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RILs development and its characterization for MLB resistance and flowering in maize (*Zea mays*)

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ABSTRACT

Maydis leaf blight (MLB) resistance and days to flowering are the important yield determining traits in maize. Breeding for MLB resistance and days to flowering can be accelerated by understanding their genetics and identifying genomic regions contributing for their expression. Two F_2s population with 338 and 349 individuals along with their recombinants inbred lines (RILs) having 283 and 277 individuals were developed from F_1 crosses HKIPC4P × CML269 and ESM113 × P72clXbrasil1117 for genetic studies of MLB resistance and flowering. The populations along with their parents were screened under artificially inoculated conditions at hot-spot sites during 2015–17. Race *O* inoculum was artificially inoculated in the leaf whorl of each plant at 4-6 leaf stage. The inoculation was repeated after 8-10 days of first inoculation to avoid any chance of disease escape. The partial dominance in F_1s, normal distribution patterns in F_2s and RILs for both the traits has indicated their polygenic nature. Correlation analysis found negative and significant association ($P \leq 0.001$) between disease scores and days to flowering across the populations. Total 250 simple sequence repeats (SSR) markers, uniformly selected from all linkage groups were used for parental polymorphism survey between parents of the populations contrasting for target traits. Of total 250 SSRs, 122 (48.8% polymorphism) were identified as polymorphic between either of the parents. Sufficient genetic variation was observed within and between different F_2s and RILs mapping populations. The information on inheritance, parental polymorphism survey and genetic materials developed will be useful for fine mapping and systematic breeding of targeted traits in tropical maize germplasm.

Key words: Genetic study**,** Maydis Leaf Blight, Polygenic traits, Simple Sequence Repeats

Maydis leaf blight (MLB) caused by the fungus C*ochliobolus heterostrophus* is a serious foliar disease of maize (*Zea mays* L.) distributed widely in warm and humid maize growing areas throughout the world. It can cause yield losses up to 40% (Fisher *et al.* 1976, Gregory *et al.* 1979). The development of MLB-resistant cultivars is an economically viable and environment friendly means of controlling the disease. The mixed types of reports such as monogenic recessive, dominant and polygenic nature of its inheritance are available in the literature (Smith and Hooker 1973, Faluyi and Olorode 1984, Zaitlin *et al.* 1993).

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The identification of quantitative trait loci (QTLs) based only on conventional phenotypic evaluation is tough. However, advent of molecular markers technology allows disease resistance to be broken up into its components by QTL mapping and thereby it became easy and routine process to discover genes involved in resistance. Therefore, to uncover the genetic basis of complex traits, it is the pre-requisite to develop genotype, phenotype and the large mapping populations. As of now there is limited information available on genomic regions governing flowering time and providing resistance to MLB disease in tropical maize germplasm. The previous genomic research for MLB resistance was mostly focused in temperate germplasm (Balint-Kurti *et al*. 2007, 2008). The five genomic loci among the identified contribute 8–23% for MLB resistance (Balint-Kurti *et al.* 2006b) in maize. These were found unlinked in tropical maize populations (Kumar *et al.* 2016). Late flowering maize lines tend to be more resistant to MLB, northern corn leaf blight (NCLB) and grey leaf spot (GLS). Further, Wisser *et al.* (2011) reported that 48, 45 and 52% variation of resistance to NCLB, MLB and GLS diseases, respectively, was ascribed due to days to flowering. The

information on QTLs for MLB resistance and flowering time are still lacking for tropical maize germplasm being grown in India. Therefore, the present study was carried out to study genetics, develop mapping populations and their characterization. This will further facilitate the fine mapping and resistance breeding in tropical maize germplasm.

MATERIALS AND METHODS

Development of populations: The late maturing MLB resistant (CML269, P72clXbrasil1117) and early maturing susceptible inbred lines (HKIPC4P, ESM113) were selected based on previous information (Kumar *et al.* 2016). They were again validated for disease reaction under artificially created epiphytotics and days to flowering during *kharif* 2014. The two different F_2 populations of size 338 and 349 were developed from F_1s crosses, viz. HKIPC4P (S) × CML269 (R); ESM113 (S) × P72clXbrasil1117 (R), attempted during 2014, respectively. Similarly two different RIL mapping populations (F_8) of size 283 [HKIPC4P (S) × CML269 (R)] and 277 [ESM113 (S) × P72clXbrasil1117 (R)] individuals were developed from F_1 crosses attempted during 2012 and advanced by following ear to row method from F_2 onwards. The parents, along with their F_1s, F_2s and RILs were used for evaluation against MLB and days to flowering.

Inoculation and data recording: Artificial inoculation was employed during field screening following Carson *et al.* (2004), with minor modifications. Cultures (Isolate-Delhi) of C*ochliobolus heterostrophus* Race *O* was prepared in conical flask containing sorghum grains (nearly 45g). Flasks with sorghum grains were soaked in water for about 3-4 hours. It was then autoclaved twice, seeded with fungus aseptically and kept for incubation at 25-27°C for 15 days. The flasks were shaken once in 2–3 days. After incubation period, the material was dried at room temperature on clean paper under shade. The grains along with mycelium were then ground into fine powder which was used in inoculation. Parents and F_1s were planted in two rows, three replications using randomized block design (RBD) at hot-spot location (Delhi). The F_2 populations were planted in the continuity of parents and F_1s during 2015. The RIL mapping populations were also evaluated for MLB disease and flowering during 2016–17 at Delhi using an augmented block design. Each genotype/population was planted in 4m row length with row-to-row spacing of 70 cm and plant-to-plant 20 cm.

Experimental materials and border rows were artificially inoculated with Race *O* inoculums in the leaf whorl of each plant at four-to-six leaf stage. The field was kept adequately moist with irrigation so as to commence the fungal growth. The inoculation was repeated after 8-10 days of first inoculation to avoid any chance of disease escape. The plants (in parents, F_1 & F_2) were rated using 1.0–5.0 disease scale (Payak and Sharma 1983), [≤ 2.0 (resistant); ≥ 4.0 (susceptible)]. However, the disease rating in RIL populations was done following 1.0-9.0 scale (Balint-Kurti *et al.* 2006b) [≤3.0 (resistant); ≥7.0 (susceptible)]. The data was also recorded on days to 50% anthesis and silking. Disease rating was done 15 days after initiation of flowering. Days to flowering was correlated with the disease severity.

Parental polymorphism survey: DNA of individual parent was extracted following Saghai-Maroof *et al.* (1984) method with minor modifications (Kumar *et al.* 2008). Markers were standardized for their annealing temperature using gradient PCR. Parental polymorphism survey between resistant and susceptible parents of RILs populations was carried out using 250 (for 283 size RILs) and 147 (for 277 size RILs) simple sequence repeat (SSR) markers (~@15-25/chromosome). The PCR products were resolved on 3% metaphor agarose gel. Allele size was scored for each marker in accordance with the 50bp *λ DNA* ladder.

RESULTS AND DISCUSSION

The average disease score under artificially created epiphytotics for resistant parents was 1.28 (CML269) and 1.44 (P72c1Xbrasil1177-2), and for susceptible one was 4.68 (HKIPC4B) and 4.52 (ESM113) on 1.0–5.0 rating scale. The disease score in resistant and susceptible parents was ranging from 1.0–2.0 and 4.0–5.0, respectively; indicating sufficient disease pressure as well as variation for MLB disease response (Table 1). The average days to flowering in the parents and their F_1 populations varied from 53.9 (susceptible) to 62.0 days (resistant), and 57.2–58.5, respectively. The ranges for disease symptoms and flowering in the parents have clearly represented their contrasting behavior for the targeted traits. Therefore, they can be the ideal parents for genetic studies as well as development of mapping populations for genomic studies. In the F_1 crosses, viz. HKIPC4B (S) × CML269 (R) and ESM (S) × P72c1Xbrasil1177-2 (R), the average disease score was 2.32 and 2.42 with a range of 1.5–3.0, and 1.5–3.5, respectively. Similarly in both the F_2 populations, the disease score ranged from 1.0 to 5.0 with an average of 3.12–3.14 (Table 1). The disease score in RILs varied from 3.3–8.5 and 3.5–8.0 on 1.0–9.0 disease rating with average score of 6.02 and 5.7 for size 283 and 277 individuals, respectively (Table 1). Further, the sufficient genetic variation was observed for days to anthesis as well as silking in both the RILs. The days to silking varied from 41.0–63.0 and 43.0–64.0 with average of 53.8 and 54.5 days in two different RILs (Table 1). The sufficient field variation for MLB disease response and flowering was also established through box-plot analysis (Fig 1).

The average disease score of F_1s clearly indicated the partial dominant nature of MLB resistance over its susceptibility. The continuous distribution was observed in F_2s and RILs populations for disease score as well as days to silking (Fig 2). This was further confirmed through chi-square test when the null hypothesis (H_o) for monogenic and digenic control was rejected at $P<0.001$ for both traits. These findings conferred the polygenic control of MLB resistance as well as flowering; therefore the breeding strategies for genetic improvement may be followed accordingly. The polygenic control for resistance to MLB has been observed in various studies (Zwonitzer *et al.* 2010, Kump *et al.* 2010,

Table 1 Descriptive statistics for disease response and days to silking in parents, their F_2s and RIL mapping populations

Genotype/population	Descriptive statistics					
	Disease score data			Days to silking		
	Min.	Max.	Mean	Min.	Max.	Mean
CML 269 (R)	1.0	1.5	1.28±0.32	62.0	63.0	62.5±0.5
HKIPC4B (S)	4.0	5.0	4.68±0.35	51.0	53.0	52.4±0.6
P72c1×brasil1177-2(R)	1.0	2.0	1.44±0.36	62.0	63.0	62.9±0.6
ESM113 (S)	4.0	5.0	4.52±0.47	55.0	56.0	55.3±0.5
HKIPC4B ×CML269 (S×R)	1.5	3.0	2.32±0.33	57.0	58.0	57.2±0.2
ESM113×P72c1Xbrasil1177-2 (S×R)	1.5	3.5	2.42±0.45	58.0	60.0	58.5±0.6
F_2-(HKIPC4B ×CML269)	1.5	5.0	3.14±0.59	47.0	70.0	58.4±4.3
F_2-(ESM113×P72c1Xbrasil1177-2)	1.0	5.0	3.12±0.64	49.0	72.0	61.1±3.5
RILs (HKIPC4B ×CML269; 283)*	3.3	8.5	6.02±0.97	41.0	63.0	53.8±2.9
RILs (ESM113×P72c1Xbrasil1177-2 ; 277)	3.5	8.0	5.7±0.96	43.0	64.0	54.5±3.2

*RILs were scored using 1.0–9.0 disease rating, however remaining all were scored by 1.0-5.0 scale

Kumar *et al.* 2016). There was 4–5 days heterosis towards early flowering in the F_1 populations. This information may be useful while selecting the parents for development of different maturity group hybrids. The range of phenotypic variation for each trait has been reduced from F_2s to RILs population, this is due to fixation of many alleles while inbreeding during generation advancement.

As compared to the RILs, many outliers were found in F_2s population (Fig 1); however they might have become non-existent due to segregation distorting during the generation advancement. Still there were sufficient phenotypic variations in the RIL population after inbreeding which can be useful for fine mapping of targeted traits. Further, negative significant correlation (r = -0.43 and -0.53; $P<0.001$) was observed between disease score and days to flowering in all the populations. Late flowering maize lines tend to be more resistant to most of the foliar diseases. Several other studies have also found significant negative correlation amongst many of foliar diseases with days to flowering and maturity (Bubeck *et al*. 1993, Wisser *et al.* 2006, Kumar *et al.* 2016). The association of flowering time with disease score may be due to the pleiotropic effect and/or linkage of genomic region for such traits on same chromosomal segments. Of 250 SSR markers, 122 markers were polymorphic between resistant and susceptible parents. Around 50% of markers were found polymorphic between the parents, which was possible due to presence of large genetic diversity between them. As one of the parent of RIL

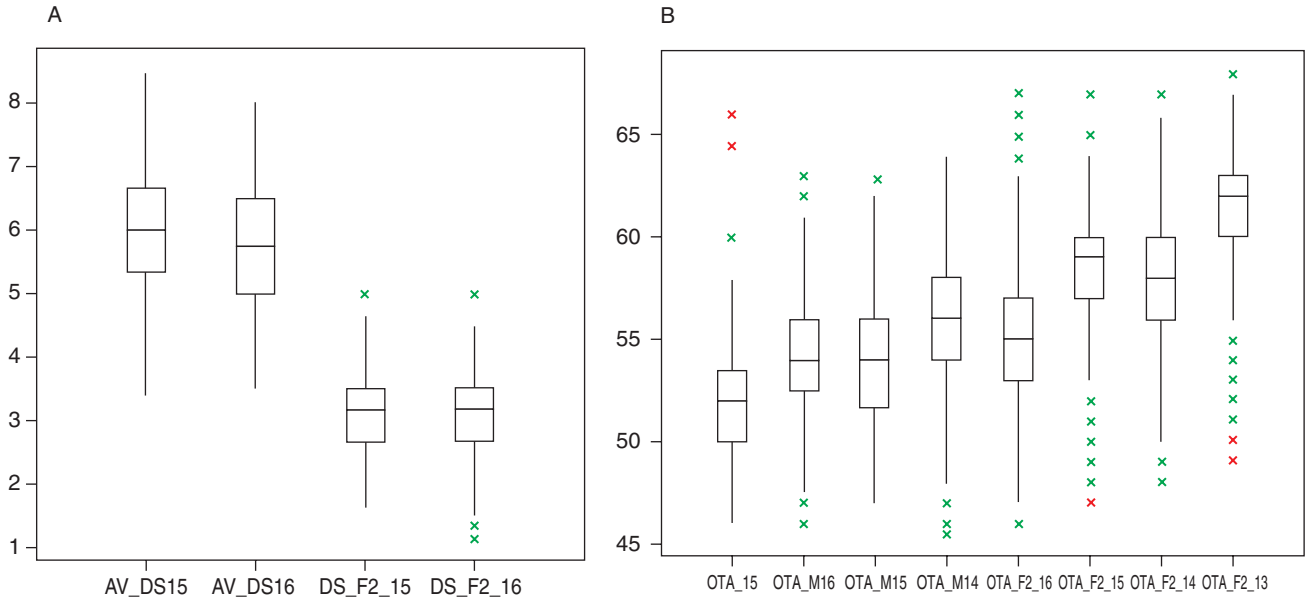

Fig 1 Box-plot showing the distribution of A: disease score (DS) and B: days to anthesis (DTA) and silking (DTS) in F_2s [(F_2_15: F_2 of cross HKIPC4B (S) × CML269 (R); F_2_16: F_2 of cross ESM113 (S) × P72c1Xbrasil1177-2)] as well as in RILs (Av_DS15, and Av_DS16) mapping populations. The sufficient variations following normal distributions have been represented for all traits.

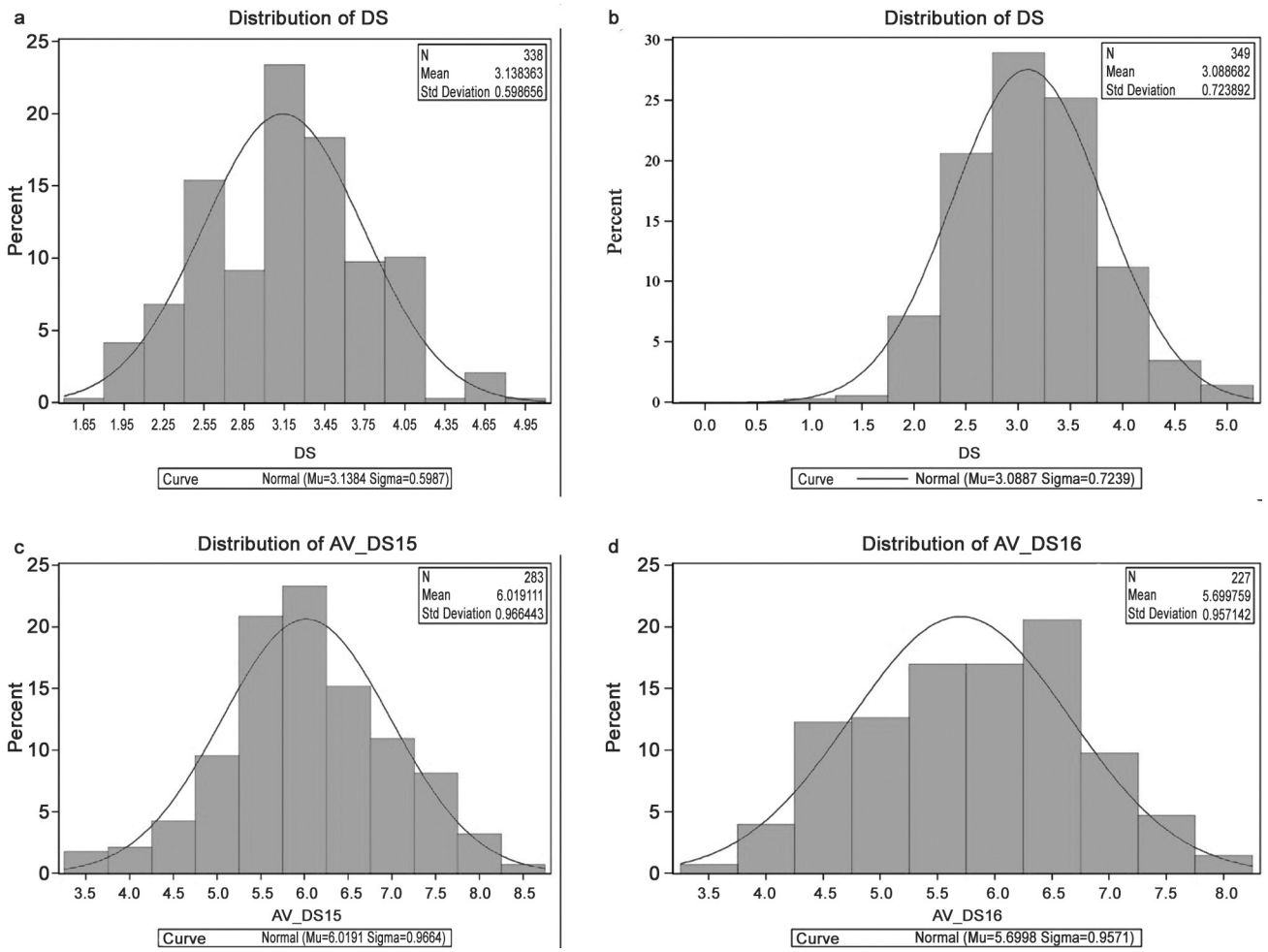

Fig 2 Distribution in F_2s (a, b) and RILs (c, d) populations for maydis leaf blight disease score. The F_2 population in a. was derived from cross HKIPC4B (S) × CML269 (R) and in b. from ESM113 (S) × P72c1Xbrasil1177-2 (R). Further, the c and d were the RILs mapping population derived from F_1 crosses HKIPC4B (S) × CML269 and ESM113 (S) × P72c1Xbrasil1177-2 (R) respectively. The continuous distribution in both F_2s as well as in RILs mapping populations has indicated the polygenic control MLB resistance in tropical maize.

mapping population is popcorn and another is normal field corn. Similarly, they belong to different maturity and have large genetic variation for other plant and kernels traits. Total of 147 SSR markers were common among both RILs for polymorphism survey. Of 147, 62 were polymorphic between the parents. Further, out of 62 markers, 23 were found polymorphic exclusively between parents CML269 (R) and HKIPC4P (S) while 13 between P72c1Xbrasil1177-2 (R) and ESM113 (S) (Table 2).

The 26 markers were identified polymorphic between both, resistant (CML269, P72c1Xbrasil1177-2) and susceptible (HKIPC4B, ESM113) parents of populations (Table 2). The markers, which were found common during polymorphism survey, may be very useful for fine mapping. The annealing temperature for SSRs markers ranged from 50°C to 62°C. The details of markers and their annealing temperature have been given in Table 2. Of total 122 polymorphic markers, 21 were distributed on chromosome (chr) 1; 16 on chr 2; 12 each on chr 3, 4; 11 on chr 5; seven each on chr 6, 7; nine on chr 8; 17 on chr 9 and 10 on chr 10. Further, there was significant negative correlation between disease resistance and days to flowering. Multiple genomic regions for MLB resistance in maize (bin 3.04, 6.06 and 9.03-9.04) have been reported consistently in a series of mapping studies in various genetic backgrounds (Balint-Kurti *et al.* 2007, Belcher *et al.* 2011), but could not differentiate between resistant and susceptible parents of various populations developed from Indian based tropical germplasm lines (Kumar *et al.* 2016). Wisser *et al.* (2011) has reported 45 to 52 % variation of resistance to maize foliar diseases due to days to flowering and found common genomic regions for both the traits, hence, our findings corroborate with them. The inheritance studies and various mapping populations developed here could be valuable assets for their fine mapping and systematic breeding efforts in Indian maize breeding programme.

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Table 2 Detail of SSR Markers standardized and used for parental polymorphic survey in different RILs mapping populations

Chromosome number	No. of polymorphic markers between parents of different RILs mapping populations	
	RILs (HKIPC4B ×CML269; 283)	RILs (ESM113×P72c1Xbrasil1177-2 ; 277)
1	bnlg1124*, phi097*, bnlg1458*, phi109275*, bnlg176*, umc1021&, umc2124*, bnlg2086 º, umc1917*, phi038*, phi308707*, umc2241*, phi227562*, umc1676@, bmc1866 º, bnlg2238^, bnlg1629+, bnlg1866*, bnlg1597*, bnlg1615*, phi037+	phi109275*, umc1917*, bnlg176*, umc2124*, bnlg1458*, umc1676@
2	phi96100º, umc1845*, Zca381*, nc133*, bnlg108*, nc003*, bnlg1940*, phi090*, umc2077@, umc2245$, mmc0491^, umc1560*, umc2205*, bnlg2077^, bnlg1606^, bnlg1265*	phi96100*, bnlg2248*, umc1845*, bnlg1940*, umc2077@
3	phi029*, phi036*, umc2000&, phi046*, umc1012*, umc1209@, umc1521@, bnlg1523º, bnlg1456º, umc1674º, umc1970º, bnlg1449^	bnlg1536+, phi046*, phi036*, umc1521@, bnlg1449^, bnlg1523º
4	phi072*, phi021*, phi076*, umc2284^, dupssr28#, umc1574$, bnlg1126º, bnlg1937*, umc1559*, umc1649*, umc2039*, umc2138*,	phi096*, dupssr28#
5	zag557*, nc130*, phi109188*, umc1332*, phi085*, umc1072*, bnlg1006^, umc2161*, umc2198@, umc1287^, bnlg105*	umc2303*, phi109188*, nc130*, phi008*, umc1332*, umc1056*, bnlg1006^
6	phi075*, zct161*, zag249*, bnlg1443*, phi089*, umc1859*, umc2141*	zag249*, bnlg1238@, umc2324*, bnlg1443*
7	phi112*, phi034*, phi114*, phi051*, umc1695*, dupssr13^, umc1066*	phi034*, umc1378*
8	bnlg1863@, umc1130*, mmc0181º, bnlg1252*, bnc1599*, phi015*, umc1673@, phi080$, phi420701*	bnlg1350*, mmc0181º, phi080$
9	Umc1267*, phi022*, phi065*, umc1094*, phi040º, umc1231*, bnlg128*, phi028@, phi067*, umc1893$, bnlg1159+, dupssr6º, umc1310º, umc1357*, umc2337*, umc1751*, umc2133º,	umc1417*, phi040º
10	Umc1152*, umc1061*, bnlg1762*, umc1962^, dupssr31º, bnlg1762$, phi118º, bnlg1450*, bnlg210^, phi063*	phi118º, bnlg1124*

Annealing temperature ^ = 50Cº, * = 55ºC, # = 57ºC, @ = 58ºC, $ = 59ºC, º = 60ºC, + = 62ºC, & = 52º C

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