Mycopath (2018) 16(1): 15-22 **Research Article**

Identification of charcoal rot infecting pathogen of sunflower from Pakistan and detection of resistance source

[*]Misbah Iqbal Qamar, Muhammad Usman Ghazanfar and Muhammad Imran Hamid

Department of Plant Pathology, College of Agriculture, University of Sargodha, Sargodha, Pakistan
[*]Corresponding author's email: misbahqamar230@gmail.com

Abstract

Macrophomina phaseolina (Tassi) Goid was isolated from sick field of sunflower (*Helianthus annuus* L.) grown from Sargodha, Pakistan. Round to oblong shape sclerotia were recorded in morphological characterization. The isolate MIQ was highly virulent in pathogenicity test. Nucleotide evidence of internal transcribed spacer (ITS) regions (ITS1 and ITS2) from MIQ was submitted under the GenBank accession no. MH277017. In evolutionary tree, nucleotide evidence was compared with the charcoal rot of sunflower, mash, potato, sesame, black gram and cotton reported from Pakistan, China and Korea. MIQ shared the clusters with sunflower as it exhibited 99% genetic homology with charcoal rot of sunflower. Seventy nine germplasms of sunflower (61 from NARC and 18 from CA Sargodha) were screened during two consecutive years 2016-17 and 2017-18, against MIQ to figure out the best resistance sources under the field conditions. During the first year, the screening results revealed 0, 2, 5, 9, 8, 35 and 20 germplasms/lines found to be immune, highly resistant, resistant, moderately resistant, moderately susceptible, susceptible and highly susceptible respectively. During the second year, this response was changed to 0, 2, 3, 5, 6, 41 and 22 germplasms/lines respectively. Out of 61 lines received from NARC, none of the lines showed immune, highly resistant while 17576 and 17586 were found resistance. 5, 4, 32 and 18 were moderately resistance, moderately susceptible, susceptible and highly susceptible respectively. Out of 18 lines, B8555 and B2728 found to be highly resistant, B-208, B-302 and Rafinjan Black Iran exhibited resistant response and B-224, HA-259, HA-65 and Pervenate responded as moderately resistant. During the second year, the change in resistance to the susceptible response of NARC (17576, 17586, 17551, 17565, 17555 and 17546) and COA (Pervenat) lines/ germplasms were recorded. Thus the germplasms of COA, Sargodha consisted of a greater number of resistant lines as compared to that of NARC, Islamabad and can be used in sunflower breeding program against destructive charcoal rot disease of sunflower.
Keyword: Charcoal rot, Nucleotide evidence, Resistance sources, Sunflower.

Introduction

Sunflower is native to North America. It is placed at 4th position in oilseed crops (Rodridguez *et al.,* 2002) and it was first time sown in Pakistan during 1960. Sunflower is well adopted in the cropping system of Pakistan and its commercial cultivation began in 1965. The cultivated area of sunflower is increasing in the country due to its adoptability to a wide range of climatic conditions, high oil contents and maturation period maximum 110 days (Khan *et al.,* 2003). Average yield of sunflower is nearly half (1241 kg ha^{-1}) in Pakistan as compared to world leading countries of the world (Shah *et al.,* 2005). Many biotic and abiotic factors are responsible for this yield reduction in Pakistan. The major diseases which attacks are phomopsis stem canker, sclerotinia wilt, downy mildew, charcoal rot, rust and verticillium wilt (Gulya *et al.,* 2016). Among all these diseases, charcoal rot caused by *M. phaseolina* is the serious threat to sunflower crop and causes 90% yield loss in the country (Khan, 2007). It was first time recorded in Pakistan in 1984 and spread across the regions of Punjab, Sindh and Khyber Paktunkhwa. This pathogen is prevailing in seed and soil and causes early death of plants and lower stem or root. Round about 500 species (Jalil *et al.,* 2013) in the world and 67 in Pakistan were reported as the host of *M. phaseolina*. In circumstances of water stress soils, it can also live for 10 months. The population of active sclerotia is directly related to the cruelty of the disease (Khan, 2007). Morphological, biochemical and serological identification of *M. phaseolina* is confusing due to their limited specificity and variation among isolates and nucleotide evidence of internal transcribed spacers (ITS) regions provide reliable identification of charcoal rot of sunflower (Saleh *et al.,* 2010; Amer and Budak, 2017). To explore the sources of resistance is the only viable solution to mitigate the effect of this disease as the chemical control is not effective to minimize the losses inflicted by this disease (Ijaz *et al.,* 2013; Shoaib *et al.,* 2018). Development of resistant varieties (Ambrósio *et al.,* 2015) is the cheapest source for the management of this disease, but there is a scarcity of resistance in the

commercial cultivars as most of the existing cultivars are susceptible to this the disease. Only moderate level of resistance can be found in cultivated sunflower germplasms (Ijaz *et al.,* 2013; Jalil *et al.,* 2013). Great variation in the physiology, morphology, pathogenicity and genotypes of *M. phaseolina* have been reported worldwide (Edraki and Banihashemi, 2010; Sánchez *et al.,* 2017). Different techniques like restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) are applied to the better understanding of genetic variation in *M. phaseolina* (Mayék-Pérez *et al.,* 2001; Purkayastha *et al.,* 2006). In spite, of all these techniques it is difficult to distinguish the isolates *M. phaseolina* from exact hosts or geographical due to the heterogeneous nature (Khan *et al.,* 2017) which includes the lack of a strong association between the geographical origin and genotype propose (Jana *et al.,* 2005). On the other hand, the knowledge of genetic and biological diversity reported in *M. phaseolina* isolates in others hosts, have a better understanding of the biology and population dynamics of this fungus, with the aim to obtain cultivars with a durable and stable resistance to this disease (Sánchez *et al.,* 2017). The objectives of the present study were to determine the nucleotide evidence of charcoal rot infecting sunflower from Pakistan and the screening of advanced lines/ varieties to find out the resistance sources against charcoal rot of sunflower.

Materials and Methods

Isolation of pathogen

The infected stem samples showing typical symptoms of the disease were collected from the sunflower field, Department of Plant Breading and Genetics, College of Agriculture (COA) Sargodha. The samples were washed with running tap water and dried on a double layer of sterile filter paper. The stem was cut into 3 cm small fragments and surface sterilized with 1% sodium hypochlorite for 1 min. The sterile fragments were dipped in sterilized distilled water for 1 min. and dried on a double layer of sterilized filter paper. The fragments were transferred to Potato Dextrose Agar (PDA) media and incubated at 28 ± 2 ºC for 5 days. Hyphal tip method was used for the purification and fungal colonies were identified as described by Dhingra and Sinclair (1978).

Pathogenicity test

Sunflower seeds of susceptible line UCS-5RR were obtained from COA and sown in pots as complete randomized design (CRD). A 9 mm disk from each isolate was shifted on PDA at 28 ± 2 °C for two weeks and fungal mycelium was harvested with 10 mL sterile distilled water. After 30 days of sowing, the suspension (100 g mycelium L^{-1} of water) was drained near the plant roots. The damaged roots were measured with the help of a scale (0 to 2) after 40 days of inoculation. Four plants were selected for a single isolate and experiment was repeated twice. Sterile distilled water was used for negative control (Khan *et al.,* 2017). The highly virulent isolate was used for the preparation of mass culture, screening of germplasms of sunflower, sequencing and sequence analysis.

Molecular confirmation

The genomic DNA of highly virulent isolate (MIQ) was extracted with phenol extraction methods and the internal transcribed spacer (ITS) regions were amplified with universal sense (5′ TCC GTA GGT GAA CCT GCG G 3′) and antisense (5′ TCC TCC GCT TTA TTG ATA TG 3′) primers (White *et al.,* 1990). The PCR reaction mixture (50 µL) was comprised of DNA of MIQ (3 µL), polymerase enzyme (2 µL), sense and antisense primers, (5 µL each), PCR reaction buffer (15 µL), dNTPS (10 µL), magnesium chloride (5 µL) and diethyl pyro-carbonate (DEPC) treated water (5 µL). The PCR reaction mixture was heated at 95 °C for 7 min and 30 cycles of denature (95 °C), annealing (55 °C) and extension (72 °C) for 30 s. was conducted. The final extension was performed at 72 °C for 5 min. and the results were visualized at 1% (w/v) agarose gel stained with ethidium bromide. The negative reaction (without DNA) was included in the mixture. The PCR product of MIQ was sequenced in sense and antisense direction from Microgen Korea and submitted in the public database of the National Center for Biotechnological Information (NCBI). Basic Local Alignment Standard Tool (BLAST) was used to compute the genetic homology of MIQ with previously reported isolates of *M. phaseolina*. Molecular Evolutionary for Genetic Analysis (MEGA) was used to compute the evolutionary history of MIQ by using the maximum likelihood method based on the Tamura-Nei model and (Tamura *et al.,* 2013).

Preparation of mass culture

Barnyard millet (*Echinochloa frumentacea*) grains (50 g) were soaked in 1000 mL sterile distilled water for 24 h and autoclave at 121 ºC for 30 min. The grains were allowed to cool down and 4 plugs of 3 cm mycelial disc from seven days old pure fungal culture of MIQ were transferred to sterile millet grains and incubated at 28 ºC for 15 days. The inoculum was spread at the time of seed bed preparation with the ratio of 3-4 g per meter (Shukla *et al.,* 2015).

Screening of available germplasms/lines

During the year 2016-17 and 2017-18, the inoculum of MIQ was applied in the field and 79

sunflower germplasms/lines, 61 from National Agricultural Research Centre (NARC) and 18 were from College of Agriculture (COA), University of Sargodha, Sargodha were sown on 27-03-2016 and 27-02-2017 in the field of Plant Pathology at COA, Sargodha (Table 1). From a single advance line, 20 sunflower seeds were sown with 2 ft row to row and 9 inch plant to plant distance. The earthling was performed at a height of one foot. All the agronomical practices were conducted as and when required. Inoculation of spore suspension (100 g mycelium L^{-1} of water) was applied at the time of sowing and after 60 days. Percentage disease incidence was computed with the help of the formula and the level of resistance or susceptibility of each line was determined (Table 2) as described by Aziz-ur-Reman (1992).

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

Results and Discussion

The fungus *M. phaseolina* has shown heterogeneity with regard to physiology, ecology, morphology and generic characteristics (Babu *et al.,* 2010; Ndiaye *et al.,* 2010) which favor its survival and adaptation to variable environments (Rayatpanah *et al.,* 2012). A total number of 17 fungal isolates were morphologically identified. Round to oblong shape along with irregular edges of sclerotia were recorded and range of sclerotia was 18-40 in 9 mm Petri plate. The morphological identification was similar to *M. phaseolina* (Tanaji *et al.,* 2017). The morphological identification of fungal pathogens is confusing and experts are required to determine the pathogen at the species level (Khan *et al.,* 2017). The new molecular approaches were further adopted for the reliable confirmation of fungal pathogen. Among all isolates, MIQ was found to be highly virulent in pathogenicity test. A 650 bp DNA fragment of MIQ was recorded on gel electrophoresis and a total number of 558 nucleotides were submitted in the public database of NCBI under the GenBank accession no. MH277017. The molecular weight of MIQ was 19667.04 and amino acid composition was Ala (A) 8.5%, Arg (R) 11.3%, Asn (N) 5.6%, Asp (D) 2.3%, Cys (C) 2.8%, Gln (Q) 4.0%, Glu (E) 1.7%, Gly (G) 7.9%, His (H) 2.3%, Ile (I) 4.5%, Leu (L)14.1%, Lys (K) 5.1%, Met (M) 0.6%, Phe (F) 4.0%, Pro (P) 7.3%, Ser (S) 6.2%, Thr (T) 4.5%, Trp (W) 0.6%, Tyr (Y) 2.3%, Val (V) 4.5%, Pyl (O) 0.0% and Sec (U) 0.0%. BLAST tool confirmed 99% genetic homology of MIQ with previously reported isolates (GenBank accession no: KT862031 and GenBank accession no: KT862032) of charcoal rot infecting sunflower from China. From Pakistan, multi-gene sequence analysis of different fungal pathogens was recommended, but nucleotide evidence of ITS regions provides the sufficient information to determine the charcoal rot of sunflower (Amer and Budak, 2017). The nucleotide and amino acid composition will play a significant role for the researchers and breeders to develop resistance sources against the charcoal rot of sunflower from Pakistan. In evolutionary tree, the nucleotide evidence of MIQ was compared with the charcoal rot of sunflower (GenBank accession No: KT862031 and KT862032), seasame (GenBank accession no: JX945162), potato (GenBank accession no: LT797496), mash (GenBank accession no: LT222228), black gram (GenBank accession no: LT799974) and cotton (GenBank accession no: KP174124, KP174125 and KC422671) reported from Pakistan, China and Korea. The evolutionary tree of *M. phaseolina* was computed into 5 different clusters of sunflower, potato, mash and seasame, black gram and cotton (Figure 1). The isolates which have maximum genetic homology was recorded in same clusters and MIQ was observed in sunflower clusters as it was exhibiting 99% genetic homology with *M. phaseolina* causing charcoal rot of sunflower from Pakistan. The present findings are partially in accordance with the earlier results that the *M. phaseolina* isolates of same host were genetically similar and differed distinctly from the isolates of other hosts (Purkayastha *et al.,* 2006; Su *et al.,* 2001). The existence of genetic diversity among the isolates from the same provinces might be due to the movement of *M. phaseolina* through infested seeds and soil (Aghakhani and Dubey, 2009). The results of Rayatpanah *et al.* (2012) are corroborating the present findings as they studied seventy isolates of *M. phaseolina* obtained from different hosts, including sunflower and soybean from the northern oilseed planting regions of Iran. These results revealed that a significant pathogenic and genetic variability within the Iranian isolates of *M. phaseolina*.

The artificial inoculum of MIQ was introduced on infected soil and silvery grey lesions with black, spherical micro-sclerotia on stem, premature death and reduction in head diameter were recorded on all screened genotypes of sunflower. These were the typical symptoms of *M. phaseolina* causing charcoal rot of sunflowers in Pakistan (Hussain *et al.,* 2016). All the germplasms were found infected and the maximum resistance response was recorded from COA while not a single germplasm was found to be immune against the disease in both years (Table 4). The variation in percentage disease incidence was recorded due to their different genetic makeup. During the first year of screening, only two lines from COA (B-8555 and B-2728) were exhibiting 1-10% (HR) infection and considered as highly resistant while no lines of NARC were recorded in this category. Three lines (B-208, B203 and Rafinjan Black Iran) from COA and two lines (17576 and 17586) were showing 11-30% (R) response. 31-40% (MR) infection was recorded in 3965, 17551, 17565, 17572, 17582, B-224, Prevent, HA-259 and HA-65. 41-50% (MS) infection was recorded in 17555,

17556, 17546, 3964, Bds-3, R-356, V-160 and COA-B12 these lines were moderately susceptible to disease. 32 lines from NARC (3961, 3962, 3968, 3969, 17545, 17547, 17548, 17549, 17550, 17552, 17553, 17554, 17561, 17566, 17568, 17569, 17573, 17574, 17575, 17577, 17578, 17579, 17581, 17584, 17585, 17587, 17588, 17589, 17592, 17594, 17595 and 17596) and 3 lines from COA (B-64, B-291 and B-385) were showing 51-70% infection (S). The sunflower lines which showed 71-100% (HS) highly susceptibility against disease were 3960, 3963, 3966, 3967, 17544, 7557, 17558, 17559, 17560, 17562, 17563, 17564, 17570, 17571, 17580, 17590, 19591, 17593, UCS-5RR and HA-314 and only last two germplasms were from COA. During the second year (2017-18) of screening, only one COA line Pervenat showed moderately susceptible response while it was moderately resistance in the first year. 17576 and 17586 were resistance in first year and recorded as susceptible during the second year. 17551 and 17565 were recorded as susceptible while these were moderately resistance in the first year. 17555 and 17546 were recorded as susceptible instead of moderately susceptible. The response of sunflower line/germplasms was changed from resistance to susceptible in the second year (Fig. 2).

The sources of resistance in local and exotic germplasms of sunflower against *M. phaseolina* is very scare in Pakistan (Ahmad, 1991) hence, the researchers need to put focus to find out the resistance germplasms against this notorious disease. Charcoal rot disease has a wide host range and different germplasms/liness of sunflower were found infected (Dawar and Ghaffar, 1998; Anis *et al.,* 2011). Although it is monotypic and no physiological races have been reported, it has high genetic variability resulting in a wide host range, which in turn means that crop rotation is not an effective strategy to combat the disease (Ijaz *et al.,* 2012). Our results are in accordance with Jalil *et al.* (2014), they reported significant variation in twenty four accessions of sunflower to charcoal rot with none of them was found immune (0%) or highly resistance (1-10%) contrary to these results, our study showed that two lines B-8555 and B-2728 were found highly resistance. Recently, (Hussain *et al.,* 2016) screened sixteen different sunflower genotypes against charcoal rot disease in Bahawalpur and found cultivars (14013, 14052, 14082 and 14095) resistant, (14009, 14021, 14035, 14041, 14048 and Check-1) moderately resistant, (14005, 14092 and check-2) moderately susceptible, (14001 and 14071) susceptible and (14068) highly susceptible responses further, no line was immune to charcoal rot disease was recorded. Six sunflower cultivars when grown in naturally infested soil, showed a susceptible response (Anis *et al.,* 2011) as compared to autoclaved soil. The results reported by (Hafeez and Ahmad, 2001) are in line with our findings as they found one genotype SF-187 was highly resistant among the seventeen different genotypes of sunflower screened for their resistance to charcoal rot under artificially inoculated field conditions. This resistance response of different germplasms/lines of sunflower is becoming susceptible to charcoal rot disease (Fig. 2) while B-8555 and B-2728 showed highly resistance response during both years of artificial screening (Table 2). If these lines possess desirable agronomic characters and these can be release directly as commercial cultivars. Development of resistant varieties is more reliable, cost effective, environment friendly and important method for proper management of *M. phaseolina* (Mahtab *et al.,* 2013). Development of resistant varieties is the cheapest source for management. The use of resistant cultivars is considered as one of the most important methods (Mahtab *et al.,* 2013).

Conclusion

Morpho-molecular characterization and pathogenicity test confirmed *M. phaseolina* as the causal agent of charcoal rot of sunflower. Charcol rot sunflower transferred from seeds and phylogenetic analysis confirmed that this pathogen might be transferred from China to Pakistan as progressive farmers are exporting the sunflower material without any quarantine measures. The nucleotide evidence of ITS regions from MIQ (GenBank accession no. MH277017) will provide reliable information to researchers working on this destructive pathogen. B-8555 and B-2778 were highly resistance against *M. phaseolina* and further recommended to farmers. The sowing of resistance source will increase the economic condition of the growers. It will increase the yield of sunflower in the country which will play a significant role in the GDP.

Table 1: List of cultivars/ lines from different sources for screening against *Macrophomina phaseolina* disease.

Source	Germplasms/lines
National Agricultural Research Centre (NARC)	3960, 3961, 3962, 3963, 3964, 3965, 3966, 3967, 3968, 3969, 17544, 17545, 17546, 17547, 17548, 17549, 17550, 17551, 17552, 17553, 17554, 17555, 17556, 17557, 17558, 17559, 17560, 17561, 17562, 17563, 17564, 17565, 17566, 17568, 17569, 17570, 17571, 17572, 17573, 17574, 17575, 17576, 17577, 17578, 17579, 17580, 17581, 17582, 17584, 17585, 17586, 17587, 17588, 17589, 17590, 17591, 17592, 17593,

	17594, 17595 and 17596
College of Agriculture University of Sargodha (COA)	B-64, B-208, B-224, B-291, B-385, B-302, B-8555, B-2728, Bds-3, R-356, V-160, UCS-5RR, UCA-B-12 Pervenat, Rafinjan Black Iran, HA-314, HA-259 and HA-65

Table 2: Disease rating scale of M. *phaseolina*.

Disease incidence	Level of resistance/ susceptibility
0	Immune (I)
1-10	Highly resistant (HR)
11-30	Resistant (R)
31-40	Moderately resistant (MR)
41-50	Moderately susceptible (MS)
51-70	Susceptible (S)
71-100	Highly susceptible (HS)

Table 3: Summary of response of sunflower varieties/lines to charcoal rot disease during 2016-17 and 2017-18 at College of Agriculture.

Disease rating	Response	Response of sunflower Germplasm/lines during	
		2016-2017	2017-2018
	Immune	Nil	Nil
1-10	Highly resistant	B-8555 and B-2728	B-8555 and B-2728
11-30	Resistant	17576, 17586, B-208, B-302 and Rafinjan Black Iran	B-208, B-302 and Rafinjan Black Iran
31-40	Moderately resistant	3965, 17551 , 17565, 17572, 17582, B-224, HA-259, HA-65 and Pervenat	3965, 17582, B-224, HA-259 and HA-65
41-50	Moderately susceptible	17555, 17556, 17546, 3964, Bds-3, R-356, V-160 and UCA-B-12	3964, Bds-3, R-356, V-160, UCA-B-12 and Pervenat
51-70	Susceptible	3961, 3962, 3968, 3969, 17545, 17547, 17548, 17549, 17550, 17552, 17553, 17554, 17561, 17566, 17568, 17569, 17573, 17574, 17575, 17577, 17578, 17579, 17581, 17584, 17585, 17587, 17588, 17589, 17592, 17594, 17595, 17596, B-64, B-291 and B-385	3961, 3962, 3968, 3969, 17545, 17547, 17548, 17549, 17550, 17551, 17552, 17553, 17554, 17555, 17561, 17565, 17566, 17568, 17569, 17572, 17573, 17574, 17575, 17576, 17577, 17578, 17579, 17581, 17584, 17585, 17586, 17587, 17588, 17589, 17592, 17594, 17595, 17596, B-64, B-291and B-385
71-100	Highly susceptible	3960, 3963, 3966, 3967, 17544, 17557, 17558, 17559, 17560, 17562, 17563, 17564, 17570, 17571, 17580, 17590, 17591, 17593, UCS-5RR and HA-314	3960, 3963, 3966, 3967, 17544, 17546, 17557, 17558, 17559, 17560, 17562, 17563, 17564, 17570, 17571, 17580, 17582, 17590, 17591, 17593, UCS-5RR and HA-314

Table 4: Category wise numbers of sunflower cultivars/ lines from different sources after artificial inoculation with. *M. phaseolina.*

No.	Level of resistance/ susceptible	NARC 2016	COA 2016	NARC 2017	COA 2017
1	Immune (0)	0	0	0	0
2	Highly resistant (1-10)	0	2	0	2
3	Resistant (11-30)	2	3	0	3
4	Moderately resistant (31-40)	5	4	2	3

5	Moderately susceptible (41-50)	4	4	1	5
6	Susceptible (51-70)	32	3	38	3
7	Highly susceptible (71-100)	18	2	20	2
	Total	61	18	61	18

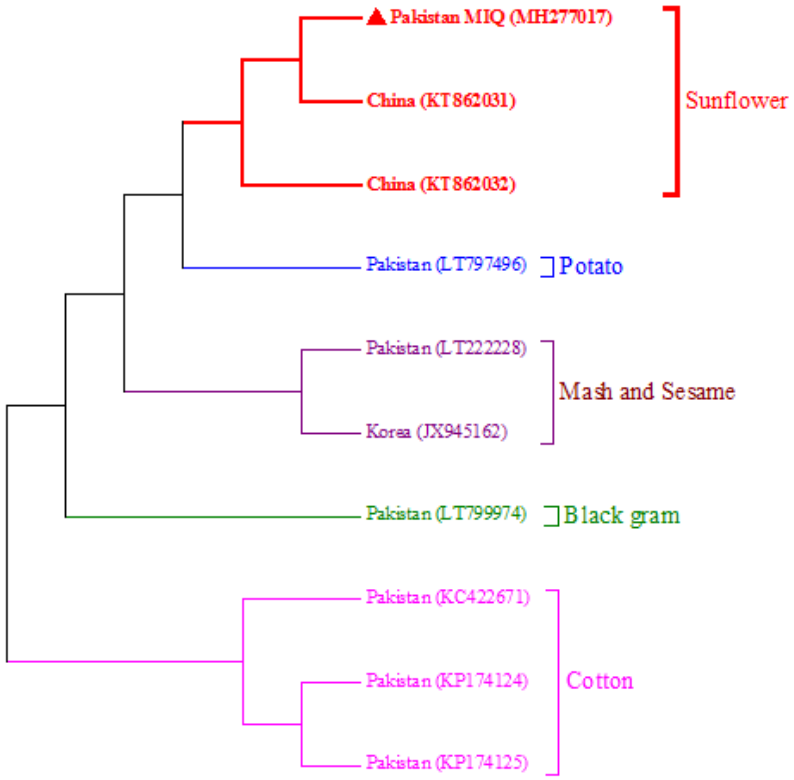

Fig.1: Phylogenetic analysis of charcoal rot of sunflower in Pakistan.

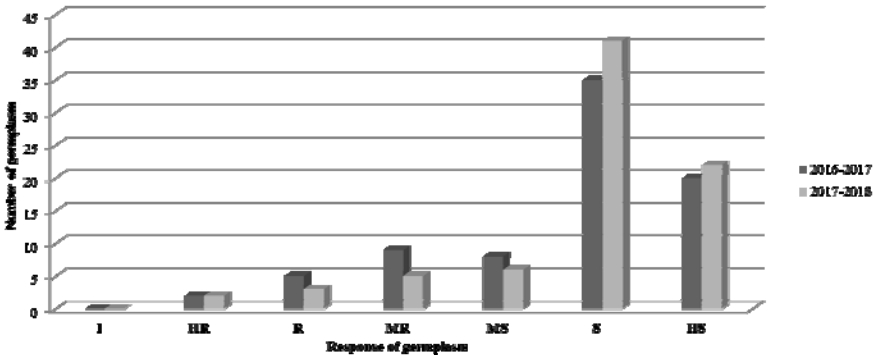

Fig. 2: Screening response of sunflower lines /germplasms against charcoal rot disease.

References

Abbès S, Ouanes Z, Salah-Abbes J, Houas Z, Oueslati R, Bacha H, Othman O, 2006. The protective effect of hydrated sodium calcium aluminosilicate against haematological, biochemical and pathological changes induced by zearalenone in mice. *Toxcion,* **47**: 567-574

Aghakhani M, Dubey S, 2009. Determination of genetic diversity among Indian isolates of *Rhizoctonia bataticola* causing dry root rot of chickpea. *Int. J. Geon.*, **96**: 607-619.

Ahmad I, 1991. National Symposium on the status of Plant Pathology in Pakistan. 3-5 December,

1991 Karachi, Pakistan. pp. 53.

Ambrósio MM, Dantas AC, Martínez-Perez E, Medeiros AC, Nunes GH, Picó MB, 2015. Screening a variable germplasms collection of *Cucumis melo* L. for seedling resistance to *Macrophomina phaseolina*. *Euphytica,* **206**: 287-300.

Anis, M, Zaki M J, Dawar S, 2011. Screening of sunflower (*Helianthus annuus* L.) varieties against charcoal rot fungus, *Macrophomina phaseolina* (tassi) goid. *Fuuast J. Biol.,* **1**: 43-46.

Aziz-ur-Rehman, 1992. Screening of sunflower germplasm against Charcoal rot disease and quantitative determination of the growth responses of sunflower cultivars against the disease. M.Sc. (Hons.) Thesis, Dept. of Plant Pathology, University of Agriculture, Faisalabad, Pakistan.

Babu BK, Reddy S, Yadav M, Mishra S, Saxena A, Arora K, 2010. Genetic diversity of *Macrophomina phaseolina* isolates from certain agro-climatic regions of India by using RAPD markers. *Indian J. Microbiol.,* **50**: 199-204.

Dawar, S, Ghaffar A, 1998. Effect of sclerotial inoculum, density of *Macrophomina phaseolina* on charcoal rot of sunflower. *Pak. J. Bot.,* **30**: 287-290.

Edraki V, Banihashemi Z, 2010. Phenotypic diversity among isolates of *Macrophomina phaseolina* and its relation to pathogenicity. *Iran. J. Plant Pathol.,* **46**: 93-100.

Gulya TJ, Mathew F, Harveson R, Markell S, Block C, 2016. Diseases of sunflower. In: McGovern R., Elmer W. (eds) Handbook of Florists' crops diseases. Handbook of Plant Disease Management. Springer.

Hafeez A, Ahmad S, 2001. Screening of sunflower germplasm for resistance to charcoal rot. Sar. J. Agri., **17**: 615-616.

Hussain MA, Aslam MN, Bakht A, Usman R, Sajjad M, Ahmad S, 2016. Host status of sunflower (*Helianthus annuus* L.) against Charcoal rot caused by *Macrophomina phaseolina* in arid region of Bahawalpur, Pakistan. *Int. J. Biosci.,* **9**: 303-310.

Ijaz S., H.A. Sadaqat and M.N. Khan, 2012. A review of the impact of charcoal rot (*Macrophomina phaseolina*) on sunflower. *J. Agric. Sci.,* **1**: 1- 6

Jalil S, Sadaqat HA, Tahir HN, Shafaullah A, Ali N, 2013. Response of sunflower under charcoal rot (*Macrophomina phaseolina*) stress conditions. *Int. J. Sci. Eng. Res.,* **1**: 89-92.

Jalil S, Sadaqat HA, Tahir HN, Shafaullah HA, Ali N, 2013. Response of sunflower under charcoal rot (*Macrophomina phaseolina*) stress conditions. *Int. J. Sci. Eng. Res.,* **1**: 2347-3878.

Jana T, Singh N, Koundal K, Sharma T, 2005. Genetic differentiation of charcoal rot pathogen, *Macrophomina phaseolina*, into specific groups using URP-PCR. *Can. J. Microbiol.,* **51**: 159-164.

Khan AN, Shair F, Malik K, Hayat Z, Khan MA, Hafeez FY, Hassan M, 2017. Molecular identification and genetic characterization of *Macrophomina phaseolina* strains causing pathogenicity on sunflower and chickpea. *Front. Microbiol.,* **8:** 1309-1313.

Khan SH, Shuaib M, 2007. Identification of sources of resistance in mung bean (*Vigna radiata* L.) against charcoal rot *Macrophomina phaseolina* (Tassi) Goid. *Afr. Crop Sci. Conf. Proc.,* **8**: 2101-2102.

Khan SN, 2007. *Macrophomina phaseolina* as causal agent for charcoal rot of sunflower. *Mycopath,* **5**: 111-118.

Khan SN, Ahmad I, Ayyub N, 2003. A reevaluation of geographical distribution of charcoal rot on sunflower crop in various agroecological zones of Pakistan. *Mycopath,* **1**: 63-66.

Mahmoud A, Budak H, 2011. First report of charcoal rot caused by *Macrophomina phaseolina* in sunflower in Turkey. *Plant Dis.,* **95**: 223.

Mahtab R, Alireza R, Abas A, Masoud N, 2013. Study on reaction of sunflower lines and hybrids to *Macrophomina phaseolina* (Tassi) Goid. causal agent of charcoal rot disease. *World Appl. Sci. J.,* **21**: 129-133.

Mayék-Pérez N, López-Castañeda C, González-Chavira M, Garcia-Espinosa R, Acosta-Gallegos, J., Vega O, 2001. Variability of Mexican isolates of *Macrophomina phaseolina* based on pathogenesis and AFLP genotype. *Physiol. Mol. Plant Pathol.,* **59**: 257-264.

Ndiaye M, Termorshuizen A, Bruggen C, 2010. Effects of compost amendment and the biocontrol agent *Clonostachys rosea* on the development of charcoal rot (*Macrophomina phaseolina*) on cowpea. *J. Plant Pathol.,* **92**: 173-180.

Purkayastha S, Kaur B, Dilbaghi N, Chaudhury A, 2006. Characterization of *Macrophomina phaseolina*, the charcoal rot pathogen of cluster bean, using conventional techniques and PCR-based molecular markers. *Plant Pathol.,* **55**: 106-116.

Purkayastha S, Kaur B, Dilbaghi N, Chaudhury A, 2006. Characterization of *Macrophomina phaseolina*, the charcoal rot pathogen of cluster bean, using conventional techniques and PCR-based molecular markers. *Plant Pathol.,* **55**: 106-116.

Raeder U, Broda P, 1985. Rapid preparation of DNA from filamentous fungi. *Lett. App. Microbiol.,* **1**: 17-20

Rayatpanah S, Nanagulyan G, Alav S, Razavi M, Ghanbari-Malidarreh A, 2012. Pathogenic and genetic diversity among iranian isolates of

Macrophomina phaseolina. *Chil. J. Agric. Res.,* **72**: 40-44.

Rodridguez JD, Romero-Garcia J, RodriguezGarcia R, Sanchez JLA, 2002. Characterization of proteins from sunflower leaves and seeds. Relationship of biomass and seed yield. In: Trends in New Crops and New Uses. Janich J, Whikey A (eds), ASHS Press, Alexandria. pp. 143-149.

Saleh A, Ahmed H, Todd T, Travers S, Zeller K, Leslie J, 2010. Relatedness of *Macrophomina phaseolina* isolates from tallgrass prairie, maize, soybean and sorghum. *Mol. Ecol.,* **19**: 79-91.

Sánchez S, Chamorro M, Henríquez J, Grez J, Díaz I, Santos B D, Gambardella M, 2017. Genetic and biological characterization of *Macrophomina phaseolina* (Tassi) Goid. causing crown and root rot of strawberry. *Chil. J. Agric. Res.,* **77**: 325-331.

Shah NA, Shah H, Akmal N, 2005. Sunflower area and production variability In Pakistan: opportunities and constraints. *Helia,* **28**: 165-178.

Shoaib A, Munir M, Javaid A, 2018. Anti-mycotic potential of *Trichoderma* spp. and leaf biomass of *Azadirachta indica* against the charcoal rot pathogen, *Macrophomina phaseolina* (Tassi) Goid in cowpea. *Egypt J. Biol. Pest Control,* **28**: 26-28.

Shukla N, Awasthi RP, Rawat L, Kumar J, 2015. Seed biopriming with drought tolerant isolates of *Trichoderma harzianum* promote growth and drought tolerance in *Triticum aestivum*. *Ann. Appl. Biol.,* **166**: 171-182.

Su G, Suh S, Schneider R, Russin J, 2001. Host specialization in the charcoal rot fungus, *Macrophomina phaseolina*. *Phytopathology,* **91**: 120-126.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.,* **30**: 2725-2729.

Tanaji G M, Ramesh M, Ramchandra K, Mallikarjun B, Bhale U, 2017. Cultural and morphological variability of *Macrophomina phaseolina* (tassi) goid causing charcoal rot of jowar in Solapur District of Maharastra, India. *Int. J. Curr. Adv. Res.,* **6**: 5334-5337.

Zazzerini A, Tosi L, 1989. Chlorate sensitivity of *Sclerotium bataticola* isolates from different hosts. *J. Phytopathol.,* **126**: 219-224.