Pak. J. Bot., 55(4): 1539-1544, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-4(31)

MOLECULAR IDENTIFICATION OF *COLLETOTRICHUM CAPSICI* ISOLATES AND DEVELOPMENT OF MATHEMATICAL MODEL FOR CHILLI ANTHRACNOSE DYNAMICS IN SEMI-ARID CLIMATE OF THE PUNJAB, PAKISTAN

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Abstract

Anthracnose is one of the major constraints to chilli production globally, especially in tropical and subtropical regions. Exact identification of *Colletotrichum* isolates from Pakistan and anthracnose dependence on weather parameters in semi-arid condition necessitated conducting work on molecular identification of the isolates and epidemiological aspects of the disease. DNA of five representative samples from five districts of the Punjab was extracted, purified and amplified by PCR. The purified PCR products were sequenced and submitted to NCBI GenBank database to obtained the accession numbers *viz.* MG799567, MG799563, MG799564, MG799565 and MG799566 were obtained for ITS region while MK088521, MK088522, MK088523, MK088524 and MK088525 for Beta tubulin gene. After confirmation of the isolates, sequences were uploaded on NCBI for BLAST to determine the similarity of the sequences of subjected isolates to previously reported sequences. Molecular characteristics of the pathogen resulted in confirmation of *Colletotrichum capsici* as causal agent of the chilli anthracnose in Punjab, Pakistan. Relationship of weather parameters with disease for two years in the form of mathematical model suggested that all the three factors (mean minimum and maximum temperatures and relative humidity) play significant role in disease dynamics. For most of the time period, weather conditions have been conducive for anthracnose development suggesting that researchers should pay more attention to develop and introduce anthracnose resistant chilli varieties for this zone.

Key words: Anthracnose, Epidemiology, Molecular characterization, Regression, Weather.

Introduction

Chilli (*Capsicum annuum* L.) is an important spice and vegetable crop in many countries including Pakistan which is cultivated over 65 thousand hectares across the country (Hussain *et al*., 2011). Continuing low yield of some of the crops like peanut in the potohar plateau as reported by Sher *et al.*, (2019) is constraining the farmers to switch over to some other cash crop like chilli resulting in increased area under the crop. In Pakistan, chilli is affected by number of pathogenic diseases like anthracnose, Fusarium wilt, Chilli leaf curl virus, *Pythium* and *Phytophothra* root rots etc. (Lin *et al*., 2004, Than *et al*., 2008). However, the major production constraint in sub-tropical to tropical areas is chilli anthracnose caused by *Colletotrichum* spp. Colletotrichum is the most notorious pathogen of chilli crop in Asia, the disease causes losses to chilli crop up to 80% (Poonpolgul & Kumphai, 2007, Montri *et al*., 2010, Sharma *et al*., 2005). Taxonomic identification, classification and characterization are highly important for pathologists to develop the disease management strategies and resistant varieties (Freeman *et al*., 1998). Mostly characterization of fungal pathogens are based on morphological characteristics like conidia, setae, appressoria, colony colour texture and growth rate. However, these parameters alone many times are not enough to classify the pathogen due to number of variations in genetics which may alter phenotypic characteristics of the pathogen. To beat over these complex morphological problems associated with many of the fungal pathogens, DNA based molecular characterization of pathogens had helped to solve classification complexities in many fungal genera like Fusarium and Pestalotiopsis (O'Donnell *et al*., 1998) as well as Colletotrichum (Jeewon *et al*., 2004; Photita *et al*., 2005; Sreenivasaprasad & Tahinhas, 2005). The ambiguous taxonomic status of *Colletotrichum* species has resulted in inaccurate identification which may cause practical problems in plant breeding and disease management.

Hot and humid environmental conditions support the sporulation and multiplication of the pathogen. Important abiotic factors affecting the development of disease are intensity of rainfall duration, relative humidity, leaf surface wetness, varieties and light (Riaz *et al*., 2017). Amongst them direct relationship between leaf surface wetness and development of disease due to this pathogen establish better in respect of germination of spore, attachment and penetration of pathogen into host tissues (Than *et al*., 2008). Before the application of any control strategy against the disease, epidemiology of the disease is of paramount importance. Therefore, this study was carried out to identify representative *C. capsici* isolates from five districts of Punjab Pakistan on molecular basis. Furthermore, epidemiological studies in open field in semi-arid climate were also undertaken to know the relationship of weather parameters with disease in the form of mathematical model.

Materials and Methods

Isolation and purification of pathogen: Total forty isolates were derived from 40 samples taken from forty

fields located in five districts of the Punjab including Bahawal Nagar, Kasur, Okara, Pakpattan and Rawalpindi. Isolation was carried out by using the standard protocol described by Photita *et al*., 2005. Around 5 mm piece of each sample was taken and sterilized by using 1% NaOCl. After sterilization, samples were placed on PDA for subject pathogenic growth at 28ºC. After purification, isolates were preserved at 4ºC on PDA media plates and deposited in Culture storage facility at Fungal Plant Pathology laboratory.

DNA extraction: DNA was extracted and purified by using PrepMan DNA extraction kit (Thermo Fisher) and GenJet purification kit respectively. Each source culture was derived from single conidium on PDA media. Mycelium of each isolate was used for DNA extraction. Presence of extracted DNA was confirmed by gel electrophoresis.

PCR and sequencing: The extracted DNA was amplified by PCR. Two genes of each isolates were amplified by using ITS1 and ITS4 of ITS/5.8S rRNA gene and Bt-2A and Bt-2B of partial Tubulin gene (White *et al*., 1990; Glass & Donaldson, 1995) respectively.

PCR was performed with the programmable thermo cycler (Bio-Rad T100) as per following protocols: Initial denaturation of DNA was done at 95ºC for three minutes followed by 30 cycles of denaturing at 95°C for one min. The annealing step was carried out at 72ºC for one min and final extension at 72ºC for ten minutes. The PCR amplicons were analyzed using 1% agarose gel electrophoresis where ethidium bromide was used as staining agent at 90 V for 35-40 minutes. All positive DNA were purified with GeneJet purification kit (KT-00701) following standard manufacturer protocols. Furthermore, nucleotide sequencing was done from Macrogen Korea Inc. both in forward and reverse direction. The obtained sequences were aligned and processed with BioEdit software and submitted to NCBI GenBank database (obtained accession numbers were listed in Table1).

Phylogenetic analysis: Datasets of IT'S and BT gene regions were used for phylogenetic analysis of the isolates from the major chilli growing areas of the Punjab, Pakistan. *C. acutatum* was used as out group for distinguishing the targeted isolates.

Epidemiological studies of the disease: This study was conducted under field conditions at university research farm, Koont for two years (2018 and 2019) by considering the key epidemiological factors (mean weekly minimum temperature, maximum temperature and relative humidity) affecting the disease development. Disease inoculum was sprayed at flowering and fruiting stage with concentration of 1×10^5. Before that**,** moderately susceptible cultivar namely Apollo's sowing was done in pots and trays in green house of the department in month of February. Seeds were collected from Horticultural Research Institute (HRI), National Agriculture Research Centre (NARC), and Islamabad. Thirty days old seedlings were transplanted at the farm on 28th March, 2018 and 31st March, 2019 respectively. Row-row distance was kept 1.5 feet and plant-plant distance was 1 foot.

Disease data in the field trail was noted after the interval of seven days for ten weeks in both years. Meteorological data was obtained from Pakistan Agro meteorological station near the farm.

Disease rating scale and mathematical model development: Severity of disease was recorded according to 0-9 rating scale of (Montri *et al*., 2010). Variables were taken and regression analysis was executed to evaluate the effect of different factors on disease development (ROCD=rate of change in disease) and generation of regression models by using Statistix 8.1 software.

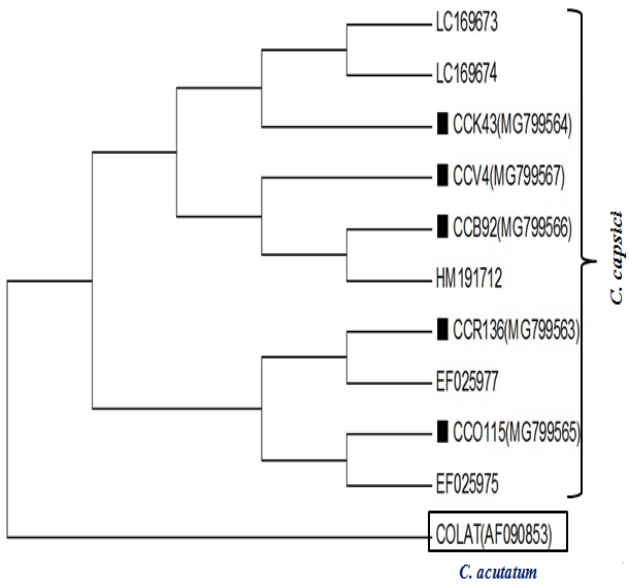
Results and Discussion

Forty isolates of *Colletotrichum* were collected and processed from symptomatic fruit samples collected in survey of major chilli growing areas of the Punjab *i.e.*, Vehari, Okara, Kasur, Bahawal Nagar and Rawalpindi of which one sample from each of the districts mentioned was chosen for the molecular identification.

Molecular studies: A total of 40 isolates of *C. capsici* were morphologically identified and characterized on the basis of defined morphological keys (data not shown here) from which the five representative isolates CCV4, CCB92, CCR136, CCK43 and CCO115 one from each district were used for molecular studies for confirmation of the isolated pathogen. The Internal Transcription Spacer (ITS) region and beta tubulin region of the genes of these isolates were amplified, through PCR and confirmed by running samples on 1% agrose gel. Purified PCR products were sequenced and sequences were submitted to GenBank and the accession numbers MG799567, MG799563, MG799564, MG799565 and MG799566 were obtained for ITS gene while MK088521, MK088522, MK088523, MK088524 and MK088525 for Beta tubulin gene (Table 1).

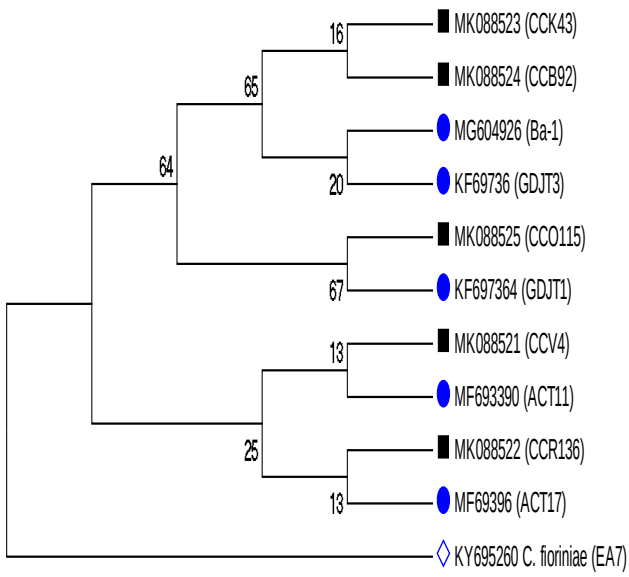
Table 1. Accession numbers obtained and primer used for molecular characterization of *Colletotrichum capsici* isolates.

Sr. No.	Accession No. ITS	Accession No. Bt	Isolate	Location	Host
1.	MG799567	MK088521	CCV4	Vehari, Punjab	*Capsicum annuum*
2.	MG799563	MK088522	CCR136	Rawalpindi, Punjab	*Capsicum annuum*
3.	MG799564	MK088523	CCK43	Kasur, Punjab	*Capsicum annuum*
4.	MG799565	MK088524	CCO115	Okara, Punjab	*Capsicum annuum*
5.	MG799566	MK088525	CCB92	Bhawalnagar,Punjab	*Capsicum annuum*



■ Shows Local Accession numbers while non labeled shows reference accession numbers

Fig. 1. Molecular phylogenetic tree constructed by using Maximum Likelihood analysis from ITS gene sequences of 5 highly aggressive *Colletotrichum capsici* isolates along with accession numbers with the available sequences on Genbank accessions. *C. acutatum* was used to root the tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replications were collapsed. Bootstrap values were set at 1000 replications. The analysis consisted of 25 nucleotide sequences in tree. Evolutionary analyses were conducted in MEGA 7.0.



Black squares represent current study/ local Accession number (isolate ID)

Blue circles represent Accession number (isolate ID) used for comparison from already reported accessions from Genbank database. *C. fioriniae* (EA7) represent Outgroup.

Fig. 2. Molecular phylogenetic tree constructed by using Maximum Likelihood analysis from Bt gene sequences of 5 highly aggressive *Colletotrichum capsici* isolates along with accession numbers with the available sequences on Genbank accessions. *C. fioriniae* was used to root the tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replications were collapsed. Bootstrap values were set at 1000 replications. The analysis consisted of 25 nucleotide sequences in tree. Evolutionary analyses were conducted in MEGA 7.0.

After confirmation of the isolates sequences were uploaded on NCBI for BLAST to determine the similarity of the sequences of subjected isolates to previously sequenced and confirmed isolates around the globe. All the five isolates showed 100% similarity to the selected comparison isolates (GenBank accession No.s HM191712, EF025977, LC169673 and EF025975 LC169674) homology to previously reported *C. capsici* isolates in term of ITS gene while approximately same trend was also followed for Beta tubulin gene (Figs. 1&2). In case of ITS gene, phylogenetic analysis of these five locally obtained sequenced isolates and five reference isolates of *C. capsici* resulted in a tree with two distinct clusters. Isolates CCV4, CCB92 and CCK43 and three reference isolates of *C. capsici* (LC169673, HM191712 and LC169674) showed 99% similarity and fell in one cluster due to genetic similarity within the isolates while the other CCR136 and CCO115 showed 100% similarity with isolates having accession numbers EF025977 and EF025975 and fell into the second *C. capsici* cluster due to sharing their genetic homology among them. *C. acutatum* was used as out group for distinguishing the targeted isolates.

In case of Bt gene sequence analysis and tree construction, same trend was observed and found 100% similarity with already isolated and identified *C. capsici* isolates (Fig. 2). Briefly, the evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura *et al*., 1993). The tree with the highest log likelihood (-1431.4432) is shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 659 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura *et al*., 2013).

A joint study on morphological (data not shown in the paper) and molecular characteristics of the pathogen resulted in confirmation of *C. capsici* as causal agent of the chilli anthracnose disease in Punjab, Pakistan. Sequence analysis using two genes ITS and BT was found fruitful and described the phylogenetic relationship of *C. capsici* isolates from Punjab, to other isolates of the same pathogen around the world and found mostly related to each other on the basis of the similarity in genetic makeup (Figs. 1&2).The sequences of selected isolates with sequences of BRIP 26974, BRIP 4703a, BRIP 4704a, BRIP 11086a and BRIP 28519a were aligned in cluster W in the same way by Thomson *et al*., 1997 and many other researcher later.

Epidemiological studies: Epidemiological study of the disease was done under field condition at University research farm Koont (33 07 00 N 73 00 36 E) by considering the epidemiological factors such as temperature and relative humidity. Inoculum was sprayed after 4 weeks of transplanting. First inoculum was sprayed at flowering stage (1×10^5) while second inoculation was performed at fruiting stage with same concentration.

Effect of mean minimum temperature, maximum temperature and relative humidity on disease development: In the year 2018, it was evident that disease steadily but continuously increased with the passage of time. Mean weekly minimum temperature remained in same range and not increased much as it fluctuated only 3ºC because optimum range of minimum temperature prevailed during the study period. Initially relative humidity, mean minimum and maximum temperatures did not increase significantly, therefore, disease severity increased only 2-3 % as shown in (Figs. 3&4) while significant progress in the disease (18.2% to 25.8%) was noticed when RH increased from 49.2 to 83.5.

Initially the minimum temperature was 23.3ºC and relative humidity was 44.6% then fruits depicted 11.5% infected area. Slight change in minimum temperature and big change in relative humidity resulted in final disease of 31.5%. most of the time RH has been higher than 70% (Fig. 2). However, during the period, mean maximum temperature remained in the range of 32.5-38ºC. Although many other factors such as variety, crop stage, fruit ripening etc. significant role in the spread of disease but hot and humid environmental conditions favour the sporulation and multiplication of the pathogen.

In the cropping season of 2019, relative humidity has been 5-10% higher due to more rainfall in monsoon season and it reached 84.5%. It remained 80% or above for 4 weeks as compared to last year when it crossed 80% only once. Due to higher rainfall, mean weekly maximum temperature remained slightly less than previous year. However, mean minimum temperature readings were almost at par which were in optimum range. As a result disease was found 5% higher than 2018 (Figs. 5&6). Mean maximum temperature for fungal growth, sporulation and germination is 33-34^{o}C and temperatures in the range 31-36^{o}C would favour infection and development of disease. The incubation and latent period would have been short and the formation of acervuli producing a large number of conidia would have led to relatively rapid disease development (Trapero-casas and Kaiser, 1992). Similar types of models were derived by Riaz *et al*., 2017 in which they found that temperature and wetness periods were the significant environmental factors influencing infection and chickpea blight development. However, effects of change in temperature (21-26^{o}C) on physiology of wheat as reported by Chang *et al*., (2023), there was no significant change in oth photosynthesis and biomass of winter wheat.

Regression analysis and model development: As the overall conditions slightly differed, therefore, data of both years (as shown in Figs. 2-5) was pooled and subjected to the regression. Mathematical model derived is presented in the following equation.

$$ROCD = -68.21+2.06T\ min. +0.31T\ max. +0.47RH$$

$$R2 = 82.9\%$$

P values for the constant, mean minimum temperature, mean maximum temperature and relative humidity were 0.01, 0.05, 0.06 and 0.00 respectively.

In the above regression equation, co-efficeints indicated the little change in minimum temperature influenced the disease development significantly as change in minimum temperature ranged only between 22.8-25.0 in first year and 22.8-25.8 in second year while maximum temperature and relative humidity affected the disease development less sharply due to more fluctuation in both of them as compared to the former one.

It was evident from the above results that the mean relative humidity had also a significant effect because the value of P is 0.00. The value of co efficient of determination (R^2) could not reach 100% because many other factors involved in development of anthracnose as the disease severity increased more rapidly at mature stage as compared to initial stage of fruit. Ten weeks' data of both years showed significant positive relationship between mean relative humidity and rate of change in disease(ROCD). The reasons for this response might be due to the prevalence of optimum range (in case of minimum temperature 22.8-25.8ºC) and (in case of maximum temperature 31.2-38ºC) during the study period as disease has been reported to develop in this range (Roberts *et al*., 2015). Other reasons might be due to some biochemical changes in fruits.

The difference in the disease between two years was not much pronounced. The site of experiments comes under the semi-arid zone with some years of more monsoon rainfall render conditions for disease development generally conducive (Riaz *et al*., 2017). Mean annual 300mm-600 mm and fertile soils in these areas results into luxurious growth of the crop. Plant canopy of the crop helps to increase the crop humidity which also remains medium to high in the atmosphere for longer period due to rains. These results demand that researchers and extension agents should pay more attention to develop and introduce anthracnose resistant chilli varieties for this zone.

Conclusion

Molecular characteristics of the pathogen resulted in confirmation of *Colletotrichum capsici* as the only causal agent of the chilli anthracnose in the area. Relationship of weather parameters with disease for two years in the form of mathematical model suggested that mean minimum and maximum temperatures and relative humidity play important role in the spread of the disease. As most of the time period, weather conditions have been conducive for anthracnose development suggest that researchers should pay more attention to develop resistant chilli varieties against the disease for this area.

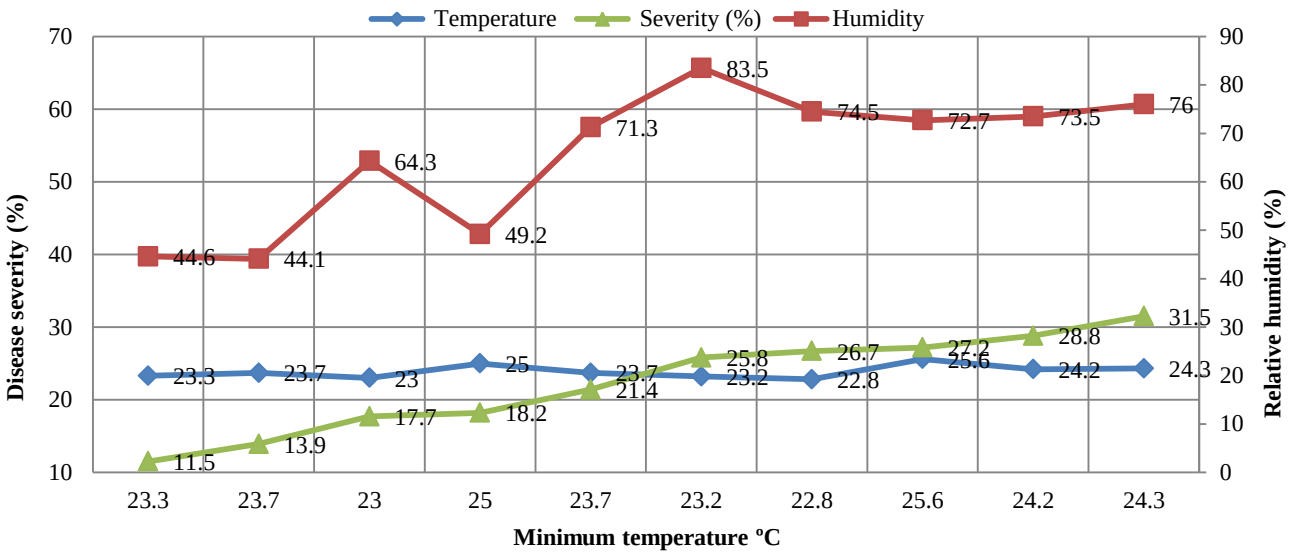


Fig. 3. Effect of mean minimum temperature and relative humidity on disease severity of chilli anthracnose during the crop season of 2018.

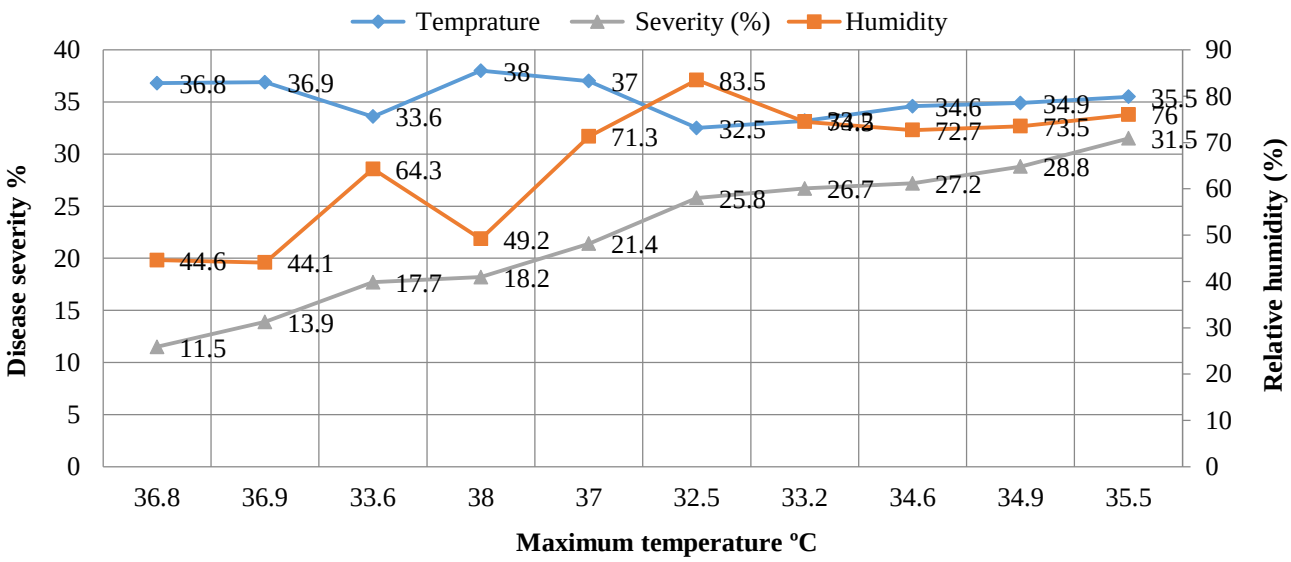


Fig. 4. Effect of mean maximum temperature and relative humidity on disease severity of chilli anthracnose during the crop season of 2018.

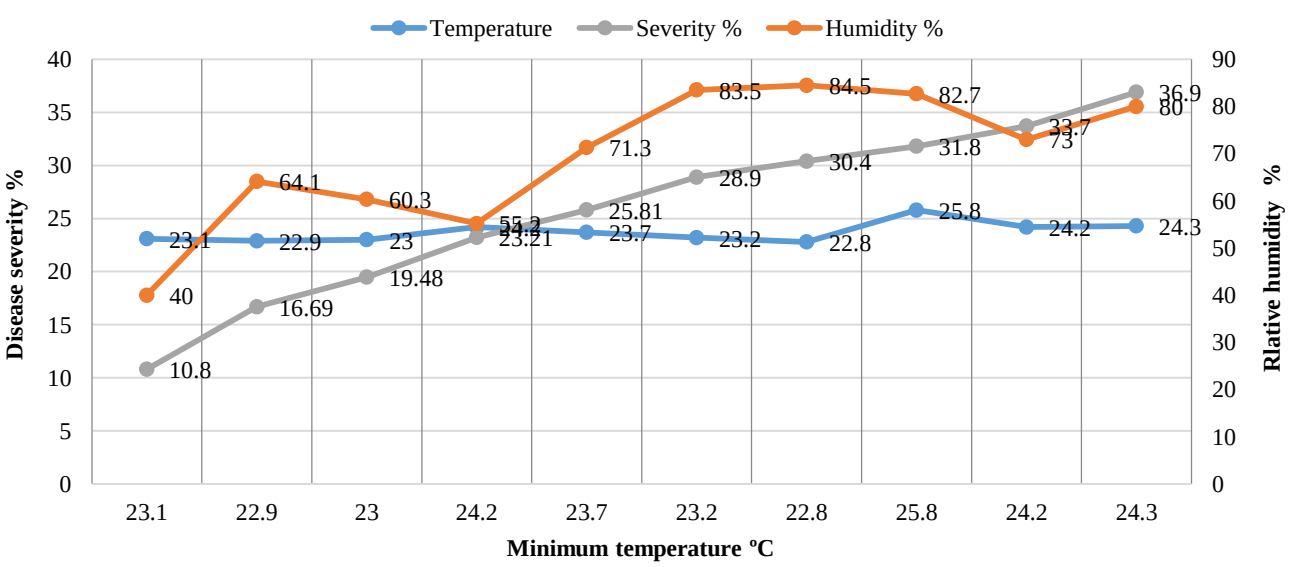


Fig. 5. Effect of minimum temperature and relative humidity on disease severity of chilli anthracnose during the crop season of 2019.

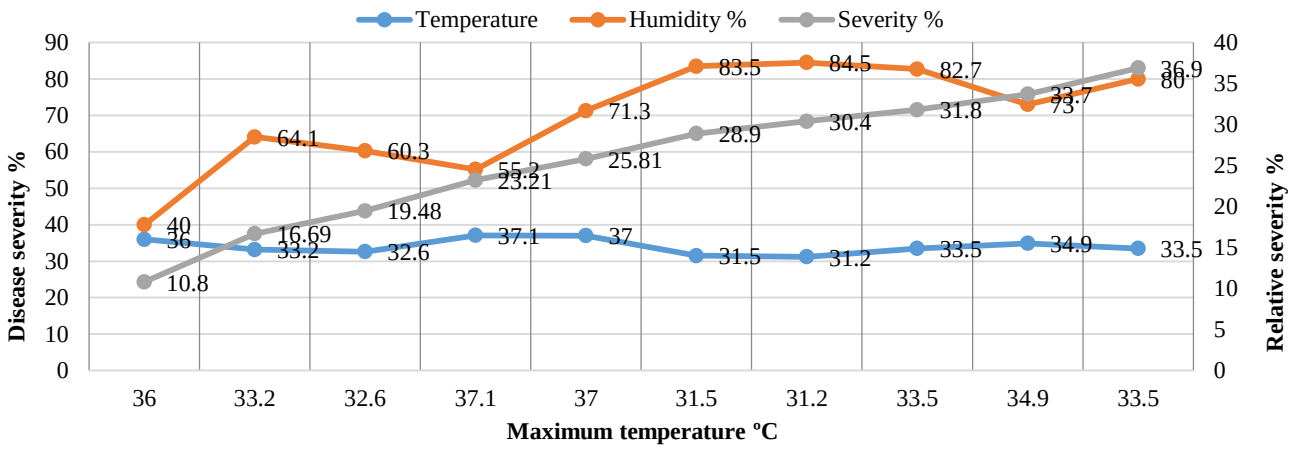


Fig. 6. Effect of mean maximum temperature and relative humidity on disease severity of chilli anthracnose during the crop season of 2019.

Acknowledgement

This research work was a part of the research project "Epidemiology, Population Biology and Management of Chilli anthracnose in Punjab, Pakistan. Authors are thankful to Agricultural Linkage Programme, Pakistan Agricultural Research Council, and Islamabad for funding the research work.

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(Received for publication 28 December 2021)