Pak. J. Bot., 53(1): 301-308, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-1(5)

MORPHOLOGICAL AND MOLECULAR MARKERS BASED SCREENING OF MAIZE HYBRIDS AGAINST NORTHERN CORN LEAF BLIGHT

SADAF NAEEM[1], MUHAMMAD TAHIR[2], KIRAMAT KHAN[1], AMJAD HASSAN[1] AND RAFIQ AHMAD[1*]

[1]Department of Biotechnology, COMSATS University Islamabad, Abbottabad Campus, 22060, Abbottabad, Pakistan
[2]Department of Botany, Government Khawja Fareed Post-Graduate College, 64200, Rahim Yar Khan, Pakistan
**Corresponding author's email: drrafiq@cuiatd.edu.pk; drrafiq@ciit.net.pk*

Abstract

Northern Corn Leaf Blight (NCLB) is a fungal disease and it is a stumbling block in maize production. Moreover, conventional field evaluation of a large number of maize hybrids for NCLB is expensive as well as time consuming. This study was done to evaluate initially the 36 maize hybrids in field condition for NCLB resistant and agronomic parameters were measured. Twelve best maize hybrids resistant to NCLB were screened at molecular level by using specific primers of Ht1, Ht2, and Ht3 and HtN genes. Finally, we compared molecular data with morphological data to pinpoint the maize hybrids with enhanced resistance against NCLB. The results showed that germination of 36 hybrids including three check hybrids (commercial ones) were not statistically different except pwx-131-6. Hybrid FRW2 x PSE.3-157-5-4-2 showed maximum yield 11.474 tons per hectare followed by check hybrid CH-III (30 k08) with 11.018 tons per hectare among all finally 12 selected hybrids. Disease ranking showed that both above high yielding hybrids were resistant to NCBL. In addition, both hybrids showed the presence of all resistant genes Ht1, Ht2, Ht3 and HtN that could be involved in resistance to NCLB. Among the commercial hybrids, CH-II(BABAR) was moderately resistant to NCLB having disease ranking 2 and all the resistant genes Ht1, Ht2, Ht3 and HtN were absent in this commercial hybrid. Globally, 4 maize hybrids i.e. PSEV.3-15-5-4-2 × PK9, FRW2 x PSEV.3-120-2-3-2, PSEV.3-157-5-4-3 x (FRW3x FRW6)sp out of 12 maize hybrids were moderately resistant while all others were resistant according to disease ranking but variation in resistant genes were observed in all maize hybrids. The morphological and molecular based screening use in this study can be used in other plants that are sensitive to NCLB particularly using Ht1, Ht2, Ht3 and HtN genes.

Key words: Maize hybrids; Agronomic parameters; Genes; NCLB; Screening.

Introduction

Maize (*Zea mays* L.) belongs to the Poaceae family and it has the highest yield potential among all cereal crops due to its large leaf area and C_4 pathway for carbon fixation that is more efficient as compared to common C_3-pathway. Major proportion of the maize is used for human consumption in poorer countries, whereas in the industrialized countries, most of the crop is used for animals (Louie, 2017). The total production of maize is affected by several factors such as weather conditions (Ahmad *et al.*, 2014; Shahzad *et al.*, 2020), hybrids cultivation, imbalance use of inputs and number of pathogens. The major factor for lower yields is sensitivity of maize to several diseases and abiotic stresses (Rahul & Singh, 2002; Arif *et al.*, 2017; Zhou *et al.*, 2019; Riffat & Ahmad, 2020). Maize crop is infected by approximately 65 pathogens (Rahul and Singh, 2002) and the most important are gray leaf spots (GLS), different types of rust, southern leaf blight (SLB) and northern leaf blight (NLB) which causes yield reduction (Rahul and Singh, 2002; Razzaq *et al.*, 2019) as well as limit maize productivity worldwide.

Northern corn leaf blights (NCLB) is the most common disease of maize caused by the *Setosphaeria turcica*. It is one of the major foliar diseases in maize and it reduces yield of maize up to 15-30% or even more (Raymundo & Hooker, 1981; Masuka *et al.*, 2017). As NCLB is a foliar disease, therefore, it prevails in humid areas (Levy and Cohen, 1983). In northern corn leaf blight, initial spots of NCBL appear in the lower leaves and grow upward. Grey-green lesions grow and become elliptical and cigar shape. Mature lesions are long, straw-grey color killing large parts of the leaves but in severe cases killing the whole plant. In Pakistan NLB occurs more in the maize growing hilly areas of Khyber Pakhtun Khwa resulting in 20% or some times more yield losses (Hafiz, 1986). Losses may exceed 50% if fungus attacks before flowering (Tefferi *et al.*, 1996). Low temperature, high humidity, heavy dews and repeated rains are the favorable environmental conditions for development and severity of disease (Jordan *et al.*, 1983). The Mid-altitude areas of 900-1600 m above sea level such as in eastern and southern Africa, Latin America, China and India have a particularly favorable climate for this disease (Renfro & Ullstrup, 1976).

NCLB is mainly controlled by resistant cultivars and that resistance could be either qualitative or quantitative. Qualitative resistance is typically race specific and inherited by single genes whereas quantitative resistance is not race specific but oligo-genic or polygenic. NCLB is a monogenic resistance conferred by *Ht* genes such as *Ht1*, *Ht2*, and *Ht3* genes (Galiano-Carneiro & Miedaner, 2017). Polygenic resistance to NCLB is expressed as a reduction in the development of disease severity (Li & Liu, 1984; Smith & Kinsey, 1993). Because the categories qualitative and quantitative refer to the distribution of a trait in a population and not to its effectiveness (Geiger & Heun, 1989) and one cannot equate qualitative with complete and quantitative with partial resistance. The level of resistance conferred can range from small reductions in pathogenicity to complete immunity. Complete resistance, also called qualitative resistance, *R*

gene resistance, major gene, monogenic, or vertical resistance, is usually conditioned by a single gene (St. Clair, 2010). In contrast, quantitative disease resistance (QDR), known also as adult-plant, durable, general, partial, polygenic, or horizontal resistance, is generally controlled by many genes each of which have small effects (St. Clair, 2010). DNA markers are defined as a small fragment of DNA revealing mutations / variations that could be used to detect polymorphism between different genotypes. Southern blotting (Southern, 1975) and PCR (Mullis, 1990) are two basic methods to detect the polymorphism.

Northern Corn Leaf Blight (NCLB) can cause more than 30% yield loss and is a stumbling block in maize production. Moreover, conventional field evaluation of a large number of hybrids for NCLB is expensive as well as time consuming. Therefore, this research was designed to study following objectives in order to find out the authentic, less expensive and less time consuming method for screening of maize against NCLB, i) To evaluate 36 maize hybrids in field condition for NCLB by using standard data scoring procedure, ii) To screen maize hybrids resistant to NCLB at the molecular level by using specific primers, iii) To compare molecular data with morphological data to pinpoint the hybrids with enhanced resistance against NCLB.

Materials and Methods

Field conditions and experimental hybrids: The experiment was conducted at CIIT COMSATS Abbottabad. The soil of the experimental site was slightly acidic (pH of 6.0 - 6.5) and temperature during growing period was 25-40°C. A plot size of two rows each 3 m long and 0.75 m between rows was used. Initially two seeds per hill were used and was later thinned to one seedling per hill. Fertilizer in the form of urea and DAP was applied (150 kg N and 75 kg P_2O_5 ha^{-1}). Half of N and whole P_2O_5 were applied at the time of sowing, while the remaining half dose of N was applied when the plant was about 30 cm tall.

Agronomic parameters: Days to flowering were measured as the number of days from seedling emergence to 50% sillking on each plot. Number of plants were counted for each plot at the time of harvest and converted to plant population / ha by using following formula:

No. of plants/ha = No. of plants x 10,000 / Plot size

Ten plants from each plot were randomly selected at physiological maturity (formation of black layer in seed) and plant height was measured in cm from base of each plant to the base of tassel with the help of measuring rod. Ear height (cm) from the base to node of ear emergence was measured by using a measuring rod on ten randomly selected plants in each plot. It is one of the most important traits as it directly affects the yield of maize crop. Disease scoring was recorded 2 weeks before harvesting by using the CIMMYT disease scale. All plants in the plot were used for successive disease assessments. Plants were rated at 10 days interval for percent incidence, the number of lesion on the ear leaf and second leaf above the ear leaf. NCLB severity rating was done as follow;

1.0 = Resistant, one or two restricted lesion on lower leaves or trace.
2.0 = Moderately resistant, slightly to moderate infection on lower leaves.
3.0 = Moderately susceptible, abundant lesions on lower leaves/ a few on middle leaves.
4.0 = Susceptible, abundant lesions on lower and middle leaves extending to upper leaves.
5.0 = Highly susceptible, abundant lesions on all leaves, plant may be prematurely killed by blight

Shelling percentage was taken for each plot at uniform moisture content of 14 % by using the formula:

Shelling (%) = Grain weight (g)/Cob weight + grain wt. × 100

Grain yield per plant was calculated from data by using the following formula:

Grain yield per plant= {grain yield/no plants}

Grain Yield per hectare was calculated from fresh ear weight by using the following formula:

Grain yield ton = FEW x shelling % (100 – Mc) x10, 000 / plot size x (100- *12) x100

where;
FEW = fresh ear weight at harvest in kg
MC = Moisture content in grain at harvest
Plot size = harvested plot size (m^2)
Shelling % = grain wt. /grain wt. +cob wt
Desired moisture content of grain = grain wt. /grain wt. +cob wt x 100

DNA extraction and amplification of target genes: DNA extraction was carried out from seed and fresh leaf samples using CTAB protocol as described by (Sahu *et al*., 2012). The exponential amplification of target DNA sequences of Ht1, Ht2, Ht3 and Htn1 genes was done through Polymerase Chain Reaction by using the 2720 thermo-cycler "Applied Biosystem". The purpose of a PCR is to produce millions of copies of a specific DNA sequence in approximately two hours (Jamil *et al*., 2020). The amplification conditions in Thermo cycler were kept as; initial denaturation at 94ºC for 5 minutes, 35 consecutive cycles of (denaturation at 94ºC for 45 seconds, annealing at 61ºC depending on each primer for 1 minute and elongation at 72ºC for 1 minute) followed by final extension at 72ºC for 10 minutes (Table 1).

Table 1. The SSR primers used to amplify Ht1, Ht2, Ht3 and HtN1 genes from maize seeds.

S. No.	Genes name	Forward and reverse primers sequences for each gene
1.	*Ht1*	5′-GAAGGTTGCTCTTCCACTGG-3′ 5′-TGGTTTGTGCAAGTGTCACC-3
2.	*Ht3*	5′-GCTGGTAGCTTTCAGATGGC-3 5′-TGTCCCTCCTCCAGTTTCAC-3
3.	*Ht2*	5′-CAATCAGGAGCCAGGGAGATG-3 5′-CTTAAACTTGTCGAGACGGTCCTG-3
4.	*HtN1*	5′-AAGAACAGAAGGCATTGATACATAA-3 5′-TGCAGGTGTATGGGCAGCTA-3

Visualization of PCR product on Agarose gel electrophoresis: Agarose gel apparatus was used to see the amplicons of Ht1, Ht2, Ht3 and HtN1. Amplicons of each gene was run on 1% agarose gel prepared in 1X TBE buffer. 1 gram of agarose was dissolved in 100 ml of 1X TBE buffer to form a 1% Agarose gel. The solution was put in microwave oven to dissolve the agarose and then let the solution cool down at room temperature and the solution was stirred while cooling. We added 2 µl ethidium bromide stock solutions in 30 ml agarose gel and then poured it into the gel rack. After the gel was prepared, a micropipette was used to inject about 5 µl of stained DNA current at 100 volt for 30 minutes was applied to the electrophoresis chamber. The DNA ladder of known size was run in the gel to precise the length of each band size.

Statistical analysis

Data on each morphological parameter was analyzed by ANOVA format for randomized complete block design. Software GENSTAT, 12 ed. was used to analyze the data and mean comparison using least significant difference (LSD) tests.

Results

Days to 50% silking: Mean comparison for days to 50% Silking of 36 maize hybrids including three commercial hybrids, CH-I(2ES), CH-II(BABAR) and CH-III(30 k08) used as a check hybrids are presented in Table 2. Highly Significant differences were observed among hybrids for days to 50% silking. According to statistical analysis two hybrids FRW2 × SHS.2-131-6 (13) and FRW2 × SHS-131-6(3) took minimum days i.e. 58 and 61 days for silking, respectively and both were statistically different from all other hybrids including commercial ones (CH-I(2ES). The hybrid Rmw8xpw was found significantly late in flowering than all experimental hybrids. Seventeen of the 33 hybrids were significantly later than the commercial hybrid CH-III (30 k08).

No. of plants ha^{-1}: Mean comparison of no of Plants ha^{-1} for 36 maize hybrids including three commercial hybrids, CH-I (2ES), CH-II(BABAR) and CH-III(30 k08) used as a check hybrids are presented in (Table 2). Number of plants per hectare was used to check the germination rate of 36 hybrids including three check hybrids. Statistical analysis showed that all hybrids including commercial ones were not statistically different except PW × SHS.2 -131-6. It means that our hybrids performed equally well in term of germination in compared with commercial hybrids. However, highest number of plants (75000 per ha) in (Table 2) were observed in FRW2 × PSEV.3-120-2-3-2 and (72917 per ha) inFRW2 × PSE.3-157-5-4-2 and minimum plant density (46875) was observed in PW × SHS.2 -131-6 that was statistically different from all other hybrids.

Plant height (cm): Mean comparison for plant height (cm) of 36 maize hybrids including three commercial hybrids, CH-I(2ES), CH-II(BABAR) and CH-III(30 k08) used as a check hybrids are presented in Table 2. Plant height differences among hybrids were significant. The Hybrid FRW3 × FRW6 showed maximum 210 cm height and PW×SHS.2 -131-6 had 212.5 cm minimum plant height and Hybrid PSEV.3-157-5-4-2×PW showed significantly maximum plant height (282.5) followed by FRW2 × PSE.3-157-5-4-2 (280). Four hybrids (FRW2 × PSE.3-157-5-4-2, PSEV.3-15-5-4-2 × PK9, RMW8 × PSEV.3 -157-5-4-2, PSEV.3-70-4×(FRW3 x FRW6)sp2) showed highest plant height compared to commercial hybrids CH-I(2ES), CH-II(BABAR) and CH-III(30 k08), while rest of the hybrids plant height was less than commercial ones.

Ear height (cm): In our experiment, mean comparison of Ear Height for 36 maize hybrids including three commercial hybrids, CH-I(2ES), CH-II(BABAR) and CH-III(30 k08) used as a check hybrids are presented in Table 2. Among all hybrids these two Hybrids showed minimum ear height, FRW2 × SHS-131-6(3) (85 cm) and PW × SHS.2 -131-6 (86.2 cm) ear height. Differences in ear height among hybrids were significant. The hybrid CH-I (2ES) had maximum ear height of 137.5 cm among all experimental hybrids. Five hybrids out of 33 have more ear height as compared to commercial hybrid CH-III (30 k08).

Disease attack: Mean comparison for NCLB of 36 maize hybrids, including three commercial hybrids, CH-I (2ES), CH-II (BABAR) and CH-III (30 k08) used as a check hybrids are presented in Table 2. Statistical analysis showed a non-significant difference among hybrids for NCLB disease. However, all the hybrids were divided into two categories on the disease raking i.e. resistant and moderately resistant (Table 2). Maximum disease ranking (2.25) was observed in FRW2 × PSEV.3-120-2-3-2-2 that showed moderate resistance against NCLB with few lesions on the lower leaves, whereas minimum disease rate (1) was observed in the hybrid PSEV.3-45-4-3-7 × (FRW3 × FRW6) that showed maximum resistance to NCLB. Six hybrids among all the experimental hybrids, including one commercial hybrid CH-II (Babar) were moderately resistant for NCLB while all others were resistant to NCBL.

Grain yield: Highly significant differences were observed among hybrids for grain yield per hectare (Table 2). A highest grain yield (11.693 ton ha^{-1}) was found for PSEV.3-70-4 × (FRW3 × FRW6) (33) followed by w2×157-5-4-2 with a yield of 11.693 ton ha^{-1} whereas the lowest grain yield (2.224 tonha^{-1}) was observed for RMW8× PW. Nine hybrids, including two commercial hybrids CH-I (2ES) and CH-III (30 k08) were not significantly different from each other, but showed a significantly higher grain yield from all other experimental hybrids.

Shelling percentage: Significant differences were observed among hybrids for shelling**.** Highest shelling % (89.92%) was observed in CH-III (30 k08) that was significantly different from all other hybrids (Table 2). The second highest shelling percentage (87.14) was observed in PSEV.3-70-4 x (FRW3×FRW6) (33) that was statistically not different from 21 maize hybrids. The lowest shelling % (77.62%) was observed in RMW8 × PW.

Table 2. Agronomic parameters of 36 maize hybrids grown at COMSATS Abbottabad during summer 2013.

S. No.	Hybrids	Days to 50 % Silking	No. of plants ha^{-1}	Plant height (cm)	Ear height (cm)	Shelling percentage	Disease NCLB	Yield/ha (tons)
1.	FRW2 x PSE.3-157-5-4-2	71.50 Hijklm	72917 Ab	280.0 D	132.5 De	84.15 Bcdefg	1.750 A	11.474 Ab
2.	PSEV.3-45-4-3 x PW	74.00 Klmn	70833 Ab	250.0 Abcd	100.0 Abcde	81.85 Gh	1.500 A	6.211 Hijkl
3.	FRW2 x SHS-131-6	61.00 Ab	61458 Abcde	228.8 Abcd	85.0 A	83.45 Cdefgh	1.750 A	7.719 Defghi
4.	PSEV.3-70-4 x FRW6	66.00 Def	69792 Ab	247.5 Abcd	108.8 Abcde	85.57 Bcdef	1.500 A	9.314 Abcdefg
5.	PSEV.3 -45-4-3-7 x PW	71.50Hijklm	71875 Ab	227.5 Abcd	96.2 Abcd	84.12 Bcdefg	1.250 A	6.475 Ghijkl
6.	PSEV.3-70-4 x FRW.3	68.00Efghi	70833 Ab	218.8 Abc	120.0 Abcde	86.47 Bc	1.000 A	9.328 Abcdefg
7.	FRW3 x FRW6	66.50 Defg	61458 Abcde	210.0 A	110.0 Abcde	84.26 Bcdefg	1.000 A	4.324 Jl
8.	FRW2 x SHS-26-2	64.50 Bcde	68750 Ab	220.0 Abc	102.5 Abcde	84.37 Bcdefg	1.750 A	7.831 Defghi
9.	PW x SHS.2 -131-6	63.00Bcd	46875 F	212.5 Ab	86.2 A	82.96 Defgh	2.000 A	5.485 Ijkl
10.	PSEV.3-157-5-4-2 x SHS.2- 131-6	63.00Bcd	71875 Ab	233.8 Abcd	96.2 Abcd	85.81 Bcde	1.500 A	8.242 Cdefghi
11.	PSEV.3-70-4 x SHS.2-131-6	62.00Bc	55208 Ce	265.0 Bcd	113.8 Abcde	82.58 Efgh	1.500 A	8.400 Cdefgh
12.	FRW2 x RMW8	69.00Fghij	70833 Ab	247.5 Abcd	102.5 Abcde	82.81 Efgh	1.500 A	7.307 Efghi
13.	FRW2 x SHS.2-131-6	58.75A	69792 Ab	245.0 Abcd	112.5 Abcde	83.25 Cdefgh	1.250 A	8.906 Bcdefgh
14.	FRW2 x PSEV.3 -45-4-3-8	70.00Fghijkl	72917 Ab	248.8 Abcd	107.5 Abcde	84.52 Bcdefg	1.250 A	10.408 Abcd
15.	RMW8 x PK9	72.50 jklmn	72917 Ab	251.2 Abcd	105.0 Abcde	80.95 Hi	1.750 A	6.226 Hijkl
16.	PSEV.3-15-5-4-2 x PK9	71.50Hijklm	60417 Abcde	272.5 Cd	112.5 Abcde	84.40 Bcdefg	2.000 A	9.235 Abcdefg
17.	FRW2 x PSEV.3-45-4-3-8-2	73.00 Jklmn	68750 Ab	241.2 Abcd	95.0 Abcd	83.90 Cdefg	1.750 A	8.674 Cdefgh
18.	FRW2 x PSEV.3-120-2-3-2-2	71.50 Hijklm	68750 Ab	245.0 Abcd	90.0 Ab	79.52 Ij	2.250 A	7.626 Defghi
19.	RMW8 x FRW2	70.50Fghijklm	72917 Ab	248.8 Abcd	100.0 Abcde	82.57 Efgh	2.000 A	8.447 Cdefgh
20.	FRW2 x PSEV.3-120-2-3-2	74.90Mn	75000 A	257.5 Abcd	92.5 Abc	78.68 J	2.000 A	9.828 Abcde
21.	SWAJ -4-9-2 x PK9	70.50Fghijklm	66667 Abcde	236.2 Abcd	102.5 Abcde	84.08 Bcdefg	1.500 A	7.263 Efghi
22.	FRW2 x SWAJ 4-9-2	69.00Fghij	60417 Abcde	237.5 Abcd	112.5 Abcde	84.36 Bcdefg	1.250 A	7.629 Defghi
23.	PSEV.3-70-4 x PK9	73.00Jklmn	67708 Abcd	268.8 Cd	117.5 Abcde	85.22 Bcdef	1.500 A	10.866 Abc
24.	RMW8 x PSEV.3 -157-5-4-2	74.50Lmn	71875 Ab	273.8 Cd	132.5 De	83.98 Bcdefg	1.500 A	7.342 Efghi
25.	RMW8 x PW	69.00Fghij	72917 Ab	237.5 Abcd	105.0 Abcde	77.62 J	1.750 A	8.447 Cdefgh
26.	PSEV.3-157-5-4-2 x PW	76.00No	70833 Ab	282.5 D	127.5 Bcde	82.50 Fgh	1.750 A	7.964 Defghi
27.	SWAJ-4-9-1 x PW	72.00Hijklmn	69792 Ab	235.0 Abcd	102.5 Abcde	84.37 Bcdefg	1.500 A	6.806 Fghij
28.	PSEV.3-45-4-3-7 x(FRW3x FRW6)	69.50Fghijk	65625 Abcde	247.5 Abcd	96.2 Abcd	85.02 Bcdefg	1.000 A	6.746 Fghijk
29.	PSEV.3-157-5-4-3 x (FRW3x FRW6)	70.00Fghijkl	72917 Ab	255.0 Abcd	108.8 Abcde	85.77 Bcdef	1.500 A	7.821 Defghi
30.	PSEV.3-70-4 x (FRW3 x FRW6)	70.00Fghijkl	68750 Ab	257.5 Abcd	110.0 Abcde	85.33 Bcdef	1.250 A	9.998 Abcde
31.	PSEV.3-70-4 x (FRW3 x FRW6)	69.50Fghijk	58333 Bcde	253.8 Abcd	112.5 Abcde	86.13 Bcd	2.000 A	8.640 Cdefgh
32.	SWAJ-4-9-2 x (FRW3 x FRW6)	66.00Cdef	70833 Ab	243.8 Abcd	100.0 Abcde	84.06 Bcdefg	1.250 A	6.142 Hijkl
33.	PSEV.3-70-4 x (FRW3 x FRW6)	68.50Efghij	70833 Ab	271.2 Cd	130.0 Cde	87.14 B	1.000 A	11.693 A
34.	CH-I(2ES)	68.50Efghij	68750 Ab	268.8 Cd	137.5 E	84.41 Bcdefg	1.500 A	9.467 Abcdef
35.	CH-II(BABAR)	66.00Def	67708 Abc	231.2 Abcd	105.0 Abcde	84.18 Bcdefg	2.000 A	7.246 Efghi
36.	CH-III(30 k08)	67.50Efgh	66667 Abcde	270.0 Cd	115.0 Abcde	89.92 A	1.000 A	11.018 Abc
	L.S.D (5%)	**2.667**	**7606.9**	**28.31**	**20.17**		**0.9260**	**1.5936**

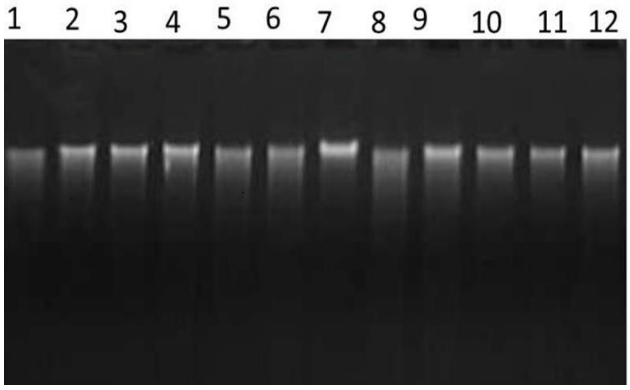

Fig. 1. Genomic DNA extracted from 12 maize hybrids and visualized at 1.5% gel electrophoresis.

DNA Extraction: The extraction of DNA from seeds of 12 maize hybrids was performed by using CTAB protocol. The quality of extracted DNA was run on a 1.5% agarose gel electrophoresis. The DNA samples run on the gel were observed under UV light on a computer screen having gel visualizing software Uvi Pro installed in it and connected to the Gel- documentation system. The image obtained after visualizing the DNA on electrophoresis, single clear bands observed in all cases, represented the good quality of DNA from each sample (Fig. 1).

Amplification of Hts Genes and Screening of maize hybrids: Analysis of amplified PCR product for Ht1, Ht2, Ht2 and HtN genes by bivariate data i.e. presence (R) or absence (S) in these 12 hybrid lines is indicated in Figure 2. All the hybrid showed Ht1 gene bands except hybrids 4, 5 and 11 (Fig. 2A). In the same way, the maximum bands of Ht2 gene were observed in all hybrids except 11 in which only one band appeared (Fig. 2B). Similarly, analysis of amplified PCR product for Ht3 gene indicated that hybrid 4 and 11 had only one band of this gene but all other hybrids had two or more than 2 band of this gene (Fig. 2C).The molecular identification of HtN gene indicated that hybrids 3, 7, 10 and 11 did not show any band of this gene but all others hybrids had HtN (Fig. 2D). The presence of any Ht gene in hybrids lines must provide some level of resistance to those lines against *Helminthosporium turcicum*. In this study, four SSR primers (Min *et al*., 2012) were used for screening inbred lines for Ht1 Ht2, Ht3 and HtN genes (R genes for NCLB resistance) respectively. Only the score able bands were included in the analysis and every single band was considered as single R gene for the genetic analysis (Table 3).

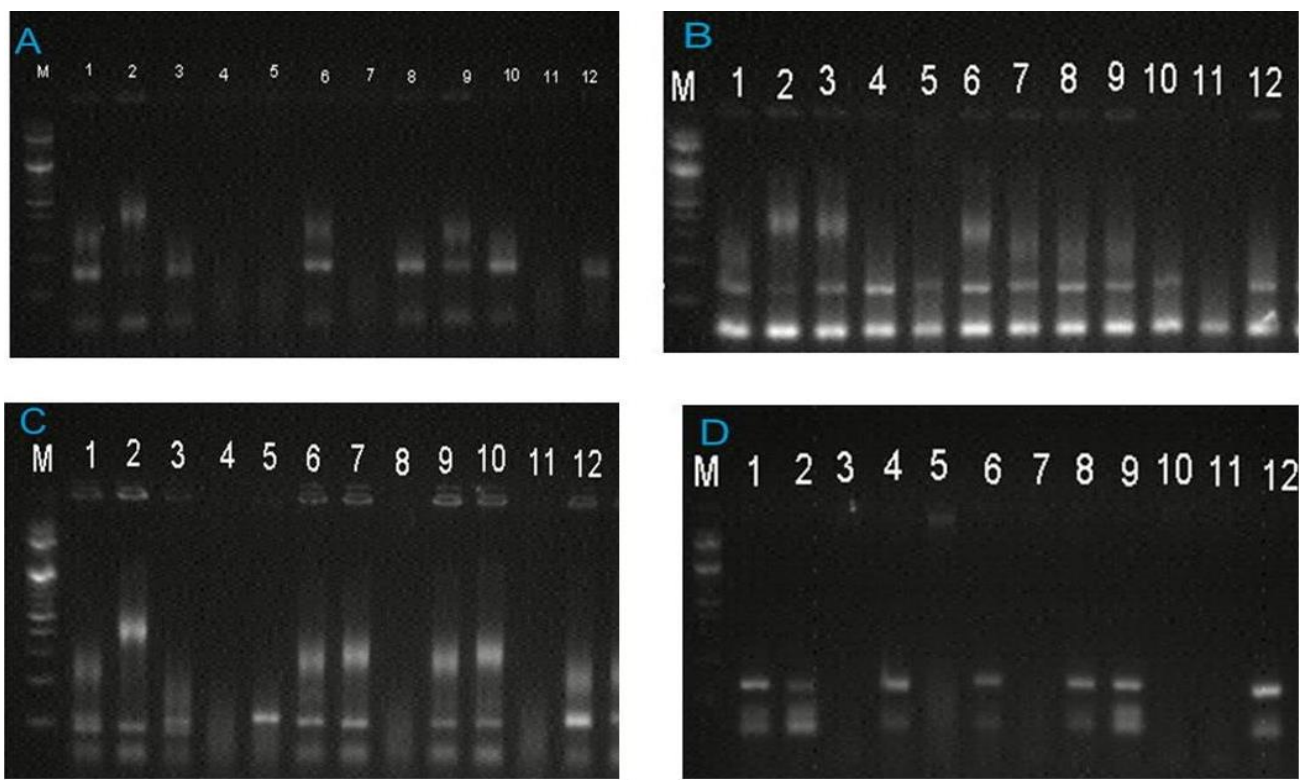

Fig. 2. Ht1 (A), Ht2 (B), Ht3 (C), HtN (D) amplified PCR product from 12 maize hybrids through RT-PCR followed by 1.5% gel electrophoresis.

Discussion

Comparison of 12 best maize hybrids selected for molecular study among the total of 36 maize hybrids is presented in table 4. Days to flowering and days to maturity in maize are among the most important traits for cultivar recommendation for a particular area, especially in high attitudes, primarily due to low temperature in later months of the summer season of the year (August, September, and October). Most of these experimental hybrids were much earlier than the exotic commercial hybrids for days to flowering.

Hybrid FRW2 x PSE.3-157-5-4-2 showed maximum yield 11.474 tons per hectare followed by check hybrid CH-III (30 k08) with 11.018 tons per hectare among all 12 hybrids and disease ranking showed that both above high yielding hybrids were resistant. In addition, both hybrids showed the presence of all resistant genes i.e. Ht1, Ht2, Ht3 and HtN that confirmed resistance. The Ht1 gene conveys a chlorotic-lesion resistant reaction in corn infected by avirulent races of *Exserohilum turcicum*, the causal agent of northern corn leaf blight (NCLB) (Pataky *et al*., 2006). The Ht1, Ht2 and Ht3 resistance gene occurs as chlorotic lesions with minimum sporulation, while the HtN induced resistance is expressed as a delay in disease development until after pollination. Turner & Johnson, (1980) reported the presence of race 1 in Indiana that was virulent on Ht1 but not Ht2. Lipps & Hite (1982) reported the presence of race 1 in Ohio that was found also virulent on Ht1 but avirulent on Ht2.

The second most important hybrid was FRW2×PSEV.3-45-4-3-8 with yield 10.408 tons per hectare. Considering the other parameters, it was resistant with 1.25 disease ranking and presence of Ht2, Ht3 and HtN genes but Ht1 gene was absent in this hybrid (Table 4). All other hybrids showed variation in yield and presence and absence of genes. Among the commercial hybrids, CH-II(BABAR) was moderately resistant to NCLB having disease ranking 2 and all the resistant genes Ht1, Ht2, Ht3 and HtN were absent in this commercial hybrid. Globally, 4 maize hybrids i.e. PSEV.3-15-5-4-2 ×PK9, FRW2× PSEV.3-120-2-3-2, PSEV.3-157-5-4-3x (FRW3x FRW6)sp out of 12 maize hybrids were moderately resistant while all others were resistant according to disease ranking but variation in resistant genes were observed in all maize hybrids.

Days to flowering were not unexpected because parentage of the experimental + commercial hybrids varied. The hybrid Rmw8xpw was found significantly late in flowering than all experimental hybrids. Seventeen of the 33 hybrids were significantly later than the commercial hybrid CH-III (30 k08). Similar differences were observed in other investigations. These results agree to those of (Hussain *et al*., 2010) and who observed maximum days (56) to 50 percent silking were taken by varieties Sahiwal-2002 and AZC-3 against the lowest (44) by EV-1097.

Germination of 36 hybrids including three check hybrids showed that all hybrids including commercial ones were not statistically different except pwx-131-6. It means that all hybrids performed equally well in term of germination compared with commercial hybrids.

Plant height of 36 hybrids were observed and four hybrids (FRW2 × PSE.3-157-5-4-2, PSEV.3-15-5-4-2 × PK9, RMW8 × PSEV.3 -157-5-4-2, PSEV.3-70-4 × (FRW3 ×FRW6)sp2), showed maximum plant height as compared to commercial hybrids CH-I(2ES), CH II(BABAR) and CH-III(30 k08), while rest of the hybrids plant height was less than commercial ones. Generally speaking, tall and leafy cultivars require low densities to maximize grain yield per area (Aldrich & Auster, 1986). It is also well known that increasing plant density increases leaf area index and consequently, water consumption (Tetio-Kagho & Gardner, 1988). The use of high plant populations under limited water supply may increase plant stress and reduce grain yield dramatically, especially if the water shortage coincides with the period of 2-3 weeks bracketing silking (Westgate, 1994). Therefore, biotic and abiotic stresses, particularly when combined with high plant density, can cause complete loss of grain production, if severe stress occurs during the tasseling and silking stage of reproduction (Herrero and Johnson, 1981; Edmeades *et al*., 1993).

Plant and ear height are very important characters not only for describing new hybrids of maize, but for green and dry matter production. The height of the main ear is a very important characteristic for breeding. Although lower ear height is unfavorable for yield and makes harvesting difficult, it does protect the stalk from excessive weight. Attempts have been made to breed in both directions, but practical experience shows that the ideal height should not be neither too high, nor too low. It is important for the ears to be at the same height within a population (Zsubori *et al.*, 2002). In our experiments, mean comparison of ear Height for 36 maize hybrids, two hybrids showed minimum ear height, FRW2 X SHS-131-6 HAD 85 CM AND PW X SHS.2 -131-6 HAD 86.2 cm. The hybrid CH-I(2ES) had maximum ear height of 137.5 cm and five hybrids out of 33 maize hybrids have more ear height as compared to commercial hybrid CH-III (30 k08).

Table 3. Ht1, Ht2, Ht3, HtNgenes presence (+) or absence (-) in 12 maize hybrids.

S. No.	Hybrids	Ht1	Ht2	Ht3	HtN
1.	FRW2 x PSE.3-157-5-4-2	+	+	+	+
2.	FRW2 x PSEV.3 -45-4-3-8	_	+	+	+
3.	PSEV.3-15-5-4-2 x PK9	+	+	+	_
4.	FRW2 x PSEV.3-45-4-3-8-2	_	+	_	+
5.	FRW2 x PSEV.3-120-2-3-2	_	+	+	_
6.	PSEV.3-70-4 x PK9	+	+	+	+
7.	PSEV.3-157-5-4-3x(FRW3x FRW6)	_	+	+	_
8.	PSEV.3-157-5-4-3 x (FRW3x FRW6)f	+	+	_	+
9.	PSEV.3-157-5-4-3 x (FRW3x FRW6)sp	+	+	+	+
10.	CH-I(2ES)	+	+	+	_
11.	CH-II(babar)	_	_	_	_
12.	CH-III(30 k08)	+	+	+	+

Table 4. Comparison of Grain yield, NCLB disease incidence and Ht1, Ht2, Ht3, HtN genes presence (+) or absence (-) in 12 maize hybrids.

Entries	Hybrids	Grain yield/ha	NCLB (1-5)	Ht1	Ht2	Ht3	HtN
1.	FRW2 x PSE.3-157-5-4-2	11.474	1.750	+	+	+	+
2.	FRW2 x PSEV.3 -45-4-3-8	10.408	1.250	_	+	+	+
3.	PSEV.3-15-5-4-2 x PK9	9.235	2.000	+	+	+	_
4.	FRW2 x PSEV.3-45-4-3-8-2	8.674	1.750	_	+	_	+
5.	FRW2 x PSEV.3-120-2-3-2	9.828	2.000	_	+	+	_
6.	PSEV.3-70-4 x PK9	10.866	1.500	+	+	+	+
7.	PSEV.3-157-5-4-3x(FRW3x FRW6)	7.821	1.500	_	+	+	_
8.	PSEV.3-157-5-4-3 x (FRW3x FRW6)f	9.998	1.250	+	+	_	+
9.	PSEV.3-157-5-4-3 x (FRW3x FRW6)sp	8.640	2.000	+	+	+	+
10.	CH-I(2ES)	9.467	1.500	+	+	+	_
11.	CH-II(babar)	7.246	2.000	_	_	_	_
12.	CH-III(30 k08)	11.018	1.000	+	+	+	+

Conclusions

Screening of maize 36 maize hybrids was done initially on the basis of morphological parameters and it was observed that Hybrid FRW2 × PSE.3-157-5-4-2 showed maximum yield 11.474 tons per hectare followed by check hybrid CH-III(30 k08) with 11.018 tons per hectare. In addition, it was observed that six hybrids among all the experimental hybrids, including one commercial hybrids CH-II (Babar) were moderately resistant for NCLB while all others were resistant to NCBL. Finally, three maize hybrids i.e. PSEV.3-15-5-4-2 × PK9, FRW2 × PSEV.3-120-2-3-2, PSEV.3-157-5-4-3 × (FRW3× FRW6) sp out of 12 maize hybrids were moderately resistant while all others were resistant according to disease ranking but variation in resistant genes were observed in all maize hybrids.

References

Ahmad, R., Y. Zuily-Fodil, C. Passaquet, O. Bethenod, R. Roche and A. Repellin. 2014. Identification and characterization of MOR-CP, a cysteine protease induced by ozone and developmental senescence in maize (*Zea mays* L.) leaves. *Chemosphere*, 108: 245-250.

Aldrich, H and E.R. Auster. 1986. Even dwarfs started small: Liabilities of age and size and their strategic implications. *Res. Organ Behave*., 8: 165-198.

Arif, K., R. Ahmad, S.A. Khan, S.A Asad, T. Ahmad, G.H. Abbasi and M. Shahzad. 2017. Molecular characterization of growth and proteolysis related genes in maize under drought stress. *Pak. J. Bot.*, 49: 2127-2132.

Edmeades, G.O., J. Bolaños, M. Hernandez and S. Bello. 1993. Causes for silk delay in a lowland tropical maize population. *Crop Sci*., 33(5): 1029-1035.

Galiano-Carneiro, A.L and T. Miedaner. 2017. Genetics of resistance and pathogenicity in the Maize/*Setosphaeria turcica* pathosystem and implications for breeding. *Front. Plant Sci.,* 8: 1490.

Geiger, H.H. and M. Heun. 1989. Genetics of quantitative resistance to fungal diseases. *Ann. Rev. Phytopathol.,* 27: 317-341.

Hafiz, A. 1986. Plant diseases. directorate of publication, Pakistan agricultural research council, Islamabad: 552 pp.

Herrero, M.P and R.R. Johnson. 1981. Drought Stress and its effects on maize reproductive systems 1. *Crop Sci.,* 21(1): 105-110.

Hussain, N., M.S. Baloch, Q. Zaman, A. Aziz and G. Sadozai. 2010. Adaptability and genetic variation in some maize hybrids. *J. Agri. Res.*, 48: 437-443.

Jamil, H.M.A., A. Ahmed, U. Irshad, A.A. Al-Ghamdi, M.S. Elshikh, I.A. Alaraidh, M.A. Al-Dosary, A.M. Abbasi and R. Ahmad. 2020. Identification and inoculation of fungal strains from Cedrus deodara rhizosphere involve in growth and alleviation of high nitrogen stress. *Saudi J. Biol. Sci*., 27(1): 524-534.

Jordan, E.G., J.M. Perkins, R.A. Schall and W.L. Pedersen. 1983. Occurrence of race 2 of E.turcicum on corn in the central and Eastern United States. *Plant Dis*., 67: 1163-1165.

Levy, Y. and Y. Cohen. 1983. Biotic and environmental factors affecting infection of sweet corn with *Exserohilum turcicum. Phytopathol*., 73: 722-725.

Li, B.D. and Z.T. Liu. 1984. The distribution pattern of *Pinusyun-nanensis*. *J. Yun. Univ.,* 1: 33-46.

Lipps, P.E. and R.E. Hite. 1982. *Exserohilum turcicum* virulent on corn with the Ht resistant gene in Ohio. *Plant Dis*., 66: 397-398.

Louie, J. 2017. Food groups. Essentials of human nutrition. 5th edition, 2016 - Oxford University Press, 273pp.

Masuka, B., G.N. Atlin, M. Olsen, C. Magorokosho, M. Labuschagne, J. Crossa and J. Macrobert. 2017. Gains in maize genetic improvement in Eastern and Southern Africa: I. CIMMYT hybrid breeding pipeline. *Crop Sci*., 57: 168-179.

Min, J.L., G. Nicholson, I. Halgrimsdottir, K. Almstrup, A. Petri, A. Barrett, M. Travers, N.W. Rayner, R. Мдgi, F.H. Pettersson, J. Broxholme, M.J. Neville, Q.F. Wills, J. Cheeseman, J. Fleckner, F. Karpe, C.M. Lindgren and K.T. Zondervan. 2012. Coexpression network analysis in abdominal and gluteal adipose tissue reveals regulatory genetic loci for metabolic syndrome and related phenotypes. *PLOS Genetics*, 8: e1002505.

Mullis, K.B. 1990. The unusual origin of the polymerase chain reaction. *Sci. Am.,* 262: 56-65.

Pataky, J.K. and T. Ledencan. 2006. Resistance conferred by the Ht1 gene in sweet corn infected by mixtures of virulent and avirulent Exserohilum turcicum. *Plant Dis.,* 90(6): 771-776.

Rahul, K. and I.S. Singh. 2002. Inheritance of resistance to banded leaf and sheath blight *Rhizoctoniasolam* f. sp. Sasakii) of maize. Proceeding 8th Asian Regional Maize Works Bangkok, Thailand: 5-8pp.

Raymundo, A.D. and A.L Hooker. 1981. Measuring the relationship between northern corn leaf blight and yield losses. *Plant Dis.*, 65: 325-327.

Razzaq, T., M.F. Khan, S.I. Awan, H. Tariq and M. Ilyas. 2019. Screening of maize genotypes under northern corn leaf blight (NCLB) epiphtotic at Rawlakot Azad Kashmir. *Pak. J. Bot.*, 51(5): 1865-75.

Renfro, B.L. and A.J. Ullstrup. 1976. A comparison of maize diseases intemperate and in tropical environments. *PANS*., 22(4): 491-498.

Riffat, A. and M.S. Ahmad. 2020. Alleviation of adverse effects of salt stress on growth of maize (*Zea mays* L.) by sulfur supplementation. *Pak. J. Bot*., 52(3): 763-73.

Sahu, S.K., Thangaraj, M. and K. Kathiresan. 2012. DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen and phenol. *ISRN. Mol. Biol.*, 14: 2012.

Smith, D.R. and J.G. Kinsey. 1993. Latent period- a possible selection tool for Exserohilumturcicum resistance in corn (*Zea-mays* L.). *Maydica*, 38: 205-208.

Shahzad, M., H. Usman, R. Ahmad, S.A. Khan, Z.A. Saqib and K.H., Muehling. 2019. Sodium in the leaf apoplast does not affect growth of maize (*Zea mays* L.) under saline field conditions. *J. Appl. Bot. Food Qual.*, 92: 117-122.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, 98: 503-517.

St. Clair, D.A. 2010. Quantitative disease resistance and quantitative resistance loci in breeding. *Ann. Rev. Phytopath*, 48, pp. 247-268.

Tefferi, A., B.J. Bartholmai, T.E. Witzig, C.Y. Li, C.A. Hanson and R.L. Phyliky. 1996. Heterogeneity and clinical relevance of the intensity of CD20 and immunoglobulin light-chain expression in B-cell chronic lymphocytic leukemia. *Am. J. Clin. Pathol.*, 106(4): 457-461.

Tetio-Kagho, F. and F.P. Gardner. 1988. Responses of maize to plant population density. II. Reproductive development, yield, and yield adjustments. *Agron, J.*, 80(6): 935-940.

Turner, M.T. and E.R. Johnson. 1980. Race of *Helminthosporium turcicum* not controlled by Ht genetic resistance in corn in the American Corn Belt. *Plant Dis.*, 64: 216-217.

Westgate, M.E. 1994. Water status and development of the maize endosperm and embryo during drought. *Crop Sci*., 34(1): 76-83.

Zhou, J., A. Nadeem., Y. A. Cheng, C.H. Qin, P. Chen, C. Zhang and L. Zhang. 2019. Effect of inoculation of strains with acc deaminase isolated from vermicompost on seed germination and some physiological attributes in maize (*Zea mays* L.) exposed to salt stress. *Pak. J. Bot.*, 51(4): 1169-1177.

Zsubori, Z., Z. Gyenes-Hegyi, O. Illйs, I. Pyk, F. Rбcz and C. Szıke. 2002. Inheritance of plant and ear height in maize (*Zea mays* L.). *Acta. Agraria. Debreceniensis*, 8: 34-38.

(Received for publication 26 January 2019)