# Inheritance Studies and Validation of Molecular Markers Associated with *Botrytis* Grey Mould in Chickpea (*Cicer arietinum* L.)

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## ABSTRACT

**Background:** The *Botrytis* grey mould (BGM) is a devastating foliar disease of chickpea. In order to develop resistant high yielding varieties, the genetic mechanism governing the inheritance of resistance against BGM must be decoded. The molecular markers associated with BGM resistance are also need to be validated for marker assisted pyramiding of BGM resistance in chickpea.
**Methods:** The present study was conducted during *rabi* seasons of 2014-18 at GBPUAT, Pantnagar. The experimental material consisted of six generations ($P_1$, $P_2$, $F_1$, $F_2$, $BC_1$ and $BC_2$) of a cross between a BGM resistant variety GL 10006 and susceptible variety H 208. The disease data was scored on nine-point (1-9) scale and were subjected to chi-square analysis. The inheritance was also studied by using 28 STMS markers at the Pulse Breeding Laboratory of GBPUAT, Pantnagar during 2017-18. The Single Marker Analysis was performed for validation of markers associated with BGM.
**Result:** The results indicated that resistance for BGM in chickpea is dominant over susceptibility. A major QTL *i.e.* TA118 along with some minor QTLs are involved in governing resistance to BGM. The marker TA118, TS72 and TA144 can be used effectively in marker assisted selection for getting desirable recombinants in chickpea breeding.

**Key words:** *Botrytis*, Chickpea, Inheritance, Markers, Validation.

## INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a popular leguminous cool season legume crop served as a high-quality diet for human consumption as its grain are rich source of protein, essential amino acids, vitamins and minerals. Globally it is second most consumed legume crop after dry bean. During the year 2017-18, in India chickpea recorded a highest ever production of 11.23 Mt from an area of 10.56 Mha with a record productivity level of 1063 kg/ha (Anonymous, 2018). Madhya Pradesh (4.60 Mt) followed by Maharashtra (1.78 Mt), Rajasthan (1.67 Mt), Karnataka (0.72 Mt), Andhra Pradesh (0.59 Mt), Uttar Pradesh (0.58 Mt) and Gujarat (0.37 Mt) are the major contributing states in chickpea production. In India, the first report of BGM on chickpea was given by Shaw and Ajrekar (1915) and later by Butler and Bisby (1931). Despite so much improvement in the productivity there was still a huge gap between the yield achieved in experimental plots and farmers' fields (Pande *et al.* 2006). There are several causes for the low productivity of chickpea but among them susceptibility to fungal pathogen is a major factor. *Botrytis* grey mould (BGM), caused by *Botrytis cinerea* Pers. ex. Fr., is one of the most devastating fungal disease in chickpea. The chickpea plants infected with *Botrytis* starts producing spores, which soon spread the disease rapidly through air (MacLeod and Sweetingham, 2000). The most severe attack of this fungus is on the flowers which leads to poor or no pod setting even if seeds are formed they remains shriveled (Knights and Siddique, 2002). The study of

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literature suggested that there are very few reports about the inheritance of resistance against BGM. The limited reports available on genetics of BGM resistance suggests that the resistance is controlled by few genes (Tewari *et al.* 1985; Chaturvedi *et al.,* 1995; Anuradha *et al.,* 2011; Nehra *et al.,* 2020). Chickpea is one of the pulses where major progress has been achieved in the use of marker-assisted selection, numerous simple sequence repeat (SSR) and SNP resources have been developed and the chickpea genetic map density has been considerably increased (Kaur *et al.,* 2013). The study of inheritance of resistance and molecular markers associated to BGM would be helpful for targeting resistance against BGM. Hence in the present study efforts are being made to find out the mechanism of

inheritance of resistance against BGM by using both field and marker data and to validate the molecular markers associated with resistance towards BGM.

## MATERIALS AND METHODS

### Experimental material and field trial

The field experiments of present study were conducted during the *rabi* seasons of 2014-15, 2015-16 and 2016-17 at Norman E. Borlaug, Crop Research Centre of the G. B. Pant University of Agriculture and Technology, Pantnagar (Uttarakhand), India (29.5°N and 79.3°E). Crosses (GL 10006 × H 208) was attempted using hand emasculation followed by immediate pollination during *rabi* 2014-15 between a BGM resistant variety GL 10006 with a BGM susceptible variety H 208. The $F_1$ seeds were planted in *rabi* 2015-16 and backcrosses were attempted with both the parents and also allowed to self-pollinate to produce seeds for $F_2$ generation. New $F_1$'s was also attempted in *rabi* 2015-16. All the generations *viz.,* $P_1$, $P_2$, $F_1$, $F_2$, $BC_1$ ($F_1$ × GL 10006) and $BC_2$ ($F_1$ × H 208) were sown in compact family block design during *rabi* 2016-17 since progeny to be tested in present experiment belong to different families, as this design facilitates comparison between progeny belonging to the same family as well as different families. The row-to-row distance was maintained at 30 cm and plant to plant at 10-15 cm. The standard package of practices for chickpea cultivation was followed.

### Disease screening and scoring

The screening for BGM was done under natural epiphytotic conditions. The plants were inoculated by spraying a spore suspension (50,000 spores/ml) of 10-days old culture of *Botrytis cinerea* at the onset of flowering to create disease pressure in field. The observations were recorded when susceptible cultivars showed the maximum score of BGM. Five plants from each parent and all the plants of $F_1$, backcross and $F_2$ generations were screened in field. At reproductive stage disease was identified and data was recorded according to per cent plant parts affected by BGM. Disease data was scored for per cent plants affected on nine-point (1-9) scale given in Table 1 (Kaur *et al*., 2013).

### DNA extraction and PCR

The molecular work was carried out in the Pulse Breeding Laboratory of the Department of Genetics and Plant Breeding, GBPUAT, Pantnagar during 2017-18. In case of inheritance study by using molecular marker data, SSR based STMS (Sequence Tagged Microsatellite Site) primers were tested for their association with *Botrytis* grey mould resistance genes in parents, $F_1$ and $F_2$ population of cross GL10006 × H208. The STMS markers are considered as more reliable as compared to SSR primers as they have high polymorphic information content and more suitable for high throughput automation. The choice of molecular markers was based on previous reports for their linkage with botrytis resistance genes. The markers used in present study were selected from the published source of Winter *et al.* (1999). A total 28 STMS primers *viz.,* TA2,TA5, TA25, TA28, TA34, TA43, TA47, TA64, TS72, TA110, TA118, TA144, TA203, TAA137, TA20, TR29, TA43, TS12, TA29, TS57, TS72, CaSTMS7, CaSTMS23, CaSTMS24, ICCM0068, ICCM0160, ICCM0178, ICCM0242a were used for inheritance study and validation. The primers that showed polymorphism among parents were used for screening in $F_1$ and $F_2$ population. In $F_1$ and $F_2$ generation plants, if the amplified band size was similar found to resistant parent than these plants were considered as resistant and if band size was similar to susceptible parent than they are considered as susceptible. For the study of inheritance and validation of markers linked with BGM resistance gene, the $F_2$ generation consisted of 84 individual plants of cross GL1006 × H208 was used for genotyping. Phenotyping was also done in same population. The primers were synthesized from Bangaluru GeNei Ltd. Assay Buffer, Taq DNA polymerase and dNTPs were purchased from Bangaluru GeNei Ltd., Bengaluru, India. The genomic DNA was extracted for molecular characterization studies by using the Cetyl tri- methyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with very slight modifications. A single PCR reaction contains a total volume of 10.0 µl and consisted of 1.0 µl DNA template (100 ng/ µl), 1.5 µl Taq buffer (10X) with 15 mM $MgCl_2$, 0.3 µl dNTPs 10 mM, 0.2 µl Taq polymerase 3U/ µl and 1.0 µl primers (50 ng) and 6 µl part $ddH_2O$ were used for each PCR reaction. The amplification reaction was carried out in thermocycler, Peq STAR, PEQ LAB Ltd., Eppendorf Ltd., Germany with a program for an initial denaturation at 94° for five minutes followed by 35 cycles of 94° for one minute, annealing temperature vary with primer (50-65°) for 1 min, extension at 72° for 1 min and a final extension at 72° for 8 min. Horizontal gel electrophoresis unit was used for fractionating PCR products on agarose gel (3.0%) and EtBr for visualization of bands under UV Alpha-imager.

**Table 1:** Disease rating scale for Botrytis grey mould (BGM) in chickpea as suggested by Kaur *et al*. (2013).

| Scale | Disease response |
|---|---|
| 1 | Highly resistant (HR) (no infection on any part of the plant) |
| 1.1-3 | Resistant (R) (minute water-soaked lesions on 1-5% leaves) |
| 3.1-5 | Moderately resistant (MR) (lesions and soft rotting on 11-25% leaves and tender shoots) |
| 5.1-7 | Moderately susceptible (MS) (rotting and fungal growth on 41-55% of the leaves and shoots) |
| 7.1-9 | Highly susceptible (HS) (extensive rotting and fungal growth on 71-100% of the leaves, shoots and stems |

### Statistical analysis

Mean disease score was calculated by using the following formula:

$$\text{Mean disease score} = \frac{\sum (\text{Infection score} \times \text{Frequency})}{\text{Total no. of plants}}$$

The disease screening data obtained from field and the genotypic data obtained from $F_1$ and $F_2$ population using the molecular markers were subjected to chi-square analysis as per standard statistical procedure for disease inheritance study. In case of validation of markers linked with BGM the Single Marker Analysis was performed with the help of QTL Cartographer software *version* 2.5.

## RESULTS AND DISCUSSION

### Inheritance studies by using field data

Promising and stable resistance against BGM is urgently required for developing high yielding and resistant varieties of chickpea. Inheritance of BGM was studied in parents, $F_1$, $F_2$ and backcross generations developed by cross GL10006 × H208. Results of field study were presented in Table 2. The mean disease score of resistant parent GL10006 was noted as 2.8 and of susceptible parent H 208 was 8.2. It was evident from these results that the parent GL10006 showed resistant reaction while the parent H 208 showed susceptible reaction. The $F_1$ plants exhibited the disease score of 4.1 and hence showed resistant reaction. These results indicated the dominance of resistance over susceptibility. The disease reaction pattern in $F_2$ generation showed the disease score from 3 to 9 with a mean of 5.19, showing segregation for resistance. The segregation of $F_2$ population showed a wide range of response to BGM. The $F_2$ generation data was found to be fit to the ratio of 3 (resistant): 1 (susceptible) indicated that inheritance of resistance to BGM is controlled by single dominant gene. In the backcross of $F_1$ with resistant parent ($BC_1$) all plants showed resistant response while in case of $BC_2$ plants segregate in ratio of 1 resistant: 1 susceptible. The results obtained from backcrosses as well as $F_2$ generation showed that resistance to BGM is under control of a single dominant gene. The results of present study agree with some of the earlier reported findings, indicating that resistance to BGM is dominant over susceptibility and govern by a single major gene (Tewari *et al.,* 1985; Bhardwaj *et al.,* 2018; Nehra *et al.,* 2020). The dominance nature of resistance against BGM is highly desirable as it facilitates easy incorporation and selection. However, Rewal and Grewal (1989) identified two genes with dominant and recessive epistasis (13:3 ratio) whereas Chaturvedi *et al.* (1995) reported duplicate dominant epistasis (15:1 ratio). To further validate the field results the molecular markers was used to confirm the obtained results.

### Inheritance study by using molecular markers along with marker validation

The selection of high yielding and disease-free chickpea lines is the key criterion for future breeding programs. The identification and evaluation of chickpea BGM resistant lines

**Table 2:** Inheritance pattern of BGM in different generation of cross GL10006 × H208.

| Parent/Cross | Generation | BGM score | | | | | Total no. of plants | Mean disease score | Observed frequency | | Expected frequency | | Expected ratio | $\chi^2$ cal | $\chi^2$ tab (0.05,1df) |
| | | 1 | 3 | 5 | 7 | 9 | | | R | S | R | S | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GL 10006 | $P_1$ | 2 | 5 | 3 | | | 10 | 2.80 | | | | | | | |
| H208 | $P_2$ | | | | 6 | 4 | 10 | 8.20 | | | | | | | |
| GL 10006 x H208 | $F_1$ | | 7 | 8 | | | 15 | 4.10 | | | | | | | |
| $F_1$ × GL 10006 | $BC_1$ | | 4 | 10 | | | 14 | 4.70 | 14 | 0 | 14 | 0 | 1:0 | 0.00 | 3.84 |
| $F_1$ × H208 | $BC_1$ | | 2 | 6 | 3 | 1 | 12 | 5.50 | 8 | 4 | 6 | 6 | 1:1 | 1.33 | 3.84 |
| GL 10006 × H208 | $F_2$ | | 35 | 70 | 27 | 12 | 144 | 5.19 | 104 | 40 | 108 | 36 | 3:1 | 0.59 | 3.84 |

aiming to link the marker with the specific QTL is a new avenue in chickpea breeding. In the present study, STMS primers were used to study the inheritance of resistance to BGM in $F_1$ and $F_2$ population of cross GL10006 × H208 of chickpea. Out of 28 STMS primers, thirteen primers *viz.,* TA203, TA47, TA43, TS29, TA118, TAA137, ICCM0068, TS57, ICM0160, TA34, TS72, TA25 and TA144 were found polymorphic. These polymorphic primers were screened in $F_1$ and $F_2$ population. The amplicon size for the resistant parent GL 10006 varied from 160 bp (TA 43) to 300 bp (ICCM 0160) whereas the amplicon size for susceptible parent H208 varied from 180 bp (TA 43) to 350 bp (TS 29) (Table 3). In $F_1$ plants all the used primers amplified band size similar to resistant parent GL10006. These results indicated the dominance of resistance over susceptibility. In case of $F_2$ generation almost all the polymorphic primers showed segregation pattern that went perfectly well with the disease scoring phenotypic data for the cross GL10006 × H208 (Table 4). When genotypic data was subjected to chi square analysis it showed goodness of fit in 3:1 ratio. These results were also pointing towards that the inheritance to BGM was under the control of major gene as evident from field phenotypic data. The role of major QTLs in controlling the resistance to BGM was also reported earlier by Anuradha *et al.* (2011) by using molecular markers in cross that involved a moderately resistant kabuli cultivar ICCV 2 and a highly susceptible desi cultivar JG 62. The thirteen polymorphic markers identified in the cross GL10006 × H 208 was further subjected to single marker analysis to find the marker trait relationship. The molecular analysis was carried out in 84 plants of $F_2$ mapping population. The $F_2$ generation was also subjected to phenotyping for BGM resistance using field screening technique similarly, the genotypic data obtained was scored by using binary scoring method. The phenotypic and genotypic data obtained from $F_2$ generation of GL10006 × H208 cross was subjected to single marker analysis using Win QTL Cartographer 2.5 to detect the association of molecular marker(s) with BGM resistance locus. The results of single marker analysis of GL 10006 × H 208 based mapping population depicted that the QTL linked with marker TA118 explained about 28.25% of total phenotypic variance whereas markers TS72, TA144, TA47, TA34, TS 57, TA 43 and TAA 137explained 7.85%, 5.48%, 2.79%, 2.71%, 1.74%, 1.55% and 0.92% of total phenotypic variance, respectively. The marker TA118 was present on the linkage group 3 (LG 3) while the marker TS 72, TA144, TA47 and TA34, TS 57, TA 43, TAA 137 are present on linkage group 4, 3, 2, 5 and 5, respectively. The results of single marker analysis results indicated that a major QTL *i.e.* TA118 along with some minor QTLs *i.e.* TS 72, TA 144, TA47 , TA34, TS 57, TA 43, TAA 137 are involved in governing resistance to BGM. The major QTL (TA118) belongs to LG 3. The amplification profile of TA 118 and TA 144 was given in Fig 1 and 2. These results are well supported by work of Anuradha *et al.* (2011) which also reported three major QTLs mapped on LG 3 and LG 6 which together accounted for 43.6% of the variation for BGM resistance

**Table 3:** Inheritance pattern of BGM in 84 plants of $F_2$ generation by using molecular markers.

| Primers | Marker sequence Forward (5'-3') | Marker sequence Reverse (5'-3') | Approximate band size (bp) GL10006 (P₁) | Approximate band size (bp) H208 (P₂) | Approximate band size (bp) F₂ | Type of band R | Type of band S | Ratio (R:S) | χ² cal | χ² tab (0.05, 1df) |
|---|---|---|---|---|---|---|---|---|---|---|
| TA118 | ACAAGTCACACATGTGTTCTCAATA | GGAAAGGTTAAGAAATTTTACAATAC | 210 | 250 | 210-250 | 70 | 14 | 3:1 | 3.111 | 3.841 |
| ICCM0068 | TCTTCTTTGCTATCTGTCTCGC | TGCATGTCAAACATTAGACAACTTT | 250 | 300 | 250-300 | 64 | 20 | 3:1 | 0.063 | 3.841 |
| TS29 | AACATTCATGAACCTACCTCAACTTA | CCATATATGAGTACACTACCTCTCGG | 300 | 350 | 300-350 | 70 | 14 | 3:1 | 3.111 | 3.841 |
| TA203 | ATAAAGGTTTGATCCCATT | TGTGCATTCAGATACATGCT | 260 | 300 | 260-300 | 57 | 27 | 3:1 | 2.286 | 3.841 |
| TAA137 | CATGATTTCCAACTAAATCTTGAAAGT | TCTTGTTTCGTTTAAACAATTTCTTCT | 200 | 230 | 200-230 | 68 | 16 | 3:1 | 1.587 | 3.841 |
| TA47 | TTTTTATAGGTGTTCTTTTTGTTGTCTTT | TCTGAATAGGAAATAAGAAAGGTAGGTT | 250 | 300 | 250-300 | 70 | 14 | 3:1 | 3.111 | 3.841 |
| TA43 | GGTTGTGGTCTCCAGATTTT | AAGAGTTGTTGGAGAGCAA | 160 | 180 | 160-180 | 65 | 19 | 3:1 | 0.254 | 3.841 |
| ICCM0160 | TTGCTTGAAACAACCTTTCG | CGGGTACAACCGTAGCAAAT | 300 | 340 | 300-340 | 69 | 15 | 3:1 | 2.286 | 3.841 |
| TA34 | AAGAGTTGTTCCCTTTCTTTT | CCATTATCATTCTTGTTTTCAA | 210 | 250 | 210-250 | 69 | 15 | 3:1 | 2.286 | 3.841 |
| TS72 | CAAACAATCACTAAAAGTATTTGCTCT | AAAAATTGATGGACAAGTGTTATTATG | 280 | 320 | 280-320 | 68 | 16 | 3:1 | 1.587 | 3.841 |
| TS57 | TCAATTTATAATCATAGAGAATCNGAGA | CCTAAAACAAATAAAATCTTAAATAATA | 280 | 310 | 280-310 | 62 | 22 | 3:1 | 0.063 | 3.841 |
| TA25 | AGTTTAATTGGCTGGTTCTAAGATAAC | AGGATGATCTTTAATAAATCAGAATGA | 250 | 300 | 250-300 | 65 | 19 | 3:1 | 1.587 | 3.841 |
| TA144 | TATTTTAATCCGGTGAATATTACCTTT | GTGGAGTCACTATCAACAATCATACT | 250 | 280 | 250-280 | 68 | 16 | 3:1 | 2.124 | 3.841 |

along with some minor QTLs. On the basis of above results the marker TA118, TS72, TA144, TA47 and TA34, TS 57, TA 43, TAA 137 were found to be associated with resistance to BGM. Anuradha *et al.* (2011) also validated the marker TA 118 and TA144 by using recombinant inbred lines (RILs) of a cross ICCV 2 × JG 62. The marker TA 144 was also validated by Ranjana *et al.* (2019) for *Botrytis* grey mould disease resistance and reported that, there is a correlation between the primers TA144 and for BGM disease resistance. Sachdeva *et al.* (2019) also reported that marker TA144 was

strongly correlated with resistance to BGM disease. Kushwah *et al.* (2021) used genome wide single nucleotide polymorphism (SNPs) markers and found five QTLs *viz.,* qbgm-3.1, qbgm-4.1, qbgm-4.2, qbgm-5.1 and qbgm-6.1 to be associated with resistance to BGM on chromosomes 3, 4, 5 and 6. Out of these five QTLs, qbgm-4.1, qbgm-4.2 and qbgm-5.1, were found to be most consistent. Their study also indicated that both major and minor QTLs were involved in governing resistance to BGM and most of these QTLs were present on linkage group 3, 4 and 5. The above
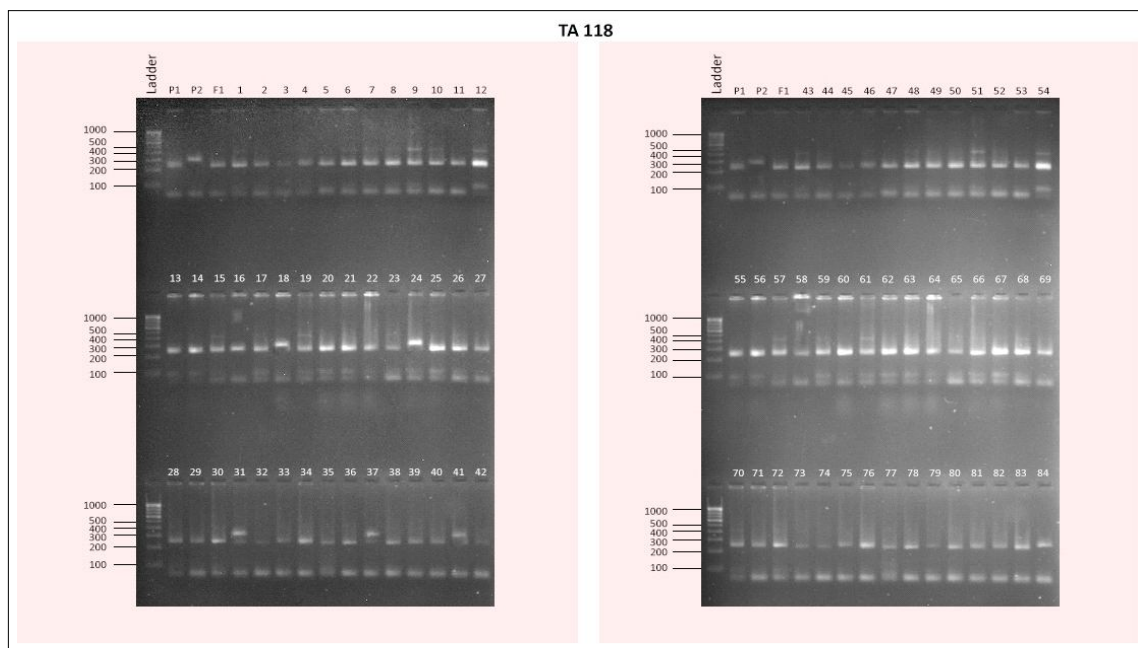


**Fig 1:** Amplification profile of primer TA118 in parents, $F_1$ and $F_2$ population in cross GL10006 × H208.
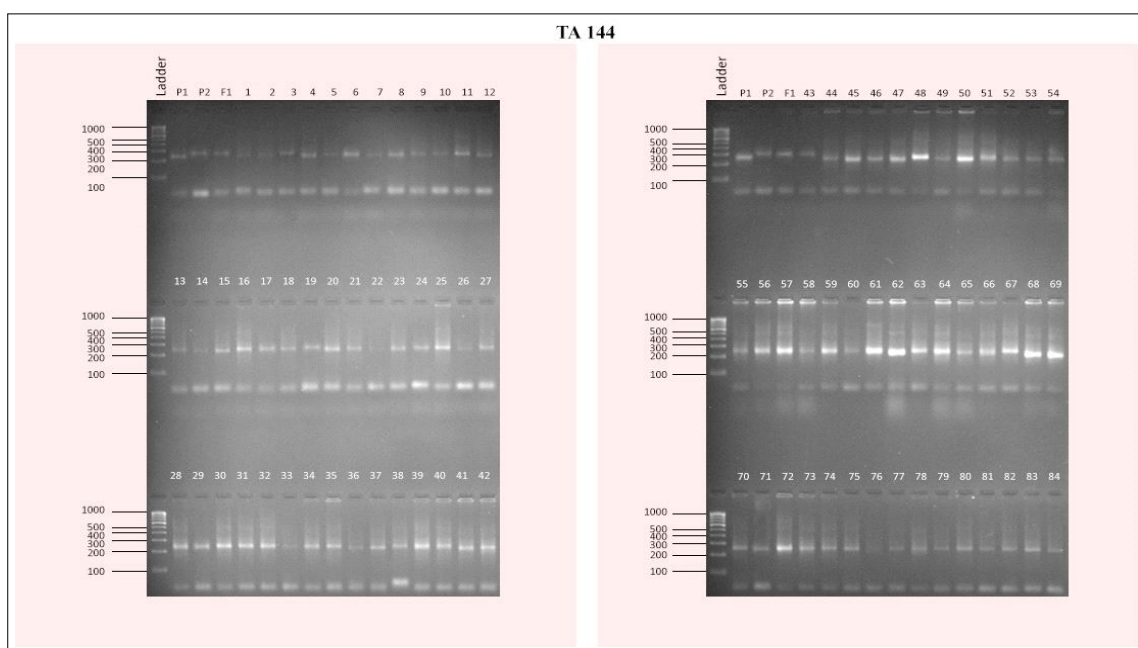


**Fig 2:** Amplification profile of primer TA144 in parents, $F_1$ and $F_2$ population in cross GL10006 × H208.

**Table 4:** Single marker analysis results of GL10006 × H208.

| Marker | Chromosome | P-value | $R^2$ |
|---|---|---|---|
| TA203 | 1 | 0.66408 | 0.58 |
| TA43 | 1 | 0.46880 | 1.55 |
| TAA137 | 1 | 0.46880 | 0.92 |
| TA47 | 2 | 0.46034 | 2.79 |
| ICCM0160 | 2 | 0.46034 | 0.20 |
| TA144 | 3 | 0.00041*** | 5.48 |
| TA25 | 3 | 0.00041*** | 0.05 |
| TA118 | 3 | 0.00041*** | 28.25 |
| ICCM0068 | 4 | 0.02154* | 0.39 |
| TS72 | 4 | 0.02154* | 7.85 |
| TA29 | 5 | 0.42672 | 0.40 |
| TA34 | 5 | 0.42672 | 2.71 |
| TS57 | 5 | 0.42672 | 1.74 |

*, **, *** Significant at 5%,1% and 0.01% level of significance, respectively.

discussion indicated that the validated STMS markers identified from present study will be useful in marker-assisted selection of desirable recombinants in resistance improvement programmes of chickpea.

## CONCLUSION

Development of resistant varieties against BGM is must for obtaining the higher yield in chickpea. In this context it becomes very important to understand the nature of inheritance of resistance of BGM. The findings of present study indicated that the resistance against BGM is dominance over susceptibility. The results of Single marker analysis results indicated that a major QTL *i.e.* TA118 along with some minor QTLs *i.e.* TS 72, TA 144, TA47, TA34, TS 57, TA 43 and TAA 137 are involved in governing resistance to BGM. The major QTL (TA118) belongs to LG 3. On the basis of above results it can be concluded that the marker TA118, TS72 and TA144 can be used effectively in marker assisted selection for desirable recombinants in chickpea breeding.

## ACKNOWLEDGEMENT

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