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Identification of SSR markers linked to Botrytis grey mould resistance in chickpea (*Cicer arietinum*)

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Summary. Botrytis grey mould (BGM), caused by *Botrytis cinerea*, is emerging as an important disease of chickpea in the northern and eastern parts of the Indian Subcontinent, including Nepal, Bangladesh, Pakistan, and in Australia. This fungus has a very broad host range, and sources of complete resistance to the disease have not been found in *Cicer arietinum* L. germplasm. Resistance to this pathogen has been identified in some wild *Cicer* species. A set of 371 lines, including 164 landraces and 207 interspecific derivative lines (derived from crosses of cultivated chickpea with *C. pinnatifidum, C. judaicum* or *C. reticulatum*) have been screened against Botrytis grey mould under field conditions, and using the cut twig method at the Punjab Agricultural University (PAU), Ludhiana, in 2015-16 and 2016–17. Strong correlations between the two screening methods were indicated by paired-t tests. The Bulked Sample Analysis (BSA) approach was used to screen DNA of the five most resistant and five most susceptible host lines using 300 simple sequence repeat (SSR) markers. Eighty-eight markers were polymorphic. Chi-square statistic values showed strong correlations of TA144, GA102, TA194, TA140 and TR2 with the resistant bulks, signifying their usability as putative markers linked to BGM resistance, and for development of BGM tolerant genotypes in chickpea. Future studies should rapidly ascertain marker trait associations, and identify and develop diagnostic markers that provide an accurate method of molecular tagging BGM resistant genes in chickpea.

Keywords. Chickpea, Grey mould resistance, SSR markers, Bulked sample analysis.

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a highly nutritious food legume, ranking as third in world importance after peas and drybeans (Bharadwaj *et al.*, 2010). Chickpea is grown on 14.56 million ha in 50 different countries, and

average yields are 1.12 t ha^{-1}(FAOSTAT, 2017). Various biotic stresses affect chickpea production globally. These include fungal, viral, and bacterial diseases, nematodes, insect pests and parasitic weeds. Transferring resistance genes to elite cultivars is one of the most efficient approaches for overcoming biotic stresses (Li *et al*., 2015). Most traits of agronomic importance are complex in nature, governed by various genes and environmental interactions (EL-Soda *et al*., 2014). The identification of linked QTLs is crucial, therefore, for understanding the molecular basis of these traits (Xu *et al*., 2010).

Botrytis grey mould (BGM), caused by *Botrytis cinerea* Pers. ex. Fr., is an economically important disease of chickpea. The occurrence of BGM has been reported in many countries, including Argentina, Australia, Canada, Columbia, Bangladesh, Nepal, Pakistan, India, Spain and the United States of America (Haware *et al*., 1998). This disease may cause yield losses upto 100% under favourable conditions (Pande *et al*., 2006a; Pande *et al*., 2002). The disease may occur at any stage of chickpea development (Hawthorne *et al*., 2006) but usually occurs at the time of flowering. Temperatures of 20-30°C and relative humidity from 70 to 100% favour BGM infections. The pathogen infects flowers most easily (Pande *et al*., 2006b, c), and infected pods may carry the infection through infected seeds to the next growing season (Nene *et al*., 2012; Matthews *et al*., 2014). Genotypes with profuse seedling growth and early flowering and canopy closure tend to develop BGM infections more often compared with other cultivars.

Botrytis cinerea has a very broad host range. Source of complete resistance to the pathogen have not been found in *C. arietinum* germplasm (Singh and Reddy, 1991), but wild *Cicer* sp. have been identified as good sources of BGM resistance. Wild *Cicer* species, including *C. judaicum, C. bijugum, C. echinospermum,* and *C. pinnnatifidum* (Singh *et al*., 1991; Haware 1998; Pande *et al*., 2002), have shown higher levels of resistance to BGM in comparison to the cultivated species. Among these wild species, *C. echinospermum* is being used for transfer of BGM resistance to the cultivated species (ICRISAT, 2007). Kaur *et al.* (2013) developed interspecific derivative lines from crosses of *C. arietinum* and *C. pinnatifidum* that were found to be highly tolerant to BGM, and this resistance can be transferred to elite lines for development of high yielding and BGM tolerant chickpea cultivars. Very few studies have been undertaken to exploit wild species and the level of diversity amongst different host plant accessions.

The present study used wild *Cicer* spp. accessions and inter-specific hybrids to identify BGM resistant genotypes and putative markers linked to BGM resistance in chickpea.

Different methods have been used for screening for BGM resistance under laboratory, greenhouse and field conditions (Gurha *et al*., 2003; Pande *et al*., 2006a, b). The cut-twig method is a non-destructive procedure for sampling, and this has been very useful in inter-specific hybridization (Kaur *et al*., 2013). In comparison to the traditional methods of breeding, time saving and cost effective approaches are now widely used in genomics and crop improvement (Sun *et al*., 2010). Marker-assisted selections and selective phenotyping can be simplified using such approaches (Xu and Crouch, 2008). Bulked BSA uses individuals with extreme phenotypes and these variants are then pooled as bulks. Bulked BSA or the Sampling-bulking method for marker development and trait mapping has been named differently as bulked segregant analysis (Michelmore *et al*., 1991) and DNA pooling (Giovannoni *et al*., 1991), and is usually achieved with molecular marker systems (Gillman *et al*., 2011; Asnaghi *et al*., 2004). Genomic regions with large differential allele frequencies between the bulks reveal association of the regions with the QTLs associated with particular trait (Deokar *et al*., 2019) (Figure 1). These QTLs are then subjected to statistical tests to verify the confidence intervals for their location (Takagi *et al*., 2013). Next Generation Sequencing-based BSA has been used for QTL mapping for different traits due to improved efficiency and affordability of NGS platforms (Deokar *et al*., 2019; Chen *et al*., 2017; Pandey *et al*., 2017; Singh *et al*., 2016; Das *et al*., 2016; Illa-Berenguer *et al*., 2015; Kaminski *et al*., 2015; Lu *et al*., 2014). BSA coupled with molecular breeding techniquesal-

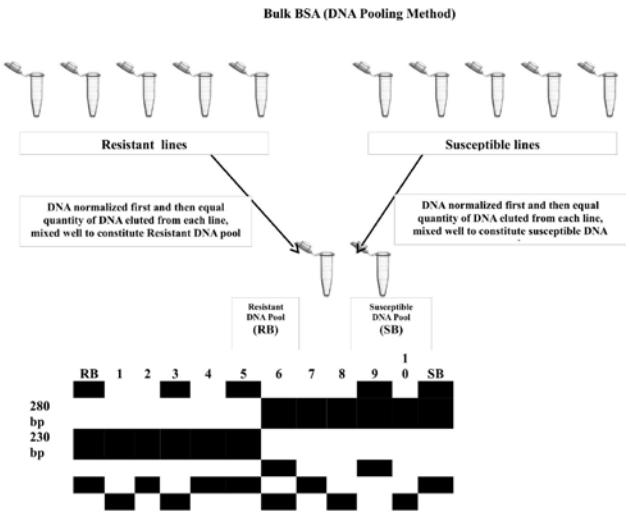

Figure 1. Figure depicting bulking sampling method where equal amount of DNA is eluted from each line after normalization and corresponding bulks generated are used for mapping genes for BGM resistance.

low rapid identification of markers, and is a promising approach for trait mapping and candidate gene discovery in plant breeding.

MATERIALS AND METHODS

Plant material

A total of 371 *Cicer* accessions, including 164 landraces and 207 F_6 interspecific derivatives lines (derived from crosses of cultivated chickpea with *C. pinnatifidum, C. judaicum* or *C. reticulatum*) were screened under field conditions and controlled conditions against *Botrytis* grey mould. The cut-twig method is non-destructive and simple, and is widely used for laboratory studies. Theaccessions were screened at the Punjab Agricultural University (PAU), Ludhiana, during 2015-16 and 2016-17. Of the 371 lines, three landraces and 23 extreme Recombinant Inbred Lines (RILs) were selected, for identification of markers linked to BGM resistance given in Table 1.

Field evaluations

The test lines were sown at plant spacings of 40 cm in 2 m length rows in fields at three replications, in a randomized complete block design (Figure 2a). The chickpea line L550 was used as a susceptible check after every eight rows in the trials. The resulting plants, at the flowering stage, were sprayed with a local isolate of *Botrytis cinerea*, in the first week of February in the evening hours. The inoculum suspension contained 10^5 conidia mL^{-1}. Isolate 24, race 510, of *B. cinerea* (Singh and Bhan, 1986) was used for screening the plants against BGM. The isolate was preserved on slants of potato dextrose agar (20g dextrose, 20g agarose, 200g potato and 1L distilled water), and were multiplied in potato dextrose broth and stored at 25°C. Following inoculation, water was applied using a sprinkler, to maintain high relative

Table 1 Plant material used in this study with, their parentage.

Name of genotype	Parentage/ Cross
GLW 42	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW 67	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW 69	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW107	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW108	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW115	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW174	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW185	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW501	GPF2 × [PBG 1 × (ICCV96030 × *C. pinnatifidum* 188) × ICCV96030]
GLW502	GL769 × *C. reticulatum*ILWC 129
GLW503	[PBG 1 × (ICCV96030 × *C. pinnatifidum* 188) × ICCV96030]
GLW504	[(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030] PBG 1
GLW505	GL769 × *C. reticulatum*ILWC 129
PAU7007	(GL769 × *C. reticulatum*129)× GL769
PAU7014	(GL769 × *C. reticulatum* 129)× GL769
C. judaicum ILWC-0*	-
C. judaicum ILWC-223*	-
GLW22	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW25	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW183	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW186	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
C. reticulatum ILWC-292*	-
GL1001	JG62 × ICCV05530
GL1002	JG62 × ICCV05530
GLW91	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
PBG-7	GPF2 × BG1084

*Chickpea landraces.

Figure 2. Figure showing screening of wild *Cicer* accessions under field conditions and laboratory conditions.

humidity. Symptoms of the disease became visible about 10 dafter inoculations, and disease severity was assessed for five plants per replication 7 d following inoculation. Disease severity was scored using a 1–9 scale, where 1 = no infection on any plant part; 3 = 1–2 lesions visible on leaves; 5 = burnt leaves with stem rotting; 7 = stem rotting with 50% dead leaves; and 9 = extensive stem rotting with fungal infection and 100% leaf death (Gurha *et al*., 2003). An average disease score was calculated.

Cut twig screening technique

Three twigs from each wild accession were cut and placed in a tray containing water in a completely randomized design in three replications. The twigs were then wrapped in moist cotton plugs and placed in test tubes (15 × 10 cm) freshly filled with tap water. Inoculation of the twigs was achieved by spraying conidium suspensions of *B. cinerea* (10^5 conidia mL^{-1}). Following inoculation, the test tubes were covered with wet polythene (Figure 2b). Incubation followed in a growth chamber, and BGM severity was recorded using the 1–9 severity scale (see above). Paired t-tests were performed to examine whether the BGM severity scores obtained under field and laboratory conditions were correlated (Table 2).

Genomic DNA extraction and pooled DNA analysis using SSRs

The CTAB method (Kumar *et al*., 2013) was used to extract DNA from young leaves of the twenty six extreme RILs. The concentration and purity of the DNA from the genotypes was further checked on 0.8% agarose gels and a nanodrop 1000 spectrophotometer (Thermo Scientific). Equal amounts of DNA from each line were taken after normalization and mixed well to constitute the resistant and susceptible bulks for bulked BSA. These bulks were screened for polymorphism using 300 simple sequence repeat (SSR) markers (Varshney *et al*. 2014; Gupta *et al*., 2012; Gaur *et al*., 2011); Bharadwaj *et al.,* 2010). These primers were custom synthesized from G-Biosciences, and the PCRs were carried out in the Chickpea Molecular Breeding Laboratory, Division of Genetics, ICAR-IARI. The 10 µL PCR mix consisting of 1 µL of 20 ng genomic DNA, 1.6 µL of 10× TBE buffer, 1µL of 10 mM dNTP mix, 1 µL forward and 1µL reverse primer and 0.3 µL of 3U μL^{-1}Taq polymerase (Genei), was amplified using a G-STORM thermal cycler (Labtech). The PCR reaction was set as per Yadav *et al.*(2011), with an initial denaturation at 90°C for 1 min and 30 seconds followed by 38 cycles including three different steps, including denaturation at 94°C for 20 sec, annealing at 50–58°C for 50 sec, and extension at 72°C for 50 seconds. This was then followed by a final extension step at 72°C for 7 min, before cooling to 4°C.Amplicons were resolved on 3% Agarose (Lonza) using 1.0× TBE buffer. The amplified products were separated on a horizontal gel electrophoresis system (Biorad) at 120 V for 3 h. Gel staining was by ethidium bromide (10 mg mL^{-1}), and visualized using the UVITECH Gel Documentation system (UVITECH Imaging System). Amplicons were scored as alleles for each locus. Allele sizes were determined by comparing with a standard 100 bp DNA ladder (Genei). Band patterns of the extreme bulks were compared with those of resistant and susceptible lines to confirm linkage of SSR markers with BGM resistance. Chi-square tests were performedto determine goodness of fit of the test lines for the phenotypic and SSR data, by comparing the observed frequency (O) with the expected frequency (E).

RESULTS

Identification of BGM resistance in chickpea accessions

The screening results for BGM resistance of the 26 extreme lines are presented in Table 2. The scoring results of field screening were in close agreement with those from laboratory assessments, similar scores were obtained for each accession under both conditions. Lines ILWC-0, ILWC-223, PBG-7 and nine RILs showed moderately susceptible to susceptible reactions to BGM under field and laboratory conditions. Line ILWC292 and thirteen RILs, including: GLW91, PAU7014, GLW42, GLW67, GLW 115, GLW174, GLW183, GLW186, GLW501, GLW504, GLW 506, GL1001 and GL1002, were resistant to moderately resistant to BGM,

Table 2 Mean Bortytis grey mould (BGM) severity scores for test host lines under field conditions and controlled conditions, and paired-t test analysis for correlating the respective BGM severity scores for the two screening methods (field and greenhouse).

Name of genotype	Disease reaction	Mean BGM score (1-9 scale)		Difference	D^2
		Field screening[a]	Lab screening[b]		
GLW42	Moderately resistant	5.0	5.5	-0.5	0.25
GLW67	Moderately resistant	5.0	4.5	0.5	0.25
GLW69	Moderately susceptible	8.0	8.0	0.0	0
GLW107	Susceptible	9.0	9.0	0.0	0
GLW108	Susceptible	9.0	9.0	0.0	0
GLW115	Moderately resistant	6.0	6.0	0.0	0
GLW174	Moderately resistant	5.5	4.5	1.0	1
GLW185	Susceptible	9.0	9.0	0.0	0
GLW501	Moderately resistant	5.5	5.0	0.5	0.25
GLW502	Moderately susceptible	7.0	7.5	-0.5	0.25
GLW503	Moderately susceptible	7.0	8.0	-1.0	1
GLW504	Moderately resistant	5.5	5.5	0.0	0
GLW505	Moderately susceptible	8.0	8.0	0.0	0
PAU7007	Moderately resistant	5.5	5.0	0.5	0.25
PAU7014	Resistant	4.0	4.0	0.0	0
C. judaicum ILWC-0	Susceptible	9.0	9.0	0.0	0
C. judaicum ILWC-223	Susceptible	9.0	9.0	0.0	0
GLW-22	Susceptible	9.0	9.0	0.0	0
GLW-25	Susceptible	9.0	9.0	0.0	0
GLW-183	Moderately resistant	5.0	5.5	-0.5	0.25
GLW-186	Moderately resistant	5.0	5.5	-0.5	0.25
C. reticulatum ILWC-292	Resistant	3.0	3.5	-0.5	0.25
GL1001	Moderately resistant	5.5	4.0	1.5	2.25
GL1002	Moderately resistant	5.5	5.0	0.5	0.25
GLW91	Resistant	3.0	3.0	0.0	0
PBG-7	Susceptible	9.0	9.0	0.0	0
$\bar{D}$					0.03846
$(\sum D-\bar{D})^2$					8.71153
SE of $\bar{D}$					0.34846
t					0.11037
df					25
Table value					2.06

[a]Average score from five plants per accession per replication, screened under field conditions.
[b]Average score from 12 plants (three plants per accession per replication), screened under laboratory conditions.
D = Difference between the field screening scores and laboratory screening scores.
$\bar{D}$ = Mean difference.
SE = standard error.
df = degrees of freedom.

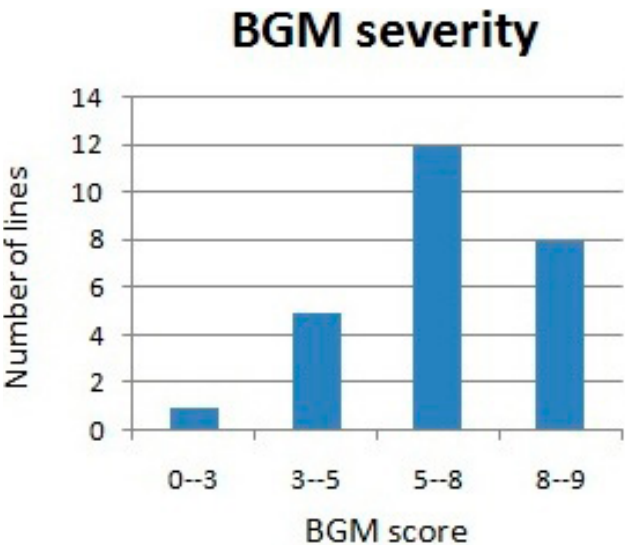

Figure 3. Frequency distribution of BGM scores of all wild *Cicer* spp. screened under field and laboratory conditions.

and could be used as resistant donors. The observed t-value of 0.1103 was much less than 2.06, (P = 0.05; d.f. = 25), showing that there was no statistically significant difference between the two screening methods (Table 2). The frequency distributions of the extreme lines for disease severity (recorded on the 1–9 scales) demonstrated a normal distribution, signifying that resistance to BGM was quantitative in nature (Figure 3).

Pooled DNA analysis using SSRs

Twenty six accession variants were selected from 371 wild chickpeas for genotyping. Of these, DNA from the five most resistant lines (ILWC 292, GLW91, GL1001, PAU7007 and PAU7014) and the five most susceptible accessions (*C. judaicum* ILWC-223, GLW22, GLW25, GLW69 and PBG7) were pooled and bulks were generated. Eighty eight markers were found to be polymorphic between the contrasting bulks, and these are list-

Table 3 List of polymorphic SSR markers used for bulked BSA in the five most Botrytis grey mould resistant and five most susceptible lines, and their corresponding bulks with their linkage groups (LGs)

Serial No.	Primer Name	LG	Serial No.	Primer Name	LG	Serial No.	Primer Name	LG	Serial No.	Primer Name	LG
1	TR43	1[a]	26	TR19	2[b]	51	NCGR209	8[d]	76	NCPGR248	*
2	TA196	3[b]	27	TS82	2[b]	52	NCPGR210	8[a]	77	NCPGR249	7[b]
3	TA144	3[c]	28	TR58	2[c]	53	NCPGR215	*	78	NCPGR250	*
4	TS5	3[b]	29	TA37	2[b]	54	NCPGR216	*	79	NCPGR252	6[d]
5	TA140	2[c]	30	GA16	2[c]	55	NCPGR218	*	80	NCPGR253	*
6	TAA104	5[b]	31	TA96	2[b]	56	NCPGR219	*	81	NCPGR254	6[a]
7	TR2	3[a]	32	TA27	2[b]	57	NCPGR220	6[a]	82	NCPGR255	6[d]
8	GA6	8[b]	33	TA21	5[b]	58	NCPGR221	3[a]	83	NCPGR267	6[b]
9	TA18	7[b]	34	CaSTMS24	2[b]	59	NCPGR224	4[a]	84	NCPGR268	7[a]
10	H3A10	5[b]	35	GA102	5[c]	60	NCPGR225	3[a]	85	NCPGR269	*
11	H2120	5[d]	36	CaSTMS22	5[b]	61	NCPGR226	6[a]	86	NCPGR272	3[b]
12	TS58	3[b]	37	NCPGR76	6[a]	62	NCPGR227	*	87	NCPGR274	6[a]
13	TAA170	4[b]	38	NCPGR78	*	63	NCPGR228	5[b]	88	NCPGR275	*
14	TA206	2[b]	39	NCPGR79	6[b]	64	NCPGR229	6[a]			
15	TA194	2[c]	40	NCPGR82	6[b]	65	NCPGR232	5[a]			
16	GAA47	4[b]	41	NCPGR84	*	66	NCPGR235	*			
17	TA3a	2[b]	42	NCPGR91	4[a]	67	NCPGR236	4[b]			
18	TR31	3[c]	43	NCPGR93	6[b]	68	NCPGR237	*			
19	TA42	7[b]	44	NCPGR95	7[b]	69	NCPGR238	6[a]			
20	TA89	4[b]	45	NCPGR96	*	70	NCPGR240	3[a]			
21	TA110	2[c]	46	NCPGR97	*	71	NCPGR241	3[a]			
22	STMS28	3[b]	47	NCPGR98	2[b]	72	NCPGR242	3[a]			
23	TR20	4[b]	48	NCPGR99	7[b]	73	NCPGR244	*			
24	CaSTMS2	4[b]	49	NCPGR100	3[a]	74	NCPGR246	*			
25	TA42	5[b]	50	NCPGR101	1[b]	75	NCPGR247	4[d]			

[a] Choudhary*et al.*, 2012; [b] Varshney *et al.*, 2014; [c] Bharadwaj *et al.*, 2010; [d] Gaur*et al.*, 2011; *Choudhary, unpublished.

Table 4 Chi-square (χ^2) test results of wild chickpeas screened using Bulked BSA for Botrytis grey mould screening.

Sno.	Marker	Observed (O)	Expected (E)	χ^2 =(Obs-Exp)^2/ Exp	χ^2 (P = 0.05)	
1	TR2	9	11	0.363636364	3.841	Strong correlation
2	TA194	11	12	0.083333333		Strong correlation
3	TA144	12	12	0.00		Strong correlation
4	GA102	11	12	0.083333333		Strong correlation
5	TA140	10	11	0.090909091		Strong correlation

Low χ^2 values indicate strong correlation with resistant allele.

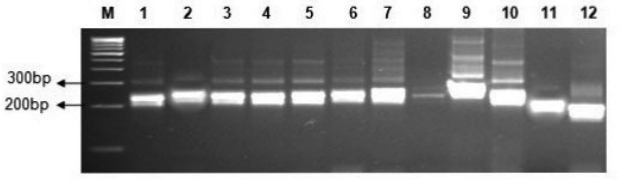

Figure 4. Gel image showing Bulked sample analysis with DNA pools generated with TA144, M: 100bp Banglore Genei ladder, 1:ILWC292,2:GL1001, 3:PAU7007, 4:PAU7014, 5:GLW91, 6:*C. judaicum* ILWC223, 7: GLW22, 8: GLW25, 9: PBG7, 10: GLW69, 11: Resistant bulk, 12: Susceptible bulk.

ed in Table 3. Most of these markers were found to be located on linkage groups (LGs) 2, 3 and 6. Anuradha *et al.* (2011) also identified three QTLs linked with BGM resistance in chickpea on LG 3 and LG 6. The BSA and chi-square statistic indicated strong correlations of TA144, GA102, TA194, TA140 and TR2 with the resistant bulks (Table 4 and Figure 4). These markers may be associated with resistance against BGM, and they could assist plant breeders in speedy development of BGM resistant cultivars.BSA combined with advanced technologies can be used to identify and develop diagnostic and constitutive markers improving the efficiency of breeding programmes and lead to animproved understanding of the molecular basis of BGM resistance.

DISCUSSION

Wild accessions serve as excellent sources of resistance to biotic and abiotic stresses, according to previous reports, and many chickpea accessions have now been identified with resistance to diseases (Madrid *et al.*, 2008). Accessions including *C. judaicum, C. pinnatifidum* and *C. echinospermum* have shown good resistance to BGM at PAU, Ludhiana, so these may be used as donors for introgressing BGM resistance in chickpea. These sources of BGM resistance were therefore used in the present study through interspecific hybridization.

Botrytis grey mould is an economically important disease of chickpea, which may cause complete yield losses under heavy rains and high humidity. It is the major production constraint limiting sustainable chickpea yields. There is an urgent need to develop varieties with resistance to BGM with greater yield stability. Screening under field and controlled conditions are time-consuming and selection of BGM-resistant genotypes may take more than 1 year or season if conventional methods are used. Screening for disease resistance may also be influenced by environmental interactions, density of pathogen inoculum, presence of some pathogens, and pathogen virulence, resulting in variable disease outcomes. Furthermore, discrepancies in scoring of disease reactions may affect the introgression of BGM resistance into elite chickpea genotypes. On the other hand, marker-assisted selection saves time in comparison to phenotypic field or greenhouse evaluations.

Bulked sample analysis and molecular markers together help discern markers associated with genes governing disease resistance in a number of plant species (Ballini *et al.*, 2008). Owing to scarcity of polymorphic markers in chickpea, BSA provides a rapid method for identifying markers linked to BGM resistance. Resistant genotypes may be identified within short periods, inferior genotypes can be excluded from the next cycle of selection, increasing the efficiency of breeding programmes.

Microsatellites combined with BSA have been used to identify molecular markers linked to genes of interest (Shoba *et al.*, 2012), and comparison of pooled DNA samples is much easier than evaluating all the individuals of different populations (Hallden *et al.*, 1997; Sweeney and Dannebeger, 1994). BSA has been successfully used in fine mapping of QTLs for *Ascochyta*blight resistance in *C. arietinum*, genes controlling powdery mildew resistance in pea (Fondevilla *et al.*, 2008), cotyledon seed colour in *Glycine max*, QTLs for rust resistance including VuUGM02, VuUGM08 and VuUGM19 in cowpea (Uma *et al.*, 2016), *Phaeoisariopsis griseola* resistance genes in *Phaseolus vulgaris* (Alzate-Marin *et al.*, 2001), 73 blast

resistance genes and 350 QTLs in rice (Ghaley *et al*., 2012),and discovery of SNPs for agronomically important traits in *Arachis hypogea* (Pandey *et al*., 2017). NGS-assisted BSA identified six candidate genes in the QTL regions on chromosomes Ca2 and Ca4 and validated for their association with *Ascochyta* blight resistance in the CPR02 population in chickpea (Deokar *et al*., 2019). A QTL-seq approach coupled with BSA identified candidate genes (Ca_04364 and Ca_04607) for 100seed weight, and one gene (Ca_04586) for total root dry weight to total plant dry weight ratio using CAPS markers in chickpea.

Anuradha *et al*. (2011) developed 126 F_{10} derived RILs) derived from a cross between a moderately BGM resistant kabuli cultivar (ICCV2) and a highly BGM susceptible desi chickpea cultivar (JG62) atthe International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. This used the single seed descent (SSD) method, and identified two QTLs for BGM resistance on LG3 and one on LG6, which after validation, can be used for marker-assisted breeding. Kaur *et al*. (2013) also developed an inter-specific population for BGM resistance using the cut twig technique. Sixty two F9 BGM resistant lines derived were evaluated for agronomically important and yield traits, and identified four lines, (GL 29029, GL29206, GL29212 and GL29081) with high degrees of resistance to BGM. These lines were crossed with the BGM-susceptible high yielding cultivar BG256 for molecular analysis, and genotyping of F_2 populations identified SSR markerspotentially linked with *Ascochyta* blight and BGM resistance genes. Of the 120 markers used, six SSRs (TA2, TA110, TA139, CaSTMS7, CaSTMS24 and TR29) were found to be polymorphic. These markers can be used for identification of markers linked to BGM resistance, and assist in marker-assisted backcrossing for resistance breeding.

In the present study, comprehensive evaluation of the test host lines and paired-t test analysis both revealed that the field and laboratory screening methods gave similar results for BGM screening of the test lines. This demonstrates that laboratory screening methods coupled with molecular marker techniques can serve as powerful tools in genetics and crop improvement. The polymorphic SSR markers identified in the present study can be used to develop chickpea cultivars with high levels of BGM resistance, that has been a challenging task due to lack of sources of high levels of resistance in cultivated chickpea. Low chi-square statistic values of five SSRs (TA144, GA102, TA194, TA140 and TR2) in comparison to the critical value at $P = 0.05$ indicated their strong correlation with the resistant bulk and BGM resistance. We therefore conclude that bulked BSA is simple and accurate method for rapidly ascertaining marker-trait associations rapidly, and may be value for molecular tagging of BGM resistant genes in chickpea.

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LITERATURE CITED

Alzate-Marin A.L., Costa, M.R., Sartorato, A., Rava, C.A., de Barros, E.G., Moreira, M.A., 2001. Use of markers as a tool to investigate the presence of disease resistance genes in common bean cultivars. *Embrapa Arroz e Feijão-Artigoemperiódicoindexado (ALICE)*.

Anuradha C., Gaur P.M., Pande S., Gali K.K., Ganesh M., Kumar, J., Varshney, R.K., 2011. Mapping QTL for resistance to botrytis grey mould in chickpea. *Euphytica* 182(1): 1–9.

Asnaghi C., Roques D., Ruffel S., Kaye C., Hoarau J.-Y., Télismart H., Girard J.C., 2004. Targeted mapping of a sugarcane rust resistance gene (Bru1) using bulked segregant analysis and AFLP markers. *Theoretical and Applied Genetics* 108: 759–764.

Ballini E., Morel J.B., Droc G., Price A., Courtois B., Notteghem J.L., Tharreau D., 2008. A genome-wide meta-analysis of rice blast resistance genes and quantitative trait loci provides new insights into partial and complete resistance. *Molecular Plant-Microbe Interactions* 21(7): 859–868.

Bharadwaj C., Chauhan S.K., Rajguru G., Srivastava R., Satyavathi, T.C., … Solanki R.K., 2010. Diversity analysis of chickpea (*Cicer arietinum*) cultivars using STMS markers. *Indian Journal of Agricultural Sciences* 80(11): 947–951.

Chen Q., Song J., Du W.P., Xu L.Y., Jiang Y., Zhang J., Xiang X.L., 2017. Identification, mapping, and molecular marker development for Rgsr8.1: a new quantitative trait locus conferring resistance to *Gibberella* stalk rot in maize (*Zea mays* L.). *Frontiers in Plant Science* 8: 1355.

Choudhary S., Gaur R., Gupta S., Bhatia S., 2012. EST-derived genic molecular markers: development and utilization for generating an advanced transcript map of chickpea. *Theoretical and Applied Genetics* 124(8): 1449–1462.

Das S., Singh M., Srivastava R., Bajaj D., Saxena M.S., Rana J.C., Bansal K.C., 2016. mQTL-seq delineates functionally relevant candidate gene harbouring a

major QTL regulating pod number in chickpea. *DNA Research* 23: 53–65.

Deokar A., Sagi M., Daba K., Tar'an B., 2019. QTL sequencing strategy to map genomic regions associated with resistance to *Ascochyta* blight in chickpea. *Plant Biotechnology Journal* 17(1): 275–288.

El-Soda M., Malosetti M., Zwaan B.J., Koornneef M., Aarts M.G., 2014. Genotype× environment interaction QTL mapping in plants: lessons from *Arabidopsis*. *Trends in Plant Science* 19(6): 390–398.

Fondevilla S., Rubiales D., Moreno M.T., Torres A.M., 2008. Identification and validation of RAPC and SCAR markers lined to the gene *Er3* conferring resistance to *Erysiphe pisi* DC in pea. *Molecular Breeding* 22(2): 193–200.

Food and Agriculture Organization of the United Nations, 2017. FAOSTAT statistics database. http://www.fao.org/faostat/en/#home

Gaur R., Sethy N.K., Choudhary S., Shokeen B., Gupta V., Bhatia S., 2011.Advancing the STMS genomic resources for defining new locations on the intraspecific genetic linkage map of chickpea (*Cicer arietinum* L.). *BMC genomics* 12(1): 117.

Ghaley B.B., Christiansen, J.L., Andersen, S.B., 2012. Genetic diversity in blast resistance of Bhutan rice landraces. *Euphytica* 184(1): 119–130.

Gillman J.D., Tetlow A., Lee J.-D., Shannon J.G., Bilyeu K., 2011. Loss-of-function mutations affecting a specific *Glycine max* R2R3 MYB transcription factor result in brown hilum and brown seedcoats. *BMC Plant Biology* 11: 155.

Giovannoni J.J., Wing R.A., Ganal M.W., Tanksley S.D., 1991. Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. *Nucleic Acids Research* 19(23): 6553–6568.

Gupta S., Choudhary S., Bhatia S., Gaur R., 2012. EST-derived genic molecular markers: development and utilization for generating an advanced transcript map of chickpea. *Theoretical and Applied Genetics* 124(8): 1449–1462.

Gurha S.N., Singh G., Sharma Y.R., 2003. Diseases of chickpea and their management. In: *Chickpea research in India*' (Massod Ali, Shiv Kumar, NB Singh, eds.), pp. 195–227.

Halldén C., Säll T., Olsson K., Nilsson N.O., Hjerdin A., 1997. The use of bulked segregant analysis to accumulate RAPD markers near a locus for beet cyst nematode resistance in *Beta vulgaris*. *Plant Breeding* 116: 18–22.

Haware M.P., 1998. Diseases of chickpea. In: *The pathology of food and pasture legumes*' (Allen D.J., Lenne J.M., eds.), pp. 473–516.

Hawthorne W., Davidson J.A., McMurray L., Lindbeck K.D., Brand J., 2006. Chickpea disease management strategy-southern region. *Disease Management Guide Series.(Pulse Australia: Sydney)*.

ICRISAT 2007. ICRISAT Archival Report 2007. International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502 324, Andhra Pradesh, India.

Illa-Berenguer E., Van Houten J., Huang Z., Knaap E., 2015. Rapid and reliable identification of tomato fruit weight and locule number loci by QTL-seq. *Theoretical and Applied Genetics* 128: 1329–1342.

Kaminski K.P., Kørup K., Andersen M.N., Sonderkaer M., Andersen M.S., … Nielsen K.L., 2015. Cytosolic glutamine synthetase is important for photosynthetic efficiency and water use efficiency in potato as revealed by high-throughput sequencing QTL analysis. *Theoretical and Applied Genetics* 128: 2143–2153.

Kaur L., Sirari A., Kumar D., Singh Sandhu J., Singh S., … Sharma M., 2013. Combining Ascochyta blight and Botrytis grey mould resistance in chickpea through interspecific hybridization. *Phytopathologia Mediterranea* 52(1): 157–165.

Kumar T., Bharadwaj C., Satyavathi C.T., Jain P.K., 2013.A high throughput, improved rapid and reliable genomic DNA extraction protocol from chickpea (*Cicer arietinum* L.). *Vegetos* 26(2): 185–190.

Li H., Rodda M., Gnanasambandam A., Aftab M., Redden R., … Slater A.T., 2015. Breeding for biotic stress resistance in chickpea: progress and prospects. *Euphytica* 204(2): 257–288.

Lu H., Lin T., Klein J., Wang S., Qi J., Zhou Q., Sun J., 2014. QTL-seq identities an early flowering QTL located near Flowering Locus T in cucumber. *Theoretical and Applied Genetics* 127: 1491–1499.

Madrid E., Rubiales D., Moral A., Moreno M.T., Millán T., … Rubio J., 2008. Mechanism and molecular markers associated with rust resistance in a chickpea interspecific cross (*Cicer arietinum* x *Cicer reticulatum*). *European Journal of Plant Pathology* 121: 43–53.

Mathews P., McCaffery D., Jenkins L., 2014. Winter crop variety sowing guide 2014. *NSW Department of Primary Industries: Sydney*.

Michelmore R.W., Paran I., Kesseli R.V., 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences* 88(21): 9828–9832.

Nene Y.L., Reddy M.V., Haware M.P., Ghanekar A.M., Amin K.S., Pande S., Sharma M., 2012. *Field Diag-*

nosis of Chickpea Diseases and their Control. Information Bulletin No. 28 (revised). International Crops Research Institute for the Semi-Arid Tropics.

Pande S., Galloway J., Gaur P.M., Siddique K.H.M., Tripathi H.S., … Kishore G.K., 2006a. *Botrytis* grey mould of chickpea: a review of biology, epidemiology, and disease management. *Australian Journal of Agricultural Research* 57(11): 1137–1150.

Pande S., Kishore G.K., Upadhyaya H.D., Rao J.N., 2006b. Identification of sources of multiple disease resistance in mini-core collection of chickpea. *Plant Disease* 90(9): 1214–1218.

Pande S., Ramgopal D., Kishore G.K., Mallikarjuna N., Sharma M., … Rao J.N., 2006c. Evaluation of wild *Cicer* species for resistance to *Ascochyta* blight and *Botrytis* gray mold in controlled environment at ICRISAT, Patancheru, India. *Journal of SAT Agricultural Research* 2(1): 1–3.

Pande S., Singh G., Rao J.N., Bakr M.A., Chaurasia P.C.P., … Gowda C.L., 2002. Integrated management of *Botrytis* gray mold of chickpea. *India: International Crops Research Institute for the Semi-Arid Tropics*.

Pandey M.K., Khan A.W., Singh V.K., Vishwakarma M.K., Shasidhar Y., … Garg V., 2017. QTL-seq approach identified genomic regions and diagnostic markers for rust and late leaf spot resistance in groundnut (*Arachis hypogaea* L.). *Plant Biotechnology Journal* 15: 927–941.

Shoba D., Manivannan N., Vindhiyavarman P., Nigam S.N., 2012. SSR markers associated for late leaf spot disease resistance by bulked segregant analysis in groundnut (*Arachis hypogaea* L.). *Euphytica* 188(2): 265–272.

Singh G., Bhan L.K., 1986. Physiological Races of *Botrytis cinerea* causing gray mold of chickpea. *Plant Disease Research* 1(1-2): 69–74.

Singh K.B., Holly L., Bejiga G., 1991. *A catalog of kabuli chickpea germplasm-an evaluation report of winter-sown kabuli chickpea land races, breeding lines and wild cicer species* (No. SB351. C45 S61).

Singh K.B., Reddy M.V., 1991. Advances in disease-resistance breeding in chickpea. *Advances in Agronomy* 45: 191–222.

Singh V.K., Khan A.W., Jaganathan D., Thudi M., Roorkiwal M., … Garg V., 2016. QTLseq for rapid identification of candidate genes for 100-seed weight and root/total plant dry weight ratio under rainfed conditions in chickpea. *Plant Biotechnology Journal* 14: 2110–2119.

Sun Y., Wang J., Crouch J.H., Xu Y., 2010. Efficiency of selective genotyping for genetic analysis of complex traits and potential applications in crop improvement. *Molecular breeding* 26(3): 493–511.

Sweeney P.M., Danneberger T.K., 1994. Random amplified polymorphic DNA in perennial ryegrass: a comparison of bulk samples vs. individuals. *Horticultural Science* 29(6): 624–626.

Takagi H., Abe A., Yoshida K., Kosugi S., Natsume S., … Innan, H., 2013. QTL-seq: rapid mapping of quantitative trait loci in rice by whole genome resequencing of DNA from two bulked populations. *The Plant Journal* 74(1): 174–183.

Uma M.S., Hegde N., Hittalmani S., 2016. Identification of SSR marker associated with rust resistance in cowpea (*Vigna unguiculata* L.) using bulk segregant analysis. *Legume Research: An International Journal* 39(1): 39–42.

Varshney R.K., Mir R.R., Bhatia S., Thudi M., Hu Y., … Riera-Lizarazu, O., 2014. Integrated physical, genetic and genome map of chickpea (*Cicer arietinum* L.). *Functional & Integrative Genomics* 14(1): 59–73.

Xu Y., Crouch J.H., 2008. Marker-assisted selection in plant breeding: from publications to practice. *Crop Science* 48(2): 391–407.

Xu Y. 2010. *Molecular Plant Breeding*. CABI, Wallingford, UK, 734 pp.

Yadav S., Bharadwaj C., Chauhan S.K., Rizvi A.H., Kumar J., Satyavathi C.T., 2011. Analysis of genetic diversity in *Cicer* species using molecular markers. *The Indian Journal of Genetics and Plant Breeding* 71(3): 272–275.