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Molecular marker validation and identification of Fusarium wilt resistant chickpea genotypes

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Abstract

***Fusarium* wilt is one of the most destructive biotic stress reducing chickpea productivity worldwide. As a step towards understanding the basis of wilt resistance in chickpea, we investigated the morpho-physiological and biochemical traits of 29 desi and 15 kabuli chickpea genotypes and screened these genotypes for fusarium wilt disease using gene based molecular markers. The number of branches per plant were significant and positively correlated with number of pods per plant (r=0.635) and number of seed per plant (r=0.556) at 1% significance level. Biochemical parameters related to stresses were also analyzed for proline (1.19-3.92 µ mol/g), sugar (23.6-37.4 mg/g), malondialdehyde (MDA) (1.1 -3.67 nmol/g) and hydrogen peroxide (H_2O_2) (10.4-21.5 µ mol/g) in seeds of these genotypes grown under normal field conditions. Molecular screening was done by using15 gene-based markers. Polymorphic Information Content (PIC) value was in the range of 0.221 to 0.695, respectively. The genotypes JG-63 and Vijay identified during the investigation could be included in the hybridization programs during development of high yielding and wilt resistant varieties. The molecular markers TA194, TA-59, TA-96, TR-19, TR-29 and TR-31 can be used as marker assisted breeding tools for screening, validation and development of fusarium wilt resistant chickpea genotypes.**

Key words: Chickpea, Fusarium wilt, proline, sugar, MDA, Polymorphic Information Content (PIC)

Introduction

Chickpea, a self-pollinating diploid (2n=2x=16) species with a genome size of 740 Mbp, is the 2nd most important food legume in the world (FAO, 2018). It is rich in protein content as well as important for soil quality due to its ability of biological nitrogen fixation. It is widely grown in countries like India (67.4%), Australia (6.21%), Pakistan (5.73%), Turkey (3.86%) and Myanmar (3.74%) (Mannur et al. 2019). In India, major chickpea growing areas are covered by Madhya Pradesh (32.97%), Maharashtra (18.36%), Rajasthan (16.70%), Andhra Pradesh (8.55%), Karnataka (8.21%), Uttar Pradesh (6.85%) and Gujarat (2.92 %). The chickpea is divided into kabuli and desi types based on seed morphology. The white colour and relatively bigger in size with thin seed coat types are kabuli. The brown with thick seed coat and smaller in size are desi types. Productivity of chickpea is, restricted due to several abiotic and biotic stresses. Major biotic stresses of chickpea include the bacterial, viral and fungal diseases. Important fungal diseases are wilt and blight caused by *Fusarium oxysporum* and *Ascochyta rabei*, respectively. Wilt of chickpea is a major limiting factor of chickpea production in the Mediterranean Basin and the Indian Subcontinent. Annual yield losses due to *Fusarium oxysporum*. f. sp. *ciceri* range from 10 to 15% but it can be devastating to individual crops and cause 100% loss under favorable conditions (Sharma et al. 2004). Till today eight distinct physiological races *i.e*., 0, 1A, 1B/C, 2, 3, 4, 5 and 6 based on variation in virulence among isolates of foc races have been reported. The genetics of six resistance races has been studied extensively (Singh et al. 1987; Sharma et al. 2004). This disease is a soil borne and its causative agent has the potential to survive in soil even in absence of host for many years. Therefore, it becomes more

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difficult to eradicate the disease completely through crop rotation or application of any chemicals.

Changes in quantity of soluble sugars, lipid peroxidation (MDA), proline and generation of active oxygen species (hydrogen peroxide) are common event associated with normal plant biochemical processes including chloroplast and mitochondrial electron transport and oxidases in the plasma membrane. These biochemical parameters are directly linked with major abiotic stresses. Chickpea pathogen grows very fast in dry soil. Analysis of chickpea genotypes in respect to proline, sugar, MDA and H_2O_2 can provide basis for resistance in field conditions.

The development of wilt resistant cultivars is both economical and environment friendly approach. Conventional breeding methods to develop wilt resistant cultivar are difficult and time consuming as compared to marker assisted selection (MAS). The use of molecular markers closely linked to wilt resistance genes can eliminate the need of creating artificial epiphytotic conditions and also save time by screening large number of germplasm lines (Pramanik et al. 2019; Adlak et al. 2019). Genomic research is accelerating breeding methods due to applications of molecular markers in crop improvement. The molecular markers for chickpea improvement, are being widely used for gene tagging, QTL mapping, genome sequencing and re-sequencing (Hiremath et al. 2012; Varshney et al. 2014; Thudi et al. 2016; Garg et al. 2018; Mannur et al. 2019). The RAPD and SSR markers have been widely used in the development of genetic linkage map of resistance genes for FOC 1–5 races. The first wilt resistance gene for H1 locus of race 1A was tagged and mapped with allele specific marker $CS27_{700}$ but other markers such as ISSR, RAPD and SSR have also been reported. The genes for fusarium wilt resistance races 1 and 4 are closely linked and ISSR marker UBC 855_{500} to a distance of 0.6 cM to wilt resistance gene (Ratnaparkhe et al. 1998a). The RAPD markers amplified the fragments linked to race 1 and race 4 of wilt resistance (Tullu et al. 1998). The marker TA27 is linked to *foc-1*, *foc-2* and *foc-3* races of fusarium wilt resistance genes (Sharma et al. 2004). The marker TA37 is linked to race-5 of foc gene with a distance of 4.4 cM (Iruela et al. 2007). Barman et al. (2014) reported close linkage (0.2 cM) of this marker with *foc-1* gene for resistance against race 1. The marker has been reported to be linked with resistant genes against all the four prevalent races of wilt viz.: *foc-1*, *foc-2*, *foc-3* and *foc-4* are linked by TA 96 within a genetic distance of 5 cM, respectively (Sharma et al. 2004). Screening of the entire reported marker for fusarium wilt of chickpea is one of the major concerns. Analysis of chickpea seed flour in respect to proline, sugar, MDA and H_2O_2 can provide basis for resistance in field conditions. To best of our knowledge, it is the first report where all these stress related parameters were studied in chickpea seeds harvested from normal field condition. Hence present work contains estimation of yield attributing morphological traits, stress related biochemical traits and screening of chickpea varieties including both *desi* and *kabuli* types for Fusarium wilt based on gene-based RAPD, DAF, ISSR and STMS markers.

Materials and methods

Plant material and experimentation

The plant material consisted of 44 chickpea genotypes received from RAK Krishi College, Sehore, Madhya Pradesh. The genotypes included both ecotypes of chick peas desi (29) i.e., JG-63, RVG-202, JGG-1, DINDORI-CHANA, BHUPDA-CHANA, JG-322, GCP-101, JG-11, RVG-203, ANNAGIRI, JG-16, JG-14, JG-6, ICCV-10, VIJAY, JG-218, RSG-888, RVG-201, ICC-4812, JAKI-9218, JG-315, GG-5, JG-74, JG-12, RVSSG-205, RVSSG-204, GBM-2, JG-62 and kabuli chana (15) i.e., KRIPA, DOLLAR, PKV-4, ICCV-2, JGK-2, BGD-128, KAK-2, JGK-5, RVKG-102, RVKG-101, JGK-1, RVSSG-30, RVSSG-37, RVSSG-24 and MNK-1.

The chickpea genotypes were grown at breeding farm, College of Agriculture, Rajamata Vijayaraje Scindia Krishi Vishwavidyala, Gwalior, Madhya Pradesh. It is located at 22°43' N Latitude and 76°54 E longitudes and altitude 618 m above the sea level. The area has sub-tropical and semi-arid climate. The experiment consisted of 44 chickpea genotypes grown in Randomized Block Design with row to row distance of 30 cm in three replications during *rabi* 2017-2018 and 2018-19. The experimental area occupied uniform topography and fertility. The uniform dosage of fertilizers was applied in the ratio of 20:40:20 (N: P_2O_5: K_2O).

Morpho-physiological traits

The data were recorded from randomly selected five plants from each plot for physiological maturity (days) from the sowing of seeds to the 90% pods leading to yellowing of plants stage, plant height (cm) from the base of the plant at the ground level to the tip of the main stem at maturity stage, number of seeds per

plant, number of pods per plant, 1000 seed weight (g), seed yield per plant (g), biological yield per plant (g) and Harvest index by dividing the total seed yield by biological yield per plant in percent.

Biochemical trait analysis

The total sugar was extracted by dehydration of glucose to hydroxymethyl furfural producing green color in 80% ethanol and estimated by the method described by Dubois et al. (1956). 100 µl of sugar extract was mixed with 0.5 ml saturated phenol (5%) and 0.5 ml of H_2SO_4 (96%) followed by incubation at 30°C for 20 min, the absorbance was measured at 490 nm. The quantity of sugar was calculated from a standard curve of glucose.

Proline levels were determined as per the method given by Bates et al. (1973). A 200 mg sample was extracted in 3% sulpho-salicylic acid followed by centrifugation at 12,000 rpm. The supernatant was mixed with 4 ml of toluene followed by vigorous shaking absorbance was measured at 520 nm.

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content, a product of lipid peroxidation (Hodges et al. 1999). Seed sample (0.5 g) was homogenized in 10 ml of 0.1% TCA. The homogenate was centrifuged at 15000 x g for 5 min. To 1.0 ml aliquot of the supernatant, 4.0 ml of 0.5% thiobarbituric acid (TBA) along with 20% TCA was added. The mixture was heated at 95 °C for 30 min followed by quick cooling in an ice bath. After centrifugation at 10000x g for 10 min, the absorbance of supernatant was recorded at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The MDA equivalent was calculated as follows:

$$\text{MDA [nmol / (mLFW)]} = \frac{A_{532} - A_{600}}{155000} \times 10^6$$

Hydrogen Peroxide (H_2O_2) content was analyzed as per the method given by Mukherjee and Choudhuri (1983). Seed (200 mg) was homogenized with 50 mM (pH 6.5) phosphate buffer and centrifuged at 8000 rpm. The absorption of reaction mixture was recorded at 410 nm.

Genotyping using gene-based markers for Fusarium wilt

Leaf samples of one-month old seedlings from chickpea genotypes were collected from experimental field. The genomic DNA from young seedlings was isolated using modified CTAB method (Murray and Thompson 1980). The quantity and quality of the DNA was checked on 1% agarose gel. The DNA of 20 ng/µl concentration was used in polymerase chain reactions (PCR).

A total of 15 gene based molecular markers for Fusarium wilt in chickpea containing two Randomly Amplified Polymorphic DNA (RAPD), one Sequence Characterized Amplified Region (SCAR), one DNA Amplification Fingerprinting (DAF), six Sequence Tagged Microsatellites (STMS) and five markers of Inter Simple Sequence Repeats (ISSR) (Ratnaparkhe et al. 1998a; b). The polymerase chain reaction were performed in 10ìl reaction mixture comprising of 1X PCR buffer, 0.1 U Taq DNA polymerase, 1 µl dNTP (1 mM), 0.5 µl of primers (10 pM) and 20 ng/µl of genomic DNA in a thermocycler (Bio-Rad, USA). The PCR protocol comprised of initial denaturation step of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, annealing cycles varied for different markers system for 30 sec, elongation at 72°C for 1 min with final extension at 72°C for 10 min. The annealing temperature of molecular markers varied from 55°C for ISSR, 54°C for STMS, 30°C for RAPD, DAF and SCAR markers. PCR amplified products of RAPD, SCAR, DAF, ISSR and STMS primer products along with standard markers were separated on 1.5% and 3% agarose gel respectively at 100 V for 2 hrs. The agarose gels contained 0.5 ìg/ml Ethidium Bromide. The amplified PCR products were visualized under UV light and photographed under Bio-Rad Gel documentation system.

The genetic profiles of genotypes were scored on the basis of difference in allele size. The major allele frequency, number of alleles per locus, polymorphism information content (PIC) and gene diversity was analyzed using Power Marker v3.25 software (Liu and Muse 2005). The dendrogram based on unweighted pair group method for arithmetic average (UPGMA) and bootstrap value of 1000 permutations was constructed using MEGA 6.0 software (Tamura et al. 2007). Based on the banding pattern data was recorded with allele pattern A/A and B/B homozygous condition and A/B for heterozygous condition and in case of no amplification (-/-) was used.

Statistical analysis

The analysis of variance (ANOVA) of morphological traits for standard error (S.E.) and coefficient of variation (CV) was calculated. The phenotypic coefficient of correlations for all the morpho-

physiological traits at maturity was analyzed using SPSS ver.19 software. The genetic similarity identified by molecular markers and taxonomic distance measured by mean genetic distance and total seed yield were analyzed using Jaccard's Similarity Index and average taxonomic distance was calculated by NTSYS-pc v2.1 software.

Results

Morpho-physiological traits analysis

The phenotypic coefficient of correlations among different traits is presented in Table 1. Significant and positive correlation was found between days to 50% flowering and days to maturity (r=0.353) and grain yield (r=0.369) at 5% significance level. Highly significant and positive correlation was found between number of pods per plant (r=0.399), number of seeds per plant (r=0.575) and harvest index (r=0.400) at 1% significance level. The number of branches per plant highly significant with number of pods per plant (r=0.635) and number of seed per plant (r=0.556) at 1% significance level (Table 1). The number of pods showed highly significant positive correlation with number of seed per plant (r=0.867), grain yield (r= 0.602) and biological yield (r=0.604) at 1% level. The negative correlation was found between number of seeds per plant and thousand seed weight (r=-0.455) and positive correlation with grain yield per plant (r=0.697) at 1% significance level.

Biochemical trait estimations

Table 2 depicts the data on biochemical analysis. Total soluble sugars and proline content. The mean value of proline content in seeds ranged from 1.19 µmol/g to 3.92 µmol/g. Highest increase was observed in fusarium wilt susceptible genotype JG-62 (3.92 µmol/g) with minimum in the genotype Vijay. The maximum sugar content was observed in wilt susceptible genotype JG-62 (37.4 mg/g) with minimum in the genotype Kripa (23.6 mg/g).

Lipid peroxidation estimated as MDA content varied among both category genotypes and it was generally higher in susceptible genotypes as compared to resistant genotypes. The highest Lipid peroxidation (3.67 nmol/g) was found in Fusarium wilt susceptible genotype JG-62 with lowest (1.1 nmol/g) for the genotypes RVG-201 and RVKG-102. Hydrogen peroxide (H_2O_2) content significantly differed amongst resistant and susceptible chickpea genotypes. The accumulation of H_2O_2 was higher in susceptible genotypes as compared to resistant genotypes. The highest H_2O_2 content (21.5 µmol/g) was observed for the genotype JG-62 with minimum (10.4 ìmol/g) for the timely sown genotypes RVKG-102 and RVSSG-24.

Biochemical estimates based hierarchical cluster analysis

According to the hierarchical cluster analysis and the content values (Table 2), the dynamic expression

Table 1. Analysis of phenotypic correlation coefficients for morpho-physiological traits in 44 chickpea genotypes

	DF_50	DM	PHT	NBP	NPP	SPP	TSW	GYD	BYD	HI
DF_50	1	0.353*	-.073	0.163	0.399**	0.575**	-.254	0.369*	0.292	0.400**
DM		1	-.333*	-.076	0.051	0.243	-.407**	-.104	-.145	0.150
PHT			1	.244	0.269	0.292	0.02	0.324*	0.300*	0.269
NBP				1	0.635**	0.556**	-0.328*	0.374*	0.361*	0.193
NPP					1	0.867**	-.411**	0.602**	0.604**	0.299*
SPP						1	-.455**	0.697**	0.671**	0.477**
TSW							1	0.250	0.259	-.002
GYD								1	0.931**	0.577**
BYD									1	0.281
HI										1

* = Significant at the 0.05 level; ** = significant at the 0.01 level

DF_50= Days to 50% flowering, DM= Days to maturity, PHT= Plant height, NBP= Number of branches per plant, NPP= Number of pods per plant, SPP= Seeds per plant, TSW= Thousand seed weight, GYD= Grain yield per plant, BYD= Biological yield per plant, HI= Harvest index per cent

Table 2. Content values of soluble sugar, proline, lipid peroxidation and Hydrogen peroxide in chickpea genotypes

S.No.	Genotypes	Soluble sugar (mg/g)	Proline (μmol g^{-1})	Lipid peroxidation (nmol g^{-1})	H_2O_2 (μmol g^{-1})
1	JG-63	28.4 ± 1.6	2.41 ± 0.34	1.23 ± 0.11	11.2 ± 1.1
2	RVG-202	29.6 ± 1.2	2.21 ± 0.47	1.34 ± 0.13	12.3 ± 1.2
3	JGG-1	30.2 ± 1.5	2.32 ± 0.32	1.40 ± 0.19	14.4 ± 0.2
4	Dindori Chana	31.4 ± 1.4	2.40 ± 0.44	1.25 ± 0.17	12.8 ± 0.4
5	Bhupda Chana	30.1 ± 1.2	2.45 ± 0.67	1.39 ± 0.16	12.7 ± 0.6
6	JG-322	28.5 ± 1.1	2.10 ± 0.78	1.28 ± 0.11	14.5 ± 1.1
7	GCP-101	26.7 ± 1.2	2.50 ± 0.56	1.36 ± 0.14	15.8 ± 1.4
8	JG-11	29.2 ± 1.3	2.71 ± 0.70	1.44 ± 0.19	10.9 ± 1.5
9	JG-16	28.5 ± 1.2	1.90 ± 0.77	1.51 ± 0.18	10.5 ± 0.5
10	RVG-203	27.4 ± 1.3	2.10 ± 0.78	1.60 ± 0.14	10.8 ± 0.6
11	Annagiri	29.1 ± 1.4	1.97 ± 0.23	1.72 ± 0.18	14.2 ± 1.1
12	JG-16	27.4 ± 1.4	1.89 ± 0.45	1.24 ± 0.15	15.9 ± 1.4
13	JG-14	29.5 ± 1.2	2.01 ± 0.55	1.40 ± 0.10	12.8 ± 1.2
14	JG-6	29.0 ± 1.3	2.15 ± 0.43	1.45 ± 0.16	11.9 ± 1.4
15	ICCV-10	30.3 ± 1.4	1.94 ± 0.87	1.62 ± 0.18	11.6 ± 1.5
16	Vijay	30.2 ± 1.5	1.19 ± 0.23	1.32 ± 0.19	11.2 ± 1.6
17	JG-218	26.4 ± 1.6	2.13 ± 0.26	1.45 ± 0.11	12.4 ± 0.7
18	RSG-888	27.8 ± 1.4	2.34 ± 0.11	1.32 ± 0.10	12.6 ± 0.8
19	RVG-201	29.6 ± 1.2	2.70 ± 0.18	1.10 ± 0.11	13.7 ± 0.9
20	ICC-4812	28.9 ± 1.1	2.10 ± 0.19	1.90 ± 0.13	13.1 ± 1.1
21	JAKI-9218	30.4 ± 1.2	1.89 ± 0.45	1.30 ± 0.12	14.2 ± 1.3
22	JG-315	28.5 ± 1.3	1.79 ± 0.56	1.20 ± 0.11	12.3 ± 0.5
23	GG-5	30.4 ± 1.4	1.86 ± 0.54	1.40 ± 0.11	13.6 ± 0.7
24	JG-74	32.1 ± 1.2	2.17 ± 0.85	1.28 ± 0.10	13.2 ± 1.2
25	JG-12	25.5 ± 1.1	2.15 ± 0.82	1.24 ± 0.11	11.6 ± 1.5
26	RVSSG-205	26.2 ± 1.4	2.10 ± 0.80	1.34 ± 0.12	11.9 ± 0.7
27	RVSSG-204	26.7 ± 1.2	1.96 ± 0.14	1.42 ± 0.14	12.8 ± 0.8
28	GBM-2	28.4 ± 1.1	1.76 ± 0.34	1.56 ± 0.12	12.6 ± 0.8
29	JG-62	37.4 ± 1.2	3.92 ± 0.41	3.67 ± 0.11	21.5 ± 0.7
30	Kripa	23.6 ± 1.3	2.11 ± 0.43	1.25 ± 0.12	12.3 ± 1.2
31	Dollar	26.4 ± 1.2	1.91 ± 0.32	1.34 ± 0.11	11.3 ± 1.3
32	PKV-4	28.5 ± 1.1	1.95 ± 0.40	1.41 ± 0.16	12.1 ± 1.4
33	ICCV-2	29.4 ± 1.2	2.09 ± 0.57	1.46 ± 0.11	13.4 ± 1.4
34	JGK-2	28.1 ± 1.3	2.14 ± 0.34	1.71 ± 0.12	14.5 ± 1.1
35	BGD-128	29.4 ± 1.4	2.43 ± 0.33	1.84 ± 0.15	11.6 ± 1.2
36	KAK-2	28.6 ± 1.2	2.21 ± 0.49	1.43 ± 0.18	13.2 ± 0.9
37	JGK-5	24.5 ± 1.3	2.16 ± 0.51	1.22 ± 0.11	12.6 ± 0.7
38	RVKG-102	26.6 ± 1.4	2.28 ± 0.60	1.10 ± 0.10	10.4 ± 0.8
39	RVKG-101	25.6 ± 1.4	2.29 ± 0.89	1.21 ± 0.11	10.7 ± 0.7
40	JGK-1	27.2 ± 1.2	2.49 ± 0.81	1.34 ± 0.11	12.3 ± 0.7
41	RVSSG-30	28.1 ± 1.4	1.31 ± 0.17	1.56 ± 0.10	11.2 ± 0.9
42	RVSSG-37	27.4 ± 1.2	1.98 ± 0.43	1.52 ± 0.11	10.9 ± 1.1
43	RVSSG-24	27.5 ± 1.1	1.77 ± 0.41	1.37 ± 0.11	10.4 ± 1.1
44	MNK-1	25.2 ± 1.1	2.19 ± 0.19	1.18 ± 0.12	11.6 ± 1.2

profile was determined and is shown in Fig. 1. Multivariate analysis based on diversity was performed using the UPGMA. The mean value of accessions falling in each cluster is presented in the generated dendrogram for all the accessions distinguished into eight clusters (I, II, III, IV, V, VI, VII and VIII). Cluster I consisted of 3 accessions encompassing JG-62, GCP-101, JG-16 as an isolated exterior group. Cluster

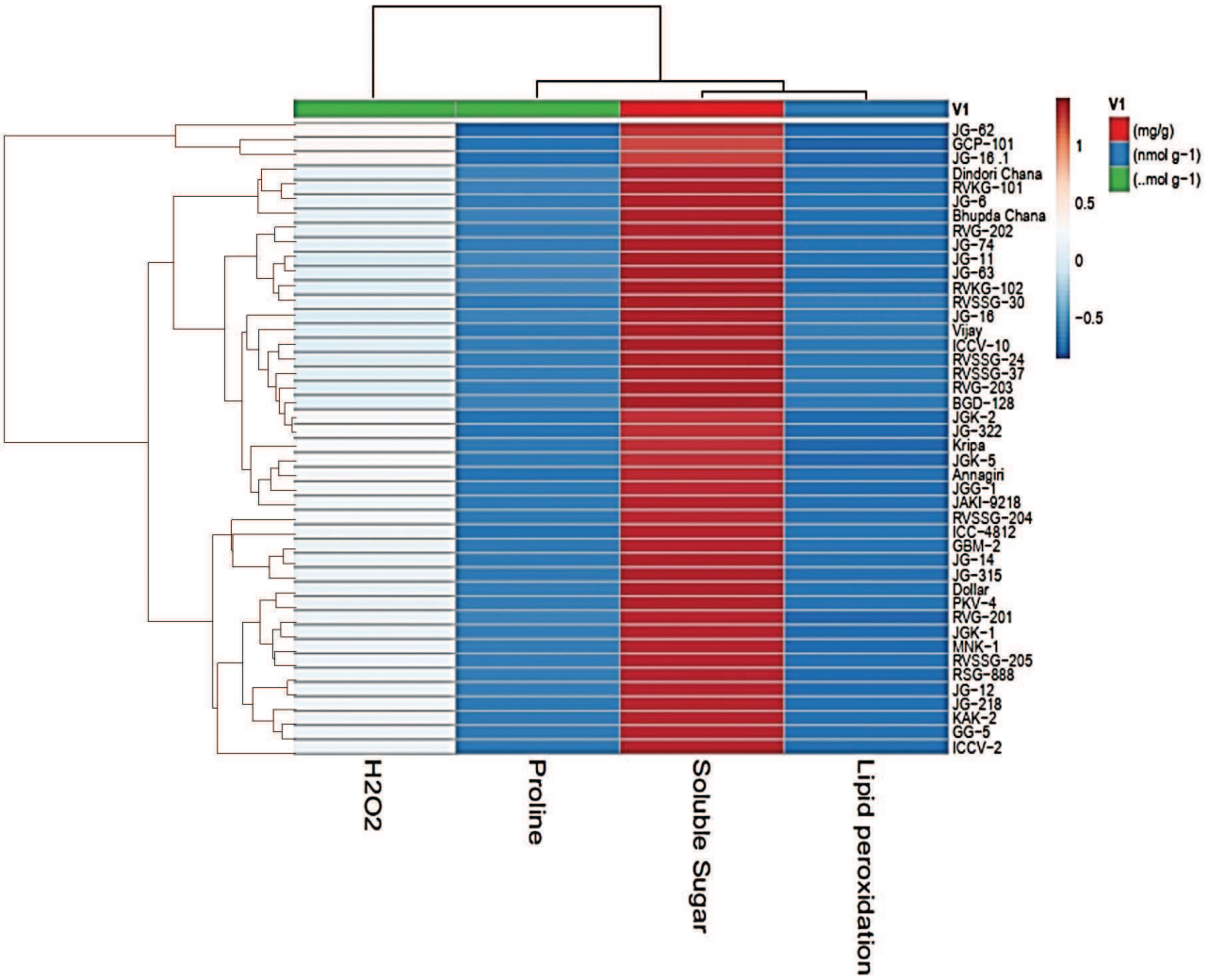

Fig. 1. Heat-map and clustering pattern of desi and kabuli chickpea genotypes for lipid peroxidation, soluble sugar, proline and hydrogen peroxide

II comprised of 4 accessions comprising of Dindori Chana, RVKG-101, JG-6, Bhupda chana. Cluster III comprised of 6 accessions namely RVG-202, JG-74, JG-11, JG-63, RVKG-102 and RVSSG-30. Cluster IV contained 10 accessions viz: JG-16, Vijay, ICCV-10, RVSSG-24, RVSSG-37, RVG-203, BGD-128, JGK-2, JG-322, Kripa. Cluster V consisted of 5 accessions JGK-5, Annagiri, JGG-1, JAKI-9218 and RVSSG-204. Cluster VI comprised of 5 accessions ICC-4812, GBM-2, JG-14, JG-315 and Dollar. Cluster VII contained 6 accessions namely PKV-4, RVG-201, JGK-1, MNK-1, RVSSG-205, RSG-888and VIII comprised of 4 accessions namely JG-12, JG-218, KAK-2, GG-5.

Molecular characterization

A total of 55 alleles were identified with an average of 3.71 alleles per locus for different markers (Table 3). We found gene diversity (0.241 to 0.736) with average value of 0.527 and polymorphic information content (PIC) range (0.221 to 0.695) with an average of 0.46. The primer TR 29 showed highest gene diversity (0.736) and PIC (0.695) values while the lowest gene diversity (0.241) and PIC (0.221) value was recorded for the primer UBC-880. The major allele frequency varied from 0.36 (TR29) to 0.86 (UBC880) with a mean value of 0.59.

The fusarium wilt susceptible variety JG 62 and resistant varieties JG 315, JG 322 and JG 36 were considered as standard for marker validation. Out of 15 primers of different categories (RAPD, DAF, ISSR and STMS), only one RAPD primer didn't get amplification. The fourteen polymorphic molecular markers were able to distinguish fusarium wilt tolerant and susceptible genotypes (Fig. 2).

Table 3. Gene based markers presenting Major allele frequency (MAF), number of alleles, gene diversity and Polymorphic Information Content (PIC) in desi and kabuli chickpea

Marker	Major allele freq	Genotype number	Allele number	Gene diversity	PIC value
CS27	0.5909	7.0000	7.0000	0.5981	0.5599
CS27A	0.6136	4.0000	4.0000	0.5527	0.4974
OPU171	0.4091	4.0000	3.0000	0.6371	0.5599
UBC825	0.4545	4.0000	4.0000	0.6663	0.6083
TA59	0.5568	4.0000	3.0000	0.5684	0.4903
TA96	0.5227	4.0000	3.0000	0.5692	0.4807
TR19	0.4773	4.0000	3.0000	0.6043	0.5223
TA194	0.6932	4.0000	3.0000	0.4491	0.3812
TR29	0.3864	9.0000	5.0000	0.7363	0.6943
TR31	0.5114	3.0000	3.0000	0.5958	0.5166
UBC811	0.8182	3.0000	3.0000	0.3130	0.2894
UBC841	0.7500	4.0000	4.0000	0.4081	0.3747
UBC864	0.7045	4.0000	4.0000	0.4401	0.3769
UBC880	0.8636	3.0000	3.0000	0.2407	0.2207
Mean	0.5966	4.3571	3.7143	0.5271	0.4695

The genetic relationships among chickpea genotypes are presented in molecular based UPGMA tree (Fig. 2). All the genotypes were grouped into 4 clusters and among them cluster 2, 3 and 4 are grouped with resistant and moderately resistant varieties for wilt resistance. Cluster 1 included five genotypes i.e., ICC-4812, RSG888, JG-62, RVSSG-204 and RVG205; Cluster II included 17 genotypes MNK-1, Dollar, RVKG-101, JGK-1, RVSKG-102; RVSSG-24, RVSSG37, RVSSG-30, JGK-2, BGD-128, KAK-2, ICCV-2, JGK-5, KRIPA, RVG201, GBM-2 and PKV-4; Cluster III included nine genotypes i.e., JGG1, Bhupda chana, Dindori chana, RVG202, JG36, JG-11, JG-322, GCP-101 and JG-6; Cluster IV included thirteen genotypes i.e., ICCV10, Vijay, JG218, JG-16, RVG-203; JAKI-9218, GG-5, JG-315, JG-14, JG-12, JG-74, JG-63 and Annagiri. The clusters based on molecular markers have been found highly associated with the degree of wilt resistance. The genotypes with the similar degree of resistance were clustered into same group. Most resistant genotypes in terms of genetic relatedness were grouped in cluster CI. Further, wilt resistant and sensitive genotypes were separated when correlation between genetic similarity index and taxonomic distance for grain yield were evaluated using Jaccard similarity index.

Discussion

The correlation coefficients for morpho-physiological traits revealed that the number of pods showed high significant positive correlations with number of seed per plant, grain yield, biological yield and number of seeds per plant demonstrated negative correlations with thousand seed weight but positive association with grain yield per plant in tune of several others (Singh et al. 2016; Kumar et al. 2017; Johanson et al. 2019) indicating significant contributions of the traits numbers of pods and seeds for higher grain yield.

Biochemical estimation revealed highest proline, total soluble sugar, lipid peroxidation and hydrogen peroxide contents and activities in fusarium wilt susceptible genotype JG-62 as compared to wilt resistant genotypes Kripa, Vijay, RVKG-102 and RVSSG-24 under study because proline and other protein constituents have been found increasing during stress conditions as reported by several others (Hayat et al. 2012). Proline acts as protective osmolyte which accumulates faster than other amino acids, shows diverse role in diseases/drought tolerance reactive oxygen species scavenger, and protection from oxidative damage and stabilizing enzymatic proteins against desiccation (Kaur et al. 2017). Disease induces oxidative stress in plants by generation of reactive oxygen species (ROS) (Farooq et al. 2009). Reactive oxygen species i.e., peroxides of polyunsaturated fatty acids generate MDA on decomposition (Davey et al. 2005). A decrease in membrane stability reflects the extent of lipid peroxidation caused by ROS. Furthermore, lipid peroxidation is an indicator of the prevalence of free radical reaction in tissues. Plants produce reactive oxygen species, which are harmful to plant growth due to their detrimental effects on the sub-cellular components and metabolism of the plants leading to the oxidative destruction of the cells and converts to hydrogen peroxide (H_2O_2) a toxic compound and its higher concentrations are injurious to plants, resulting in lipid peroxidation and membrane injury (Kaur et al. 2017).

The dynamic expression profile (Fig. 1) based on biochemical estimates-hierarchical cluster analysis done by us also confirms exterior site occupied by Cluster-I encompassing 3 fusarium wilt prone accessions JG-62, GCP-101, JG-16 probably due to presence of high antioxidants, sugar and H_2O_2 contents

leading to an inference that higher proline, total soluble sugar, lipid peroxidation and hydrogen peroxide should be neglected during selection for fusarium wilt resistant chickpea genotypes.

Gene based markers are more useful and cost-effective approach for molecular breeding of chickpea. The already reported gene-based markers as H1-the first wilt resistance gene tagged in chickpea at a distance of 7.0 cM from RAPD markers CS-27_{700} and UBC-170_{550} (syn. foc-1, Mayer et al. 1997), closely linked markers to foc-1 (Rubio et al. 2003, Sharma et al. 2004), foc-2 (Sharma and Muehlbauer 2005), foc-3 (Sharma et al. 2004), foc-4 (Ratnaparkhe et al. 1998a, b; Tullu et al. 1998), the second resistance gene for race 4 (Tullu et al. 1998), foc-5 (Ratnaparkhe et al. 1998b) were used for their validation over chickpea varieties JG 62-a fusarium wilt susceptible one and JG 315, JG 322, JG 36 resistant ones. Out of used RAPD, DAF, ISSR and STMS primers fourteen expressed polymorphic abilities to differentiate amongst 44 chickpea genotypes (Fig. 2). The primers

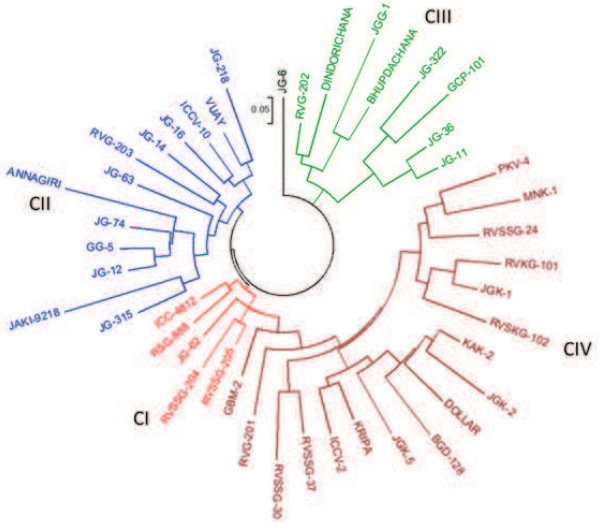

Fig. 2. UPGMA tree based on dissimilarity index for chickpea genotypes using molecular markers

TA194, TA-59, TA-96, TR-19, TR-29 and TR-31 showed high gene diversity along with PIC values and were found reproducible indicating their potentiality in the marker assisted breeding for fusarium wilt resistance. Some of these markers have also been used many others during chickpea studies (Singh et al. 2013; Singh et al. 2016; Kumar et al. 2017).

The clusters based on molecular markers have grouped the genotypes with the similar degree of resistance into same group. The most resistant genotypes in terms of genetic relatedness were grouped in cluster C-I comprising of fusarium wilt prone genotypes ICC- 4812, RSG 888, JG-62, RVSSG-204 and RVG205. However, C-II contained 13 chickpea varieties including JG 315, Jaki-9218, JG-12, CG-5, JG-72, Annagiri, JG-63, RVG-203, JG-14, ICCV-10, Vijay and JG-218 showing resistance at molecular level for fusarium wilt disease. Ratnaparkhe et al. (1998a) also found that the genes for resistance to fusarium wilt races 4 and 5 are linked. The study showed that markers linked to various genes can be rapidly identified using the ISSR markers. In our study, we used these markers and found polymorphic for tolerance and sensitive response to wilt, conferring these genotypes may possess foc 4 and 5 genes. Comparison of different studies indicated that four genes (*foc-1, foc-3, foc-4* and *foc-5*) should be in the same linkage group. Association of molecular markers for such validations have also been reported earlier (Bhardwaj et al. 2014). Further, comparative correlation studies between genetic similarity index and taxonomic distance using Jaccard similarity index for grain yield separated wilt resistant and susceptible genotypes indicating wide distance between desi chickpea JG-63 and kabuli chickpea ICC- 4812.

Thus, the present study on morpho-physio-chemical traits and molecular analysis concludes that the molecular markers TA194, TA-59, TA-96, TR-19, TR-29 and TR-31 should be used as marker assisted breeding tools for screening, validation and development of fusarium wilt resistant chickpea genotypes. The genotypes JG-63 and Vijay identified during the investigation may be included in the hybridization programs for development of high yielding and wilt resistant varieties.

Authors' contribution

Conceptualization of research (ST); Designing of the experiments (ST, VKS); Contribution of experimental materials (MY, VKS); Execution of field/lab experiments and data collection (VKS, RST, MKT, NG); Analysis of data and interpretation (RST, VKS, NG, ST); Preparation of manuscript (ST, VKS, NG, AA, RST).

Declaration

The authors declare no conflicts of interest.

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