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Multi-environment field testing to identify stable sources of resistance to charcoal rot (*Macrophomina phaseolina*) disease in tropical maize germplasm

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Abstract

The charcoal rot caused by Macrophomina phaseolina is the devastating component of post flowering stalk rot (PFSR) complex which may cause 25 to 32 % yield loss in maize. Therefore for the first time, the study was carried out with multi-environments screening of 137 inbreds at three and 48 maize hybrids at six environments under artificially created epiphytotics at hot-spot locations to identify stable sources of charcoal rot resistance in Indian maize germplasm. Analysis of variance revealed strong effect of genotype by environment interaction on disease response and therefore indicated its complex nature. The mean disease score was ranging from 2.37 to 7.20 in inbreds, and 3.63 to 6.08 in hybrids. Additive main effects and multiplicative Interactions (AMMI) analysis could identifed, DQL1020, DML339, DML1, DQL1019, CM117-1-1 in inbreds and A-7501, CMH08-287, CMH08-292, BIO-562, and CMH08-350 in hybrids as stable sources of charcoal rot resistance. Each testing site viz., Ludhiana, Hyderabad and Delhi was identified as a separate test environment for screening against charcoal rot disease in India. In this study, AMMI model offers a good tool to assess the stability of genotypes and GGE biplot found an efficient tool to identify the mega environments in multi-environment testing. The identified sources of resistance in inbreds can be used in resistant breeding and hybrids can be recommended for cultivation in charcoal rot disease prone area.

Keywords: charcoal rot disease, maize, AMMI, GGE biplot, stable genotypes

Introduction

Maize is an important world's leading crop after wheat and rice. It stands as third most important crop in India after rice and wheat with area of 9.0 mha and production 24.4 m tonnes (Yadav et al, 2015). Maize is used as food, feed and fodder crop and is subjected to extensive yield loss due to several diseases. Estimated annual loss due to major diseases in maize in India is about 13.2 to 39.5% (Payak and Sharma, 1985). Among several diseases affecting maize, post flowering stalk rot (PFSR) is a major one causing significant damage to the standing crop (Renfro and Ullstrup, 1976). This is a complex disease and number of fungi like *Macrophomina phaseolina*, *Fusarium verticillioides*, and *Harpophora maydis* causing charcoal rot, fusarium stalk rot and late wilt, respectively, involved in development of PFSR complex symptom (Khokhar et al, 2014). The *Macrophomina phaseolina* causes charcoal rot disease in nearly 500 species of plants in tropical and subtropical countries. In India, it has been found prevalent in Jammu and Kashmir, Punjab, Haryana, Delhi, Rajasthan, Madhya Pradesh, Uttar Pradesh, Bihar, Andhra Pradesh, Tamil Nadu Karnataka, and West Bengal (Kaiser, 1982). Occurrence of charcoal rot disease in India can result to yield loss of 10-42% (Desai and Hegde, 1991; Kumar et al, 1998; Harlapur et al, 2002). Since the pathogen is both seed and soil borne, hence it is very difficult to control it chemically as it does not provide protection throughout the crop growth period. Therefore deployment of genetic resistance is considered to be the effective, safer and economical way to control such type of diseases (Kumar et al, 2014; Kumar et al, 2015). Causal organism of charcoal rot i.e., Macrophomina phaseolina have shown tremendous variation in morphology and pathogenicity (Kulkarni and Patil, 1966) due to which the host genotype shows differential resistance reaction under different environments.

It is well known fact that the phenotype of a crop is a joint contribution of both genotype (G) as well as environment (E). The genotype-environment interaction (GEI) reduces phenotypic and genotypic values association and may results in bias estimates of gene

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effects. Thus, existence of GEI for traits recommends the evaluation of genotypes in multi-environment trials (MET) to determine their true genetic potential as well as stability (Yaghotipoor and Farshadfar, 2007; Alwala et al, 2010). Genotypic stability for disease response describes how consistently a genotype performs against different pathogen variants across environments (Sharma et al, 2015). Several statistical methods are available to estimates the G, E, and GEI effects, however, their efficacy to detect GEI effectively, determined the use of selected one in MET analysis (Rakshit et al, 2012). The additive main effects and multiplicative interactions (AMMI) and genotype plus genotype × environment interaction (GGE) biplot models are powerful tools for effective analysis and interpretation of multi-environment data (Samonte et al, 2005; Yan et al, 2007).

The probable interaction component and availability of powerful tools to quantifying it has necessitated the search for host cultivars possessing stable resistance with wide adaptability over different environments. Not much information is available in literature pertaining to systematic screening and identification of stable sources for charcoal rot resistance in tropical as well as temperate maize germplasm. Therefore in this study, an effort has been made to identify inbred lines and hybrids showing stable resistance against locally prevailing isolates of charcoal rot pathogen under a range of tropical environments. Effectively quantifying of GEI will enhance the efficiency of selection while breeding and use of resistant cultivars to manage disease.

Materials and Methods

Plant material and field experiments

The study consisted of two experiments. In the first experiment, a set of 137 inbred lines developed under all India coordinated research project (AICRP) on Maize in India were evaluated under artificially created epiphytotics at hotspot locations *viz.*, Delhi [E1 (during summer 2013)] and Hyderabad [during summer 2013 (E2) and winter 2012-13(E3)]. In the second experiment, 48 hybrids were evaluated at hotspot locations under artificially created epiphytotics at Ludhiana and Hyderabad during summer 2010 (E1, E2), 2011(E3, E4), and 2012 (E5, E6). Both experiments were laid out in randomized block design with two replications. The plot size was 1 row of 4 m length with row to row and plant to plant spacing of 60 cm × 20 cm in inbred lines trial and 2 rows of 4 m length with 70 cm × 20 cm for hybrids trials.

Isolates and inoculation

The *Macrophomina phaseolina* was isolated from infected stalks. Small bits cut from infected stalks were surface sterilized in 0.1 percent mercuric chloride solution for one minute followed by washing in sterile distilled water. The sterilized bit was aseptically transferred to sterilized potato dextrose agar (PDA) and incubated for 10 days to get inoculum. Round bamboo toothpicks of 6.5 cm were sterilized by boiling three times (for period of one hour at a time) in hot water. After each boiling they were thoroughly washed and dried under sun. Dried toothpicks were loosely packed in bundles and put into the bottles with enough potato dextrose broth (one - third length of toothpicks) added to thoroughly moisten the toothpicks and then they were autoclaved. The sterilized toothpicks were aseptically inoculated with pathogen culture and were used for inoculation after 10-12 days of period of growth. Inoculations were done after flowering stage of plants. For inoculating plants, the lower internodes (second/third) above soil level were opened with a jabber and the toothpick carrying inoculum was inserted into the hole. The round toothpicks effectively sealed the hole in the stalk.

Disease scoring and data analysis

Disease scoring was done in inbred and hybrids trials based on the proportion of disease symptoms present in the inoculated internodes and its subsequent spread. Based on the percent discolouration of the inoculated and adjacent internodes, an average disease score was given on a 1-9 rating scale. Plants shown up to 75% discolouration of inoculated internodes along with healthy adjacent internodes, 75-100% of inoculated along with 0-50% of adjacent, 100% of adjacent internodes to up to 50% of 3rd internodes and when symptom entered to 4th internode to up to the premature death of the plant were classified as resistant (average disease score of ≤3), moderately resistant (3.1-5.0), moderately susceptible (5.1-7.0) and susceptible genotypes (≥ 7.0) respectively.

Disease scores thus recorded were used to identify stable sources of resistance through Additive Main Effects and Multiplicative Interactions (AMMI) analysis. AMMI based stability values (ASVi) were calculated (Purchase et al, 2000; Ezatollah et al, 2011) to objectively assess the stability of genotypes using following equation:

$$ASV = \sqrt{\left(\frac{IPCA1 \text{ sum of square}}{IPCA2 \text{ sum of square}} IPCA1 \text{ sum of square}\right)^2 + (IPCA1 \text{ score})^2}$$

where $\frac{IPCA1 \text{ sum of square}}{IPCA2 \text{ sum of square}}$ is the weight given to the IPCA1-value by dividing the IPCA1 sum of squares with the IPCA2 sum of squares. The larger the IPCA score, either negative or positive, the more specifically adapted a genotype is to certain environments. Smaller ASV scores indicate a more stable genotype across environments.

Box plot analysis was done to display the variation for disease scores for inbreds and hybrids under different environments. A mega environment analysis was done using G+GE biplot analysis to identify environments with similar disease expression.

Results

Variability for disease response

The average disease reaction expressed by the 48 hybrids and 137 inbreds was ranging between 3.87 to 6.08 and 2.3 to 7.2, respectively. The mean disease score of six environments averaged over 48 hybrids and that of three environments averaged over 137 inbreds was ranging from 4.21 to 5.25 and 3.99 to 5.7, respectively. The hybrids *viz*., CMH08-350, CMH08-292, S 6304, A7501, and IMH-666 showed highest degree of disease resistance with mean disease score of 3.63 to 3.93, while the inbred lines *viz*., DQL 1020, DQL 1019, DML 339, DML 1, and CM117-1-1 showed high degree of disease resistance with mean disease score of <3.0 (Table 1). Variable degree of disease reaction was expressed by the rest of the genotypes. A box plot (Figure 1) presenting the distribution pattern of average disease scores among 48 hybrids and 137 inbreds under nine environments depicts the degree of dispersion in the population. Significant shift in the relative box position as well as median value can be observed across the environments. Few outliers were also detected on boxplot, the origin of which was tracked back to susceptible/ resistant genotypes.

Table 1 - Mean disease score, IPCA1, IPCA2, and ASVi values of inbred lines and hybrids identified tolerant to charcoal rot disease across the environments.

Genotype Name	Mean disease score	IPCAg[1]	IPCAg[2]	ASVi
Maize Inbreds				
DQL1020	2.32	-0.1586	-0.2601	0.3
DQL1019	2.43	-0.1295	-0.2541	0.4
DML339	2.47	-0.1238	-0.4485	0.4
DML1	2.57	-0.2501	-0.2712	0.5
CM117-1-1	2.93	-0.0957	-0.3042	0.4
DML306	3.23	-0.1493	-0.2120	0.4
DML327	3.60	-0.1441	-0.2771	0.4
DQL1022	3.60	0.2174	0.1290	0.4
JM-8	3.67	0.1211	0.5346	0.6
DML 330	3.80	0.0358	0.2800	0.3
DML310-A	3.83	0.4840	0.2873	1.2
DML326	3.83	-0.2757	-0.8614	1.1
DML2	3.87	0.4101	-0.2758	1.0
DML112	3.90	0.1653	0.3943	0.5
DML179	4.00	0.2162	-0.0785	0.5
VIL 29	4.20	0.1554	0.4354	0.6
DML310-B	4.27	0.3311	0.0527	0.8
DML300	4.33	0.2687	0.0076	0.6
DQL1030	4.33	-0.1732	-0.2431	0.5
HKI-323	4.33	0.0686	0.4484	0.5
LM-16	4.37	0.0805	0.0506	0.2
BML- 7	4.37	0.3987	0.0327	0.9
Maize Hybrids				
CMH08-350	3.63	-0.3743	-0.1331	0.5
CMH08-292	3.75	-0.0002	0.4535	0.5
S6304	3.85	-0.4037	-0.4138	0.8
A 7501	3.87	-0.1842	0.3032	0.4
IMH-666	3.93	0.2356	0.8747	1.0
Bisco 2668	4.01	0.3822	0.0672	0.6
BIO-562	4.05	-0.2714	0.2057	0.5
REH 2009-12	4.06	0.3472	0.4111	0.7
M 9977	4.19	0.0280	-0.7352	0.7
BIO-688	4.21	0.0274	0.6143	0.6
KNMH401061	4.21	0.5679	-0.0339	1.0
CMH08-287	4.21	-0.1908	-0.2419	0.4
HKH-317	4.24	0.5850	-0.4523	1.1
P3396	4.26	0.2877	-0.2962	0.6
BIO 151	4.27	-0.4644	-0.3051	0.8
PMH 1	4.29	-0.7703	0.2688	1.3
CMH08-433	4.32	-1.0086	0.1186	1.7
KDMH 176	4.33	0.0693	-0.6548	0.7
JH 3459	4.36	0.2928	0.0207	0.5
X35A176	4.38	-0.6387	-0.0907	1.1
JH 31404	4.38	-0.4756	-0.5899	1.0

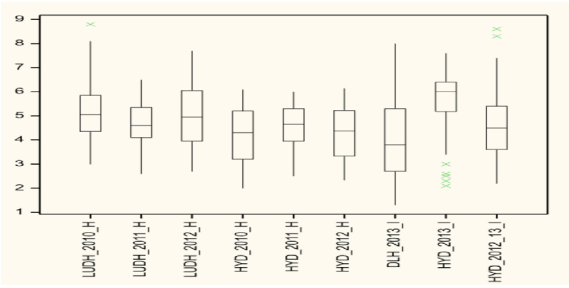

Figure 1 - Box plot showing range of variability for average disease score of 48 hybrids and 137 inbred lines screened at six [summer season 2010, 2011, and 2012 at Ludhiana (LUDH) and Hyderabad (HYD)] and three [summer season 2013 at Hyderabad and Delhi (DLH) and winter season 2012-13 at Hyderabad] environments.

Stability of genotypes: AMMI analysis

The AMMI biplot analysis was done using GenStat 17th Ed. (2014) to study the main effect, stability and interaction of genotype with environment. The analysis of variance for disease score in 48 hybrids and 137 inbreds tested over six and three environments respectively, showed significant ($p \leq 0.01$) effects of genotypes, environments and genotypes by environments interaction over the disease response (Table 2). A relatively higher proportion of total SS was attributed to GEI indicating significant interaction between genotype and environment for the expression of disease symptom. The first and second principle components (PC) together have contributed around 100% and 63.36% of the total variation for inbreds and hybrids, respectively (Figure 2). AMMI biplot plotted the main effect means on the abscissa and IPCA-1 scores of both host genotype as well as the environments simultaneously on the ordinates. It has represented the expected level of resistance and or susceptibility for any host genotype in an environment (Figure 2). Among different hybrids, A7501 (G1), CMH08-287 (G4), CMH08-292 (G16), BIO562 (G2), and CMH08-350 (G17) were identified as stable source of charcoal rot resistance across the environments, whereas Vivek Hybrid 9 (G47) and 31Y45 (G37) were consistently showing the susceptible reaction (Figure 2). The AMMI stability values (ASVi) calculated was in corroboration with the results shown in biplot. These five hybrids showed lowest ASVi values of 0.4 (A7501 and CMH08-287) to 0.5 (CMH08-292; BIO562 and CMH08-350) (Table 1), confirmed their stability for disease resistance across environments. Similarly in case of inbred lines, a good number of moderate to highly resistance lines with high degree of stability across environments were identified. Five inbreds *viz*., DQL1020 (G115), DQL1019 (G114), DML339 (G112), CM117-1-1(G136), and DML1 (G1) were identified as stable and highly resistant source against charcoal rot disease. Among the five lines, DQL 1020 has expressed the highest stability for disease resistance with ASVi value of 0.3 followed by DML339, DQL1019, CM117-1-1, and DML1 (Table 1). Eight other inbred lines have shown mean disease score of upto 3.9 but with less stability (ASVi value up to 1.2) across environments. Amongst all the genotypes, LM-16 was identified as most stable one (ASVi value of 0.2) across the environments with relatively lower degree of disease resistance. In case of inbred lines, the calculated ASVi values also corroborated with the result of AMMI biplot.

Genotype versus environment interaction

The AMMI biplot showed interaction of genotypes to specific environment (Figure 2). Hybrid PMH3 (G10) showed large positive and JKMH7004 showed negative interaction with environment E4. Large Positive interaction of IMH666 (G20), FH3525 (G46), NMH1242 (G25), and 31Y45 (G37) with environment E3, JKMH7004 (G22), Bio9681 (G8), and HKH317 (G39) with environment E5 and JH31404 (G21) and PFMH6N46 (G28) with environment E6 has shown their susceptibility in respective environments. Similarly, large negative interaction of genotypes PMH3 (G10) with E5, PFMH6N46 (G28), S6304 (G30), and JH31404 (G21) with E3 and E1, JKMH7004 (G22), Bio9681 (G8), and HKH317 (G39) with E4 and IMH666 (G20), FH3525 (G46), and X35A173 (G33) with E2 and E6 has indicated their resistance against charcoal rot disease in the environments. In case of inbred lines, majority of genotypes stayed near to origin showing lower magnitude of positive and negative interaction with different environment. Relatively larger positive interaction of DML20 (G6) and DML281 (G96) with E3 and that of DML264 (G92), DML5 (G3), DML227 (G88), and DML132 (G60) with E1 was observed in the biplot analysis. There was large negative interaction of genotype DML326 (G109) and DML167 (G78) with E2, genotype DML92 (G41) and DML38 (G10) with E1, and genotype DML61 (G25) and DML155 (G74) with E3. Large positive interaction with an environment is an indication of susceptibility in that particular environment whereas negative interaction represents the resistance.

A mega environment analysis was done with the help of software GenStat 17th Ed. (2014) to group the environments using the model considering only the effect of genotype and genotype-environment interaction (G + GE). GGE biplot analysis for hybrids (Figure 3) grouped six environments under study in three mega environments. Environment E4 (Hyderabad, summer 2011) was grouped with Environment E6

Table 2 - Analysis of variance (ANOVA) for charcoal rot disease response in inbreds and hybrids evaluated at multiple environments.

Source of variation	Inbreds		Hybrids	
	df	ms	df	ms
Genotype	136	4.3**	47	3.0**
Environment	2	214.5**	5	19.2**
Genotype × Environment	272	2.9**	235	1.7**
Error	410	1.6	287	0.4

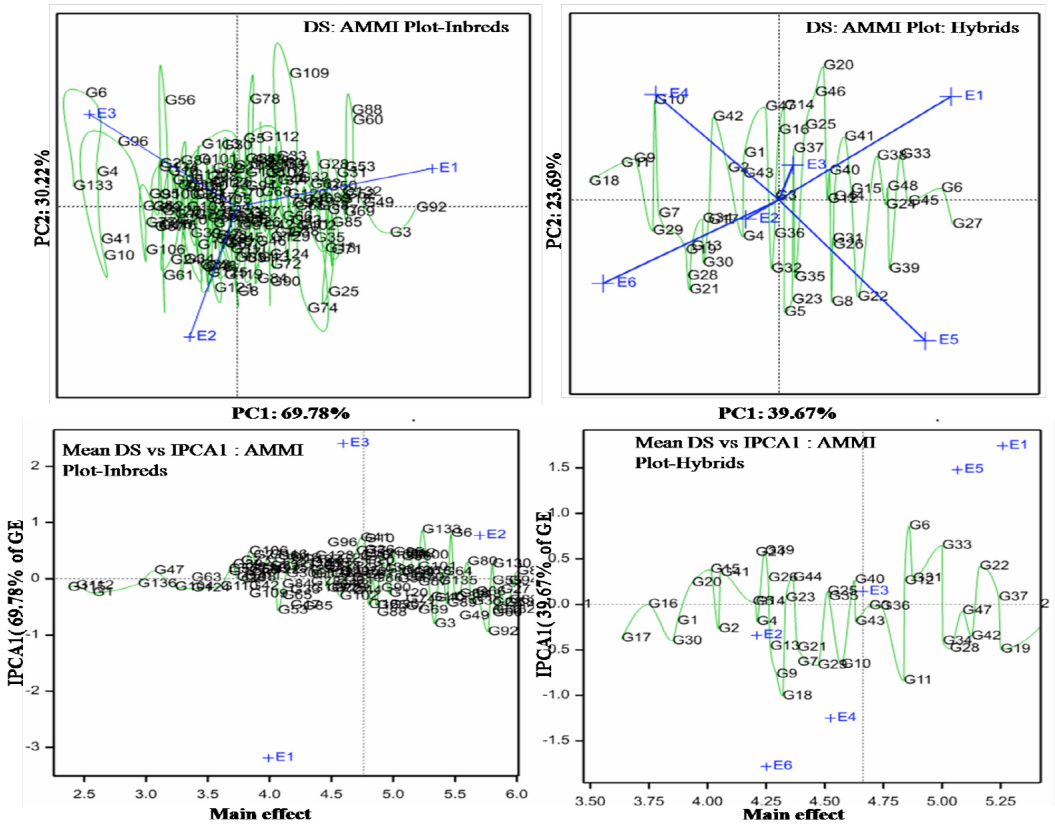

Figure 2 - AMMI biplot of principal component 1 (PC 1; along X-axis) versus PC2 (along Y-axis) and mean disease score (along X-axis) versus interaction principal component 1 (IPCA 1; along Y-axis) for 137 inbreds and 48 hybrids screened for charcoal rot disease under artificially inoculated conditions. The genotypes arranged towards the centre point and deviating less from the horizontal lines are more stable compared to the genotypes away from the centres and deviating more from the horizontal lines. In mean disease score versus IPCA1, the genotypes to the left of midpoint along the X-axis are classified as tolerant genotypes, and those to the right side are more susceptible.

(Hyderabad, summer 2012), E1 (Ludhiana, summer 2010) with E5 (Ludhiana, summer 2012), and E2 (Hyderabad, summer 2010) with E3 (Ludhiana, summer 2011). Environment E6 stood out as representative environment among E6 and E4 showing maximum differentiation among genotypes for expression of disease resistance reaction. Likewise, environment E1 showed more differentiation among genotype for disease reaction compare to E5. In case of inbred lines, all three environments stayed separate as different mega environment and no two environments could be grouped together. Environment E1 (Delhi, summer 2013) and E3 (Hyderabad, winter 2012-13) were better able to differentiate the inbred lines for their disease reaction.

Discussion

Maize cultivars resistant to charcoal rot disease under a particular environment do not necessarily exhibit a similar reaction under a different set of environments either due to the change in environment or due to change in the virulence of the pathogen. It is essential that a cultivar possess stable resistance to the disease should have wide adaptability to varied environments. Inbred lines showing stable resistance across different locations make a strong foundation for further development of breeding materials and hybrids with stable disease resistance reaction. In order to identify such stable resistance in the host cultivar, they need to be tested repeatedly under different environments. AMMI model offers a good tool to assess the stability of genotype tested across different environments (Gauch, 2006).

The significant effects due to genotype, environment and their interaction depict sufficient variability among hybrids, inbreds and test environments and therefore there is scope for selection of resistant genotypes with wider adaptability (Tonk et al, 2011). Further strong G × E interaction for disease reaction represents the complex nature of charcoal rot resistance in maize, therefore the methodologies recommended for polygenic traits improvement should be utilize while breeding. The AMMI biplots analysis has provided the graphical representation of stability of genotypes as well as their interaction with environments. Hybrids identified with stable resistance can be deployed for cultivation in charcoal rot disease prone areas. Inbred lines showing stable reaction along with

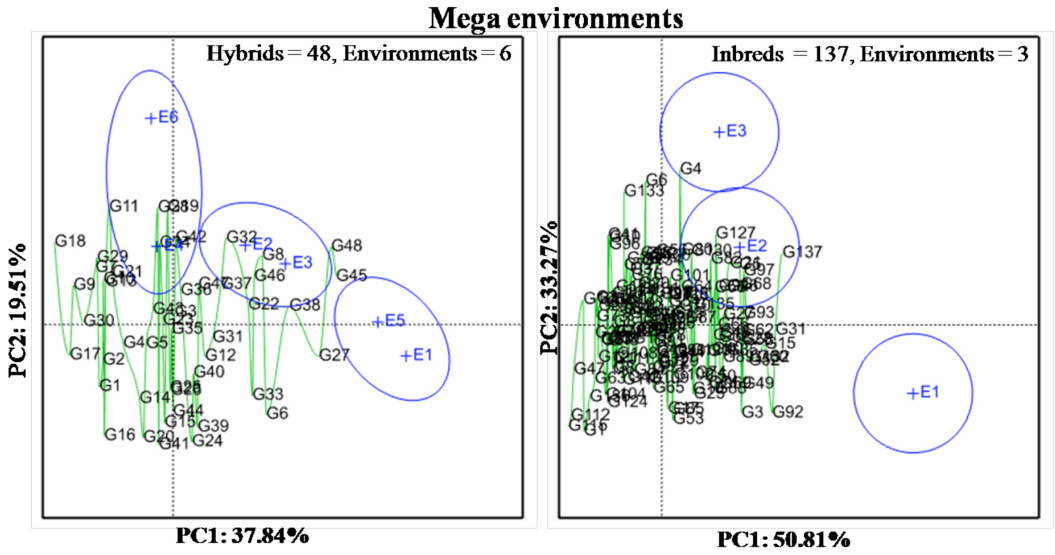

Figure 3 - Mega environments identified using G + GE biplot analysis based on average disease score of 137 inbreds and 48 hybrids screened for charcoal rot disease under artificially inoculated condition in nine environments (three for Inbreds and six for hybrids). The environments included under single circle are representing the similar type of disease response for genotypes. Each testing site *viz*., Ludhiana, Hyderabad and Delhi was identified as a separate test environment for screening against charcoal rot disease in India.

moderate to high degree of disease resistance may be used as an important source germplasm for further improvement to charcoal rot disease resistance in maize. Variation of response of genotypes in different seasons but on the same location might have occurred because of possible variation in pathotype or variable environmental conditions.

Grouping of environments into mega environments provides an idea about prevailing of similar types of growing conditions for expression of traits in various environments. This may be useful as the number of testing locations can be reduce while evaluation to draw a conclusion. This can further help to make breeding programme more efficient. Two of the identified mega environments in case of hybrids are *viz*., E1 and E5 in one and E4 and E6 in the other mega environment. The E1 and E5 represented Ludhiana locations during year 2010 and 2012; likewise E6 and E4 to Hyderabad during 2011 and 2012. Grouping of these environments displays similar performance of genotypes at same place during different years. This has further indicated that Ludhiana and Hyderabad locations are two separate environments for charcoal rot screening of tropical germplasm. Grouping of E2 and E3 (i.e., Hyderabad, summer 2010 and Ludhiana, summer 2011) might have occurred due to availability of similar climatic conditions for growth of pathotype or may be because of hidden variation as the PC1 and PC2 represent only 57.35% of total variation in the data. Similarly in case of inbred lines, all environments *viz*. Delhi, summer 2013 (E1), Hyderabad, summer 2013 (E2) and Hyderabad, winter 2012-13 (E3) represents as the different environments for disease screening. However, E2 and E3 are overlapping to some extent due to same location [Hyderabad 2012-13 (winter) and Hyderabad 2012 (summer)].The variation between E3 and E2 may be due to different growing seasons which impart different climatic conditions for disease development. Screening at Hyderabad during winter season (E3) displayed larger dispersion of disease reaction trait among genotypes, thus represents a better environment than summer season in Hyderabad (E2) for screening purpose. Environment E1 i.e., Delhi 2013 (summer) displays clearly different expression pattern of genotype for disease reaction. Thus, both Delhi and Hyderabad locations can be selected for multilocation trials to identify stable genetic material for charcoal rot resistance as they show significantly different pattern of expression of disease reaction. Continuous search for such stable resistance through repeated multilocation testing with varied levels of pathogenicity is crucial in identification of highly stable hybrids and inbred lines.

In this study, we observed strong G × E for charcoal rot disease reaction therefore methodologies available for polygenic traits improvement should be used while breeding. AMMI analysis could effectively identified the stable genotypes of charcoal rot resistance from multi-location testing which can deployed for cultivation in charcoal rot disease prone areas and for further diversification of promising maize germplasm. Further GGE biplot was found efficient to identify the mega environments which can help for efficient and effective testing of germplasm during multi-location testing. Each testing site *viz*., Ludhiana, Hyderabad and Delhi was identified as a

separate test environment for screening against charcoal rot disease in India.

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