRR (nucleotide-binding site leucine-rich repeats), zinc finger motifs, ERF (ethylene-responsive factors), autophagy-related proteins and WRKY transcription

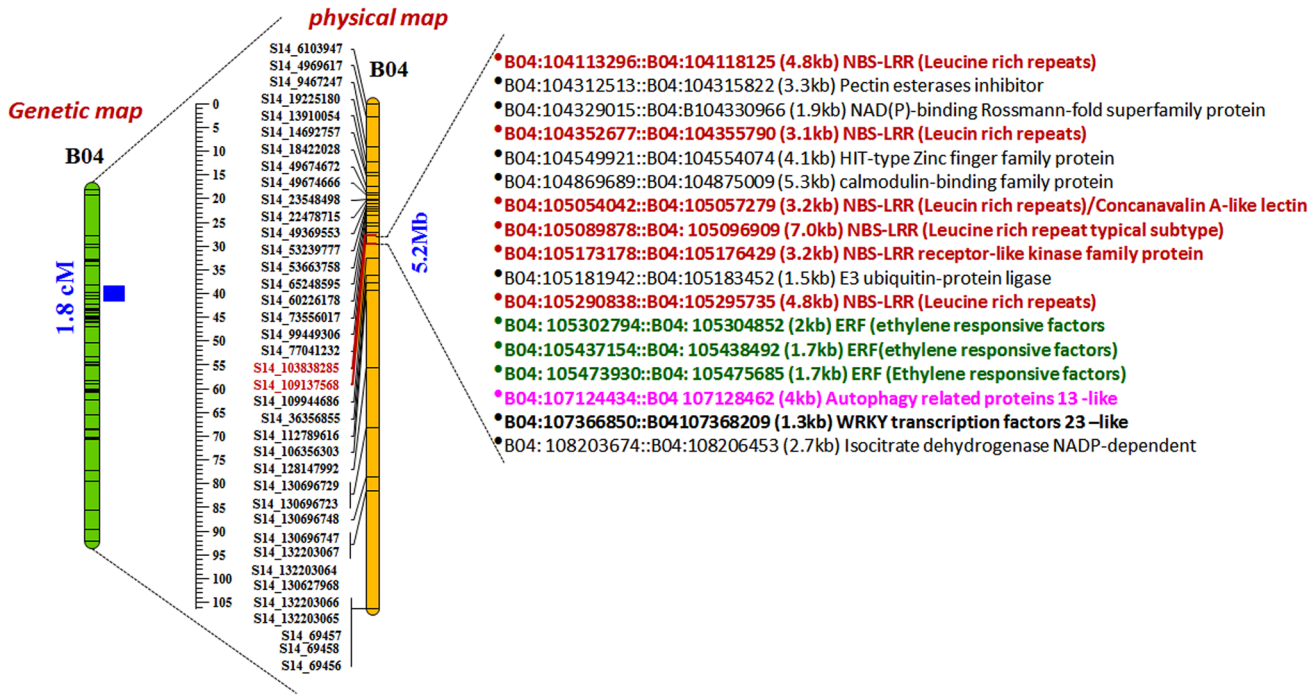


Fig. 5 Candidate genes identified in the 5.2 -Mb QTL region mapped on chromosome B04

Table 7 Disease resistance genes identified in the 5.2-Mb QTL region and epistatic QTL regions

Chr	Gene start	Gene end	Length of gene (bp)	Strand	Gene ID	Function
Genes identified in the 5.2-Mb QTL region on chromosome B04						
Araip.B04	104113296	104118125	4830	+	Araip.3TG36	Leucine-rich repeat
Araip.B04	104352677	104355790	3114	+	Araip.13LX0	Leucine-rich repeat
Araip.B04	105054042	105057279	3238	–	Araip.C6IN6	Leucine-rich repeat
Araip.B04	105089878	105096909	7032	–	Araip.WMZ64	Leucine-rich repeat
Araip.B04	105173178	105176429	3252	–	Araip.BZ3UW	LRR receptor-like kinase family Leucine-rich repeat
Araip.B04	105290838	105295735	4898	+	Araip.29WB6	Leucine-rich repeat
Araip.B04	106781989	106782734	746	–	Araip.PP2EC	Leucine-rich repeat
Araip.B04	104258699	104263382	4684	–	Araip.46XVA	Ferritin (ferric iron binding)
Araip.B04	104329015	104330966	1952	+	Araip.LXM2D	NAD(P)-binding Rossmann-fold superfamily protein
Araip.B04	104869689	104875009	5321	+	Araip.GF9L5	Calmodulin-binding family protein
Araip.B04	105181942	105183452	1511	–	Araip.XK459	E3 ubiquitin-protein ligase
Araip.B04	105302794	105304852	2059	–	Araip.U663M	Ethylene-responsive transcription factor 7-like
Araip.B04	105437154	105438492	1339	–	Araip.LL89K	Ethylene-responsive transcription factor 7-like
Araip.B04	105473930	105475685	1756	–	Araip.I6HJK	Ethylene-responsive transcription factor 3-like
Araip.B04	107124434	107128462	4029	+	Araip.VE18D	Autophagy-related protein
Araip.B04	107366850	107368209	1360	–	Araip.XVA98	Probable WRKY transcription factor
Araip.B04	108203674	108206453	2780	+	Araip.BZ4NA	Isocitrate dehydrogenase NADP dependent
Genes identified in epistatic QTL regions						
Aradu.A01	50259417	50262052	2635	+	Aradu.99WG9	Cytochrome P450
Aradu.A01	50802251	50806406	4155	+	Aradu.0IN20	Transducin/WD40 repeat-like
Aradu.A01	50805913	50807517	1604	–	Aradu.KJA6P	Riboflavin biosynthesis protein
Aradu.A01	49977606	49981865	4259	+	Aradu.U1MJX	Hydroxymethylglutaryl-CoA synthase like

factors. The majority of disease resistance genes in plants encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (McHale et al. 2006) which is largest gene family encoded by hundreds of diverse genes per genome. Interestingly, we identified 6 isoforms of NBS-LRR proteins in 5.2-Mb region which might be key potential candidate genes for stem rot resistance. The programmable cell death (PCD) is regulated by the many defence proteins containing zinc finger domain. The *Pi54* gene confers durable resistance to blast disease. Similarly, *pI8* (sunflower), *Pib* (rice), *Lr10* (wheat), *Gro1–4* (potato), *RCY1* (Arabidopsis) and *Rpg1* (barley) contain multiple number of zinc finger domains (Gupta et al. 2012). The ERF proteins involved in defence responses against pathogen infection have also been widely documented (Park et al. 2001; Shin et al. 2002; Gutterson and Reuber 2004), and overexpression of ERF genes in transgenic tobacco or Arabidopsis plants induces expression of several PR genes, resulting in enhanced resistance to various diseases caused by bacterial, fungal, viral pathogen (Zuo et al. 2007; Fischer and Dröge-Laser 2004).

WRKY transcription factors are functionally redundant and alter salicylic acid (SA) and jasmonic acid (JA) signalling in response to pathogen attack which modulates systemic defence response using effector triggered immunity (ETI) (Durrant and Dong 2004). WRKY TFs also involve in modulating the expression of several miRNA during plant defence signalling (Pandey and Somssich 2009) (ESM 3, Fig. 5).

R genes along with NBS-LRR domain play a crucial role in regulating expression of the genes involved in plant resistance (Eulgem and Somssich 2007; Ulker and Somssich 2004). The blast resistance gene Pi54 contains NFX-type zinc finger domain in their protein. The NFX1-type zinc finger proteins are a group of the human *NFX1* transcription factors (Gupta et al. 2012). Overexpressing the GmERF3 gene in tobacco showed increased resistance to *Ralstonia solanacearum*, *Alternaria alternata* and TMV under normal environmental conditions (Zhang et al. 2009).

Summary

In summary, the study has successfully performed sequencing-based genotyping leading to a good- and high-quality genetic map for stem rot disease in groundnut. Using the multi-season phenotyping, data helped in dissecting the polymorphic nature of this important disease and facilitated identification of several genomic regions for stem rot

resistance in groundnut. Most importantly, a 5.2-Mb QTL region on B04 comprising 170 genes was also discovered harbouring resistance genes such as LRR, ERF and zinc finger motifs. However, more investigation is required for further dissecting the 5.2-Mb region through fine mapping and assessing the potential of identified major-effect epistasis loci using next-generation sequencing technologies for causal resistance genes and genetic markers for use in breeding.

Author contribution statement SD, ALR, N, GPM and SC developed the RIL population, and JRD and SVC coordinated the experimental part of the project. AC performed sequencing of RIL population, and BJ and PPT phenotyped the RIL population. SSG, MKP and PS contributed to data analyses, SSG and MKP wrote the first manuscript, and RKV wrote the final version of the manuscript. RT initiated and coordinated the project. All authors reviewed the manuscript and provided suggestions.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Human or animal rights This study does not include human or animal subjects.

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