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Research Papers

Modified Method of Screening Maize Inbred Lines to Late wilt Disease Caused by *Harpophora maydis*

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Abstract

Development of rapid screening protocol and reliable scale for phenotyping breeding/germplasm lines for responses to LWD is a pre-requisite for identifying stable sources of resistance. We propose a rapid, dependable, labour and resource efficient screening protocol and reliable scale for phenotyping germplasm / breeding lines for responses to LWD. Using the proposed screening protocol, 155 maize inbred lines were phenotyped for their responses to LWD during 2014 and 2015 late rainy seasons. A wide range of response scores (3 to 8.5) suggested good discriminating ability of the proposed method. Significant rank correlation (0.86) indicated the consistency of responses of inbred lines evaluated across two years and also indicated the absence of genotype × environment interaction (GEI). Seven inbred lines were found resistant to LWD in both the years of evaluation. Identified sources of resistance are suggested for use in development of LWD resistant inbred lines / hybrids and segregating populations to map the genomic regions conferring LWD resistance using DNA markers.

Late wilt disease (LWD) of maize caused by *Harpophora maydis* is known to be an important biotic production constraint in Asia (India), Africa (Egypt) and Europe (Hungary, Portugal, Spain) (Chalkley, 2016). It is considered as endemic in major maize growing areas (Degani and Cernica, 2014). The LWD was first reported in Egypt in 1963 (Samra *et al*., 1963). Subsequently, LWD was reported from different maize growing areas *viz*., Tanzania, Pakistan, Hungary and Kenya (Freeman and Ward, 2004), Egypt and India (Ward and Bateman, 1990), Portugal and Spain (Molinero-Ruiz *et al*., 2010), Romanaia (Bergstrom *et al*., 2008), and Israel (Drori *et al*., 2013) and is distributed widely in Iberian Peninsula (Ortiz-Bustos *et al*., 2015). Substantial economic losses caused by LWD have been reported from various parts of the maize growing areas of the world. In Egypt, it is reported that some fields experienced 100% infection and in India 70% incidence and economic losses upto 51 per cent (Johal *et al*., 2004).

LWD symptoms generally appear when the plants are about to tassel. However, their appearance may vary from just prior to tasseling until shortly before maturity (Samra *et al*., 1963). Leaves of the infected plants turn pale green and roll inward and appear as though suffering from lack of water (Sabet *et al*., 1970) and eventually become dry (Samra *et al*., 1963). Drying symptoms ascends upwards and cause stem discoloration of the vascular bundles to a yellow-brown hue (Sabet *et al*., 1966). Rotting symptoms are also reported from lower inter-nodes and roots (Sabet *et al*., 1970). Because of the delay in appearance of initial symptoms until flowering, this disease has been designated as late wilt (Samra *et al*., 1963). The fungus is both soil (Samra *et al*., 1963) and seed borne (Mohammed *et al*., 1966; EL-Shafey and Claflin, 1999). Pathogen survives parasitically on Lupine under field conditions (Botros *et al*., 1990).

Genetic resistance is the most economical and eco-friendly approach to mitigate production losses caused by LWD. Development of maize cultivars resistant to LWD requires (among others) identification of stable sources of resistance. Development of an economic and rapid screening protocol and reliable scale for phenotyping breeding/ germplasm lines for responses to LWD is a pre-requisite for identifying stable sources of resistance. The method of screening and the scale used for phenotyping of breeding / germplasm lines for responses to stalk rots caused by *Macrophomina phaseolina* and *Fusarium moniliforme* also being used for LWD caused by *H. maydis* (Shekhar and Kumar, 2012). However, the LWD symptoms caused by *H. maydis* are different from those caused by other pathogens. In this article we propose a method of

inoculation of pathogen causing LWD different from that proposed by Shekhar and Kumar (2012). We also propose phenotyping scale modified from that proposed by Payak and Sharma (1983).

Material and Methods

One hundred fifty five inbred lines representing heterotic groups which were developed in different breeding programs in India and across the globe by Monsanto were screened for responses to LWD. Ten seeds of the each inbred line were dibbled in a single row of 3 m length following randomized complete block design with two replications at Mega Breeding Station, Monsanto India Ltd. (MIL), India. The inbred lines were screened for their responses to LWD infection by artificial inoculation of known concentration of pathogen spores at 65 days after sowing (DAS) during 2014 and 2015 late rainy seasons.

Isolation and mass multiplication of the pathogen (*H. maydis*)

Maize stalks showing symptoms typical to LWD were collected from the field. Infected stalks were split into small fiber tissue and surface sterilized in 4 *per cent* sodium hydrochloride solution. The same were washed twice in sterile distilled water, dried and plated on 39 per cent Potato Dextrose Agar (PDA) medium. Petri plates were incubated in Biological Oxygen Demand (BOD) incubator for 5 days for the development of pathogen colonies. The pathogen colonies were examined for morphological and fruiting body characteristics typical of *H. maydis* (Fig. 1). The mycelia of *H. maydis* were placed on Potato Dextrose Agar (PDA) for pure culture. The mycelia were aseptically transferred to sterile 24 per cent Potato Dextrose Broth (PDB) in conical flasks for mass multiplication. These conical flasks were incubated for 15 days for development of mycelia mat. On the 15th day, the mycelial mat was grounded and filtered to obtain pathogen spore suspension (Fig. 2).

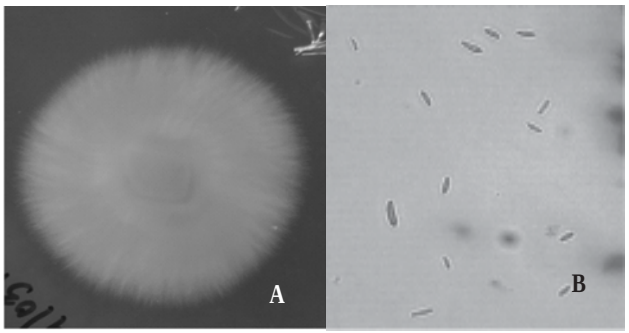

Fig. 1 : A) Characteristic rhizoidal growth of the mycelia of *H. maydis*
B) Pathogen suspension containing spores of *H. maydis* observed under microscope (10X)

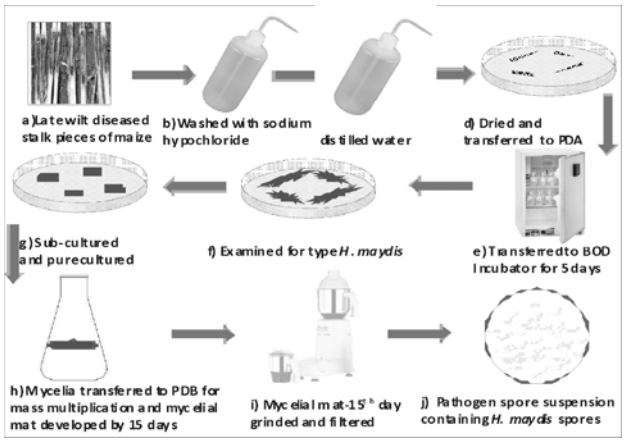

Fig. 2 : Schematic representation of isolation, mass multiplication and preparation of the suspension of the pathogen *H. maydis* for inoculation

Preparation of Inoculum

The spore suspension was observed under microscope and the concentration was adjusted to 4×10^6 spores per ml was adjusted using Haemocytometer. Whenever spore concentration was high, it was diluted with sterile distilled water to maintain desired concentration of spores. Spore suspension containing 4×10^6 spores per ml of pathogen constituted the inoculum.

Phenotyping responses of inbred lines to LWD

Two ml of the inoculum was injected to second inter-node from the base of 155 inbred lines' stalks using specialized injectors at 65 DAS. LWD symptoms were observed 20-25 days after inoculation. For disease phenotyping, inbred lines' stalks were split opened 30 days after inoculation. Disease severity and intensity was recorded on plot basis using 1-9 rating scale (Table I). The scoring pattern is developed based on spread of inter-node discoloration inside the maize stalks from the point of inoculation (Payak and Sharma, 1983). Higher the discoloration, higher is the rating. Unlike other stalk rot causing organisms, *H. maydis* apart from causing stem discoloration, also disintegrates

TABLE I

Scale used for scoring maize inbred lines for their responses to late wilt caused by H. maydis (Modified from Payak and Sharma, 1983)

Scale	Description of response of inbred lines to LWD
1	Indicates 25 per cent of inoculated inter node discolored
2	26 – 50 per cent of inoculated inter node discolored
3	51 – 75 per cent of inoculated inter node discolored or 26 – 50 per cent discoloration with high disintegration of the stalk
4	76 – 100 per cent of inoculated inter node discolored or 51 – 75 per cent discoloration with high disintegration of the stalk
5	Discoloration of less than 50 per cent of adjacent internodes or 76 – 100 per cent discoloration of inoculated inter node with high disintegration of the stalk
6	Discoloration of more than 50 per cent of adjacent internodes or less than 50 per cent discoloration with high disintegration of the stalk
7	Discoloration of more than two internodes or < 2 internodes but high disintegration of the stalk
8	Discoloration of more than three internodes or < 3 internodes but high disintegration of the stalk with complete fibrousness
9	Discoloration of more than 3 internodes and plants immaturely killed

the plant tissue and make it fibrous and finally leaves the vascular bundle in a most disorganized state (Payak *et al*., 1970) (Fig. 3). Unlike macrophoemina, which spreads to lower internodes and reaches to cobs (Khokhar *et al*., 2014), *H. maydis* fungus rarely moves to next internodes. Hence, there is every possibility that the genotypes will be wrongly classified. Disintegration and fibrousness of the plant tissue in addition to discoloration upon inoculation were considered for assigning the scores and classifying the inbred lines. Based on the responses to LWD and the proposed phenotyping scale (Table I), the 155 inbred lines were classified into different response groups (Table II).

TABLE II

Classification of inbred lines into different response groups based on their scores of responses to late wilt disease

Sl.No	Score	Response
1	1	Highly Resistant
2	>1 to 3	Resistant
3	>3 to 6	Tolerant / Moderately susceptible
4	>6 to 7	Susceptible
5	>7 to 9	Highly Susceptible

Fig. 3: Split open plant stalks after inoculation with *H. maydis* fungus : A) Disintegration and fibrousness of the inter node plant tissue but the discoloration not spread to the next inter node, B) Fungus discolored the inter node completely but the stem tissue is intact – no disintegration and fibrousness, C) complete discoloration, disintegration and fibrousness of plat tissue in susceptible varieties and D) response of a resistant inbred line.

Statistical analysis

The inbred lines were ranked based on their ssigned score in each year. Spearman rank correlation coefficient (r_s) was calculated for assessing consistency of inbred lines for the disease responses

to LWD over two years as r_s = 1- [{6 x ("d_i^2+CF)} / (N^3-N)], where, d_i = (Rank of a inbred line screened during 2014 - Rank of that inbred line screened during 2015) and N = Number of paired observations; CF=" {(t^3-t) / 12} with't' being the order of each tie.

The estimate of r_s was examined for its statistical significance using 't' test. Pooled analysis of variance was performed to partition the total variability of inbred lines for their responses to LWD into those attributable to genotype, year and genotype x year for interaction responses to LWD.

Method of inoculation

Method of inoculation followed in the present study elicited good response of the inbred lines to LWD as indicated by the wide range of scores from 3 to 8.5 which suggested good discriminating ability of the method used in the present study. While Payak *et al*. (1970) suggested the use of only tooth pick method, Degani and Cernica (2014) suggested inoculation using both tooth pick and injection method for screening germplasm/breeding lines for responses to LWD. The reported soil inoculation (Sabet *et al*., 1966; Sabet *et al*., 1970; Abd El-Rahim *et al*., 1998) and other modified methods (Singh and Sriadhana, 1986) are cumbersome, highly demanding in terms of resources, time and labour. Inoculation of pathogen suspension into stalks as is followed in present study is not only labor and resource efficient but also large number of plants could be screened in less time.

Results and Discussion

Response of inbred lines for LWD

The inbred lines differed significantly for their responses to LWD as indicated by the significant mean sum of squares attributable to inbred lines. Significance of mean sum of squares attributable to 'year' indicated possible differential influence of weather variables that prevailed during the crop growth period during 2014 and 2015 late rainy seasons on the responses of inbred lines to LWD. Non-significance of the mean squares attributable to 'inbred lines × year' interaction suggested the absence of genotype × environment interaction (GEI) (Table III). A fairly high and significant rank correlation (0.86) not only indicated consistency of the inbred lines for their response to LWD over two years but also confirmed the absence of GEI. These results suggested the effectiveness of selection of inbred lines for resistance to LWD.

The results suggested that none of the lines are highly resistant in both the years of evaluation. However, 9 and 13 inbred lines were resistant during 2014 and 2015, respectively. Fairly large a large number of inbred lines were tolerant to LWD in both 2014 and 2015. Pooled data indicated resistant response of only seven inbred lines and tolerance of 114 inbred lines (Table IV). The inbred lines that were found resistant to LWD in both the years are CV131061, CV183821, CV169435, CV239917, CV138811, CV270316 and CV266706 (Table V). The classification of inbred lines

Table III

Pooled Analysis of variance of maize inbred lines for responses to late wilt disease caused by H. maydis

Source of replication	Degrees of freedom	Sum of squares	Mean sum of squares	Calc. F	Table. F
Replications	1	26.014	26.01	38.74	
Inbred lines	154	946.01	6.14**	9.15	1.37
Years	1	7.68	7.68**	11.44	6.72
Inbred lines*Years	154	74.57	0.48	0.72	1.37
Error		309	207.48	0.67	
Total		619	1261.76		

**Significant at P = 0.01

TABLE IV

Number of inbred lines with different responses to late wilt disease caused by H. maydis

Score	Response	No. of inbred lines		
		2014	2015	Pooled
1	Highly Resistant	0	0	0
>1 to 3	Resistant	9	13	7
>3 to 6	Tolerant	108	118	114
>6 to 7	Susceptible	24	19	27
>7	Highly Susceptible	14	5	7

TABLE V

List of maize inbred lines found resistant to LWD dring2014 and 2015 late rainy seasons and across years

2014		2015		Pooled	
Identity of the inbred line	Mean disease score	Identity of the inbred line	Mean disease score	Identity of the inbred line	Mean disease score
CV131061	3	CV196707	3	CV131061	3
CV183821	3	CV331660	3	CV183821	3
CV169435	3	CV131061	3	CV169435	3
CV239917	3	CV204636	3	CV239917	3
CV216067	3	CV183821	3	CV138811	3
CV138811	3	CV169435	3	CV270316	3
CV282871	3	CV239917	3	CV266706	3
CV270316	3	CV212411	3		
CV266706	3	CV138811	3		
		CV198231	3		
		CV270316	3		
		CV266706	3		
		CV325943	3		

into five different response groups based on the proposed scale is amply justified by the significance of mean disease scores of the inbred lines classified under each response group as indicated by the 'F' test (Table VI).

The most practical method of controlling the disease in the field is the use of resistant varieties (Samra *et al*., 1963; El-Shafey *et al*., 1988). Identified sources of resistance to LWD are suggested for use in improving the parental lines of hybrids proven to be high yielders or can be used in deriving new resistant hybrids. The resistant sources can also be used for developing segregating populations to map the genomic regions conferring LWD resistance using DNA

TABLE VI

Estimates of means disease scores of inbred lines classified based on their responses to late wilt disease

Classification	(<3)	(>3 - 6)	(>6 - 7)	(>7)	F	P-value
Count	7	114	27	7	85.84	1.82E-32

markers. Once validated, linked markers could be used as surrogates of LWD resistance while breeding for resistant maize inbred lines / hybrids.

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