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OCCURRENCE OF TOMATO EARLY BLIGHT DISEASE AND ASSOCIATED *ALTERNARIA* SPECIES IN PUNJAB, PAKISTAN

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ABSTRACT

Early blight disease of tomato, triggered by *Alternaria* species, is one of the destructive diseases of tomato in Pakistan and around the globe. In this study, a survey was conducted during 2016-17 to estimate the early blight disease incidence and severity, and the identification of fungal species associated with the disease from the eight districts in Punjab Province of Pakistan: Gujranwala, Lahore, Multan, Bahawalpur, Sahiwal, Muzaffargarh, Rajanpur and Faisalabad. The disease incidence and severity from the eight districts were further associated with the different environmental variables. Eight *Alternaria* isolates, representing eight districts, were characterized on morphological basis and the four most pathogenic isolates on molecular (ITS rDNA) basis. The pathogenicity of the most pathogenic isolate was first evaluated by detached leaf assay and then compared with attached leaf assay. Bahawalpur district was found to be the most infected with maximum 75 % disease incidence and Rajanpur was the least affected region with 9% disease incidence while maximum disease severity index was recorded from Multan with 24% and lowest from Rajanpur with 1%. The disease incidence showed positive correlation with relative humidity and negative correlation with temperature while the rainfall and UV showed no influence. Out of four molecularly identified isolates of *Alternaria*, only one isolate was identified as being *A. solani* and the remaining three belonged to *A. alternata* species. *A. solani* showed the highest (score 3.33) level of pathogenicity over all the *A. alternata* isolates (0.66-2.66). This study concluded that the early blight disease is of high importance with great prevalence in Pakistan due to highly favorable environment especially relative humidity (55-61 %) and temperature (14-36 °C). Here, two *Alternaria* species are the main cause of early blight disease and out of them *A. alternata* is the most common while *A. solani* is the most pathogenic fungus.

Keywords: Tomato; Early blight disease; *Alternaria* species; Environmental variables; Pathogenicity
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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the second largest non-grain food crop next to potato around the world (Foolad, 2007; Gondal *et al*, 2019; Kassie *et al.,* 2020). Its worldwide production is estimated at 161.7 million metric tons and has estimated value of $59 billion USD worth (Adhikari *et al*., 2017; Lee and Kennedy, 2020). Pakistan's position in tomato production is 34th among countries of the world with over 572837 tons annual production (FAOSTAT 2017; Anjum *et al*., 2020) Unfortunately, the average yield per hectare in Pakistan is very low (9.6-10.5 tons per hectare) as compared to the rest of the world and, thus, is ranked 142nd in yield per hectare (Gondal *et al*., 2012; Ahmad *et al*., 2019; Junaid and Musharaf, 2020). The low yield in Pakistan occurs despite tomato being cultivated in Pakistan throughout the year, possible due to variation in climatic conditions among its provinces (http://www.finance.gov.pk , 2016).The low yield may be due to any of the various diseases of tomato caused by fungi, bacteria, viruses and nematodes known all over the world (Koike, 2007; Ghani *et al*. 2019; Gupta *et al.*, 2020). Among these disease, early blight, caused by several *Alternaria* species, is a particularly destructive disease on tomato and other solanaceous crops. The disease can occur at all stages of plant growth and in this way can affect all above ground parts of tomato, including the leaf, stem and fruit (Blancard, 2012; Akhtar *et al.*, 2019; Mphahlele *et al*., 2020). The typical symptoms on the leaves are circular lesions, 1.5 cm in diameter with dark concentric circles. A leaf blight level generally initiates on the mature and lower leaves and progresses upwards through the canopy (Adhikari *et al*. 2017). Infected leaves are weakened and can die and drop from the plan, resulting in substantial yield loss (Foolad *et al*., 2008; Ghorbanpour *et al.,*2018; Mahawar *et al.*, 2020).

Many *Alternaria* species, including *A. solani* Sorauer 1896, *A. linariae* Simmons 2007, *A. tomatophila* Simmons 2000, *A. alternata* Keissl 1912, *A. arborescens* Simmons 1999 and *A. tenuissima* Wiltshire 1933 are reported from different countries affected by tomato early blight (Ramezani *et al.*, 2019; Ayad *et al.*, 2019; Adhikari *et al.*, 2020). Bashir *et al*. (2014) reported *A. metachromatica* Simmons 1994 as the cause of early blight of tomato in Lahore District of Pakistan. Considerable physio-

logical, morphological and virulence variation has been reported within and among *Alternaria* species. (Zheng *et al.*, 2015; Upadhyay, 2019: Al-lami *et al.*, 2020). The sexual stage of *Alternaria* is still unknown, and the source of high genetic variability is not well understood. It has been hypothesized that the high genetic diversity within *Alternaria* occurs from parasexual activity, and that the large amount of asexual reproduction causes frequent mutations to occur (Van Der Waals, 2004; Odilbekov *et al.,* 2019). Species with greater genetic variation are expected to adapt better and evolve more quickly to the constantly changing of environment, which affects disease ecology and disturbs the disease management strategies. In case of *A. solani*, life cycle is polymorphic, which result in large populations of spores that are produced at various times throughout the growing season, allowing for many genetic mutation/recombination opportunities that could lead to a relatively high level of diversity (Meng *et al*., 2015; Mphahlele *et al*., 2020). Recently, Stewart *et al.* (2014) disclosed phylogenetically distinct lineages within *A. alternata* causing citrus brown throughout the world collection of isolates, and Huang *et al*. (2014) presented a new phylogenetic reconstruction for the same pathogen in China. These studies demonstrate the power of phylogenetic approaches to find out the divergence among populations of fungal plant pathogens that lack a known sexual stage and may discover cryptic species, independently evolving lineages (Ozkilinc *et al*. 2018).

Ecological and environmental conditions play an important part in determining the outcome of the interaction between *Alternaria* and its tomato host (Kumar, 2017). The environment is encouraging for early blight disease development. This disease progresses quickly when environmental conditions alternate between wet and dry weather (Parmar *et al.,* 2020). *Alternaria* spores, humidity and temperature are the main factors that contribute to the occurrence of this disease (Escuredo *et al.,* 2011; Gupta *et al.*, 2020; Chohan *et al* 2019). This disease can spread rapidly in conducive environments due to its polycyclic infection and short disease cycle which may lead to 80% yield losses (Ding *et al.*, 2019; Upadhyay, 2019; Kaur *et al.*, 2020). Many scientists correlated meteorological variables with the abundance of airborne spores in the atmosphere of climatically different areas (Escuredo *et al*., 2011). Pathogen infection and conidia germination can be intensified when relative humidity is high (Van der Waals *et al*., 2001; Vloutoglou and Kalogerakis 2000; Gullino *et al.,* 2020).

Previously, two independent studies were conducted on early blight disease in Peshawar and Faisalabad districts by Ahmad *et al*. (2014) and Akhtar *et al.* (2011) respectively in which the disease incidence has been reported as 50% and 100%. Similarly, the comprehensive field survey was also conducted during two growing seasons (2014 -2015), in District Gilgit- Baltistan of Pakistan where the disease incidence ranged from 10.22% to 44.16% in first growing season and during later season it ranged 14.03–49.16%, whereas 5.37–16.40% and 6.52–26.94% severity was recorded. So far, no survey on the early blight disease has been conducted in the province of Punjab, Pakistan. Considering the significance of early blight, a study was conducted to determine the importance of the disease in the tomato growing areas of Punjab, Pakistan. This study had the following specific objectives: 1) Determine incidence and severity of early blight in eight districts of Punjab, Pakistan; 2) identify the *Alternaria* species associated with early blight disease in the eight districts and characterize representative isolates on morphological, molecular and pathogenicity basis; 3) determination of impact of environmental factors on disease incidence and severity.

MATERIALS AND METHODS

Survey and Disease Assessment: Assessment of tomato early blight disease (at fruiting stage) was carried out in major tomato growing areas of Punjab Province including Muzaffargarh, Faisalabad, Sahiwal, Multan, Bahawalpur, Rajanpur, Lahore and Gujranwala (Fig. 1). The study was conducted in March (the month when the crop is at fruiting stage and showing maximum disease) 2016 and 2017. In each region, 3 fields, having regular tomato crop production, were selected. 10 to 15 plants were taken randomly from a $2m^2$ quadrat in each field. Disease incidence was computed according to James (1974): Disease incidence (%) =Number of Diseased Plants /Total Number of Plant Inspected ×100.Further, the disease severity index was measured on randomly selected 5 infected plants / 2 m^2 quadrat according to Singh *et al.* (2011), later adapted by Akhtar *et al*. (2019) with slight modification: Disease Severity Index (%) = sum of all disease ratings/Total plants observed ×100/5.

Sample collection and fungal isolation: Leaves, fruits and stems with early blight symptoms (concentric rings, Bull's eye or target spot symptom) were randomly collected from all the eight districts (Fig. 2). Total 104 Infected (Table.1) plant parts were surface sterilized using 1% sodium hypochlorite solution (NaOCl), thoroughly washed with distilled water and air dried in sterile environment Initially, infected plant parts were put on water agar media plates and incubated for 3 to 4 days at room temperature until sporulation occurred according to the instructions determined by Holm *et al.* (2003). Then, isolates were purified by single spore technique and sub-cultured using a sterile glass needle under stereomicroscope with aseptic conditions and incubated at 24± 2 °C for 10 days under diurnal light. Then, cultures were placed at 4°C for short term and *on silica* gel for long term storage as described by Perkins (1962).

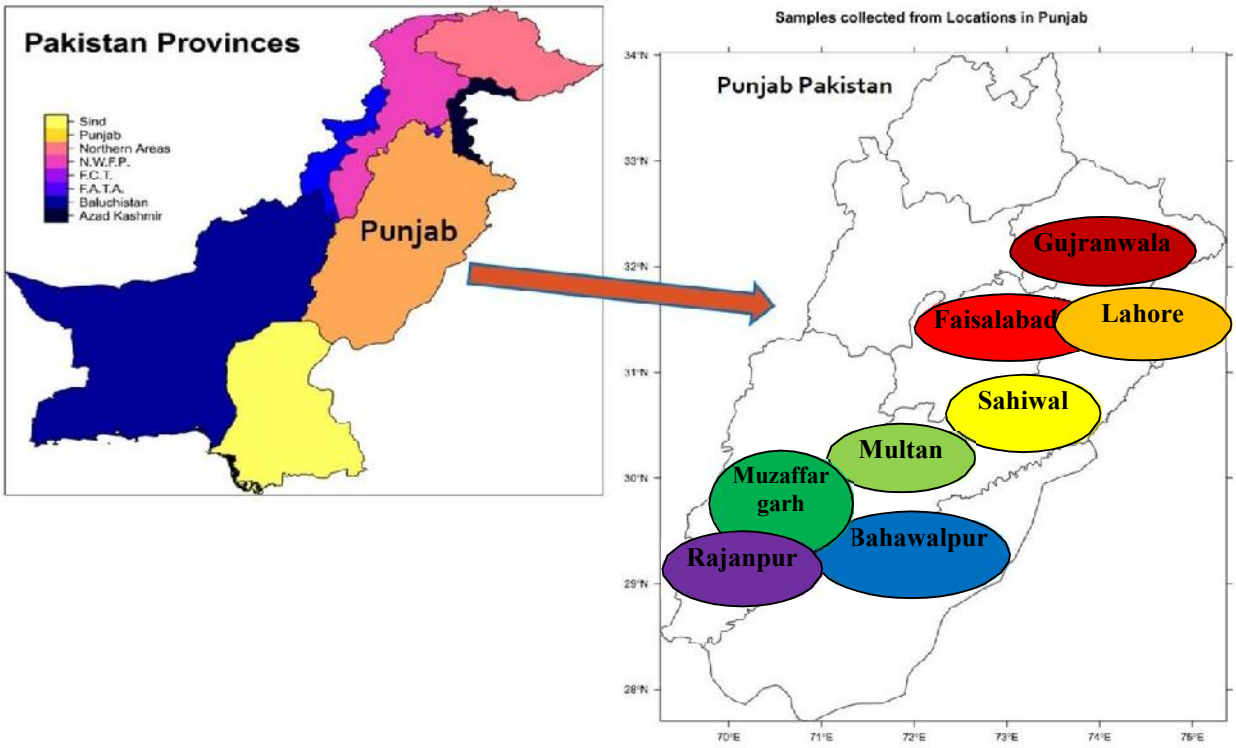

Fig. 1: Survey sites of early blight of tomato diseases in eight districts of Punjab, Pakistan

Fig. 2: Incredible symptoms of early blight of tomato with concentric lesions on leaf (a), stem (b) and fruit (c)

Table 1: Information of all the samples collected from 24 fields of 8 districts and number of isolates recovered from diseased plant parts with macroscopic symptoms of early blight.

Districts	Nb. of fields	Nb of infected samples			Nb. of *Alternaria* spp. isolates	
		Leaves	Stems	Fruits	*A. alternata*	*A. solani*
Gujranwala	3	10	-	-	6	-
Faisalabad	3	12	-	-	4	-
Lahore	3	10	2	2	6	-
Multan	3	4	-	-	6	-
Rajanpur	3	12	2	2	3	-
Muzaffargarh	3	12	2	2	-	6
Sahiwal	3	12	2	2	6	-
Bahawalpur	3	12	2	2	8	-

Identification of *Alternaria* species: All isolates were grown on potato dextrose agar (PDA) and incubated at 24± 2 °C for 10 days under diurnal light. They were identified on morphological characteristics by using the criteria as described by Ellis (1971), Akhtar *et al.* (2004) and Simmons (2007). One isolate from each locality that tentatively representing same conidial morphology as all the other isolates of same locality, was selected. This representative isolate from each locality was used for pathogenicity test and only one most virulent isolate was retested in attached leaf assay, DNA based identification using ITS region of rDNA was used to confirm the identity for the most pathogenic *Alternaria* isolates as determined in a detached leaf assay. For this purpose, spores and mycelium at the sporulating area of a culture were harvested with sterilized deionized water (containing 0.01% Tween 20), by softly scraping the culture surface with a sterile spatula. Qiagen DNeasy Plant Mini Kit, Cat. No .69104 was used to extract DNA, according to the prescribed instructions. The primer set ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (White *et.al* 1990) was used to amplify the ITS region from pathogenic isolates. The PCR reaction mixture contained 3 µl DNA, 12.5 µl PCR master mix, 1 µl of each primer (forward and reverse) and 7.5 µl double distilled water to make the total volume of 25 µl. The PCR protocol was followed as described by Pavón *et al*. (2012) with slight modification made by Ramezani *et al*.(2019). The amplified PCR products were analyzed by electrophoresis on 1.5% agarose gel and sent for sequencing to MACROGEN (Seoul, South Korea). The obtained ITS rDNA sequences were edited using BioEdit software then compared against sequences available at the National Center for Biotechnology Information (NCBI, http: //www.ncbi.nlm. nih.gov/genbank/) using Basic Local Alignment Search Tool (BLASTn, http://blast.ncbi.nlm. nih.gov/Blast.cgi) data base. Phylogenetic analysis of ITS rDNA sequences of fungal isolates along with reference sequences obtained from NCBI BLASTn data base were carried out in MEGA X Software (Kumar *et al.,*2018). Phylogenetic tree was then obtained by first aligning the sequences using Muscle program (Edgar 2004). Phylogenetic trees were constructed using maximum likelihood (ML) method and Tamura-Nei model (Tamura and Nei,1993), with 1000 pseudo replicates to measure branch strength of the tree, was constructed.

Pathogenicity Test

Detached leaf assay: Seedlings of tomato cultivar "Rio-Grande" susceptible to early blight disease (Chohan *et al*., 2015) was cultivated in pots holding soil compost (2:1) in a greenhouse range from 25 to 28 °C and relative humidity from 70 to 90%. Eight representative isolates were assessed for relative pathogenicity (virulence) on tomato via a detached leaf assay as described by Foolad *et al*. (2015). For pathogenicity test eight representative *Alternaria* spp. isolates were grown on PDA for 10 days at 24± 2 °C under diurnal light. For detached leaf assay, fully expanded healthy leaves with same size were detached at the base of the petiole from 35 days old plants, leaves were disinfected with 1% sodium hypochlorite solution for 2 minutes and rinsed 4 times with sterile distilled water. Afterward leaves were placed on paper towel and air dried in a laminar air flow-hood. They were placed abaxial side up on Whatman filter paper in sterile petri plates (90 mm dia.). Each leaf was punctured on the abaxial side with a sterile needle at its central point where it was inoculated with a mycelial agar plugs (5 mm in dia.) taken from the margin area of 10 days old culture grown on PDA media . Petri plates were kept wet by adding 2 ml of water and placed in the incubator in the dark for 48 h at 22 °C, then placed under a cool-white fluorescent diurnal light with a 12 h photoperiod for 7 days, and moistened when required. There were 3 replications for each fungal isolate. Disease severity was determined according to rating scale described by Weber and Halterman (2012) with modification: 0 = no symptoms, 1 = necrosis around the lesion, 2 = necrosis covering to 1/3 and 2/3 of leaf, 3 = whole leaf necrosis, 4 = petiole necrosis

Attached leaf assay: Pathogenicity of the most pathogenic isolate was retested by attached leaf assay as described by Tymon *et al.* (2016). The experiment was performed in greenhouse on 35 days old tomato plants, with 24°C to 30°C growth for occurrence of the disease. Four plants were used in the experiment and three leaves were selected from each plant. To perform this test, the mycelial plugs (5 mm) of virulent isolate (AA4) were taken from ten days old culture grown on PDA and were ground in 1 ml sterilized water and poured on the punctured disinfected healthy leaves of the plants. The same size of mycelial plugs was used as in detached leaf assay. The symptoms developed and the disease severity was measured after 15 days according to Weber and Halterman (2012) disease rating scale as given above.

Meteorological Data: Meteorological data were obtained from websites of national and international data centers as https://www.worldweatheronline.com ; https://www.timeanddate.com/weather/pakistan and www. http://namc.pmd.gov.pk. The variables that were examined included mean, minimum and maximum temperature (°C), relative humidity, rainfall, UV light and wind (Table 2)

Statistical analysis: The data collected during field survey and greenhouse experiments were evaluated by Analysis of Variance (ANOVA) and Fisher's least significant difference (LSD) test, at 0.05% level of significance to separate treatment means, using Statistix 8.1 analytical software (McGraw-Hill 2008). Correlation analyses were performed to evaluate the effect of meteorological factors on the disease incidence and disease severity index

RESULTS

Prevalence of tomato early blight in Punjab, Pakistan: Early blight disease was observed in all the surveyed areas, but the intensity of disease differed from one area to another. Highest mean disease incidence (75 %) was recorded in Bahawalpur followed by Multan (74%), Muzaffargarh (72 %), Sahiwal (61%) Gujranwala (51 %), and Lahore (36 %) Faisalabad (34 %) and Rajanpur had the lowest disease incidence (9 %) (Fig. 3). Highest disease severity index was recorded in Multan (24.39%) followed by Muzaffargarh (23 %), Bahawalpur (21 %), Sahiwal (19 %), Gujranwala (11 %), Lahore (10 %), and Faisalabad (6 %) and lowest recorded in Rajanpur (1 %) (Fig. 4).

***Alternaria* species associated with tomato early blight disease:** A total of 45 *Alternaria* isolates were obtained from typically early blight lesions on tomato plants (Table.1). One representative isolate from each locality was selected based on similar conidial morphology as all other isolates of that locality. In total, eight representative isolates were selected. One isolate out of eight in the genus *Alternaria* was found as being *A. solani* and the remaining isolates belonged to *A. alternata* (Fig. 5). Growth of *A. solani* on PDA medium was sparce, smooth, circular and greenish black, while that of *A. alternata* was dense, black grey and brown. The growth was dense for only *A. alternata* on PDA media. Data presented in Table 3 shows comparison of the eight isolates in terms of colony diameter after 10 days of incubation. Highest radial growth was observed for isolate AA2 collected from district Multan and lowest for AB4 collected from Rajanpur. Under microscope, the isolates of *Alternaria* that had short chains of two to three conidia on the hyphae with short beak have morphotypes of *A. alternata*. Moreover, these isolates produced conidia sized 24 to 33 µm with small beaks sized 4 to 6 µm characteristic of *A. alternata*. They had three to seven transverse septa and zero to 4 longitudinal septa. In case of *A. solani*, conidiophores arose singly, usually flexuous or straight, simple and septate, rather yellowish brown or pale brown, smooth. In *A. solani*, beak size of conidia was 68 to 88 µm. They were straight or solitary, slightly flexuous, obclavate or with the body of the conidia ellipsoidal or oblong narrowing to a beak. The beak was usually the equivalent length as or somewhat elongated as the body, pale brown with 2–11 transverse and none to a few longitudinal or oblique septa.

The four most pathogenic isolates, based on detached leaf assay, were also identified by analysis of ITS sequencing. The sequences obtained for each of the isolates had more than 98% similarity with *Alternaria* species in GenBank. Our BLAST results for the four isolates matched exactly with our morphological study. Two Phylogenetic trees constructed based on genetic similarity can be seen in Fig. 6 and 7. According to the morphological and molecular based observations the pathogenic isolates of *Alternaria* were known as *A. solani* and *A. alternata* whose cultures were submitted as AA4 and AA1, AA2, AA3, respectively, in the Fungal Ecology and Bio-Control lab culture collection of the Department of Plant Pathology, Bahauddin Zakariya University Multan, Pakistan.

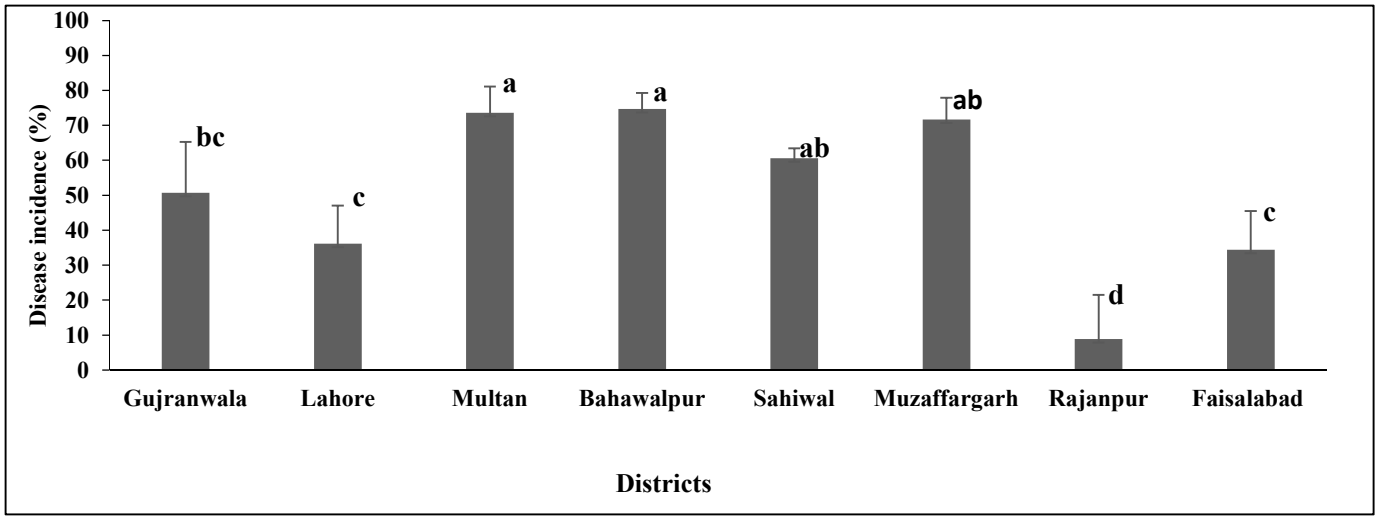

Fig. 3: Early blight disease incidence (%) means from different districts of Pakistan during 2016-17. Error bars represent the standard deviation. The means with same letters are not statistically different according to LSD test at $p < 0.05$.

Table 2: Meteorological data for Maximum, minimum, mean temperature, mean relative humidity, mean rainfall, mean wind and mean were taken during survey (March of 2106-17)

	March-2016							March-2017						
Districts	Max.T (°C)	Min.T (°C)	Mean Temp (°C)	Mean RH (%)	R.F (mm)	Wind speed (km/h)	UV In-dex	Max. T (°C)	Min.T (°C)	Mean Temp (°C)	Mean RH (%)	R.F (mm)	Wind speed (km/h)	UV Index
Gujranwala	29	15	22	60	26	9	7	33	16	25	7	10	8	7
Lahore	32	12	22	59	14	10	7	34	13	24	60	8	8	8
Multan	35	14	25	57	17	10	8	36	12	24	57	2	9	8
Bahawalpur	31.5	16	24	61	13	8	8	32	14.	23	55	0	7	8
Sahiwal	31	17	24	62	2	10	8	32	15	24	60	1	9	8
Mzaffargarh	35	15	25	63	27	10	8	33	16	25	59	0	10	8
Rajanpur	35	17	26	63	13	11	8	46	23	35	38	1	10	8
Faisalabad	39	25	32	63	24	10	7	32	14	23	59	5	8	8

Max. T: Maximum Temperature, Min. T: Minimum Temperature, Mean Temp: Mean Temperature, R.H. Relative Humidity, R.F (mm): Rain Fall (Millimeter),

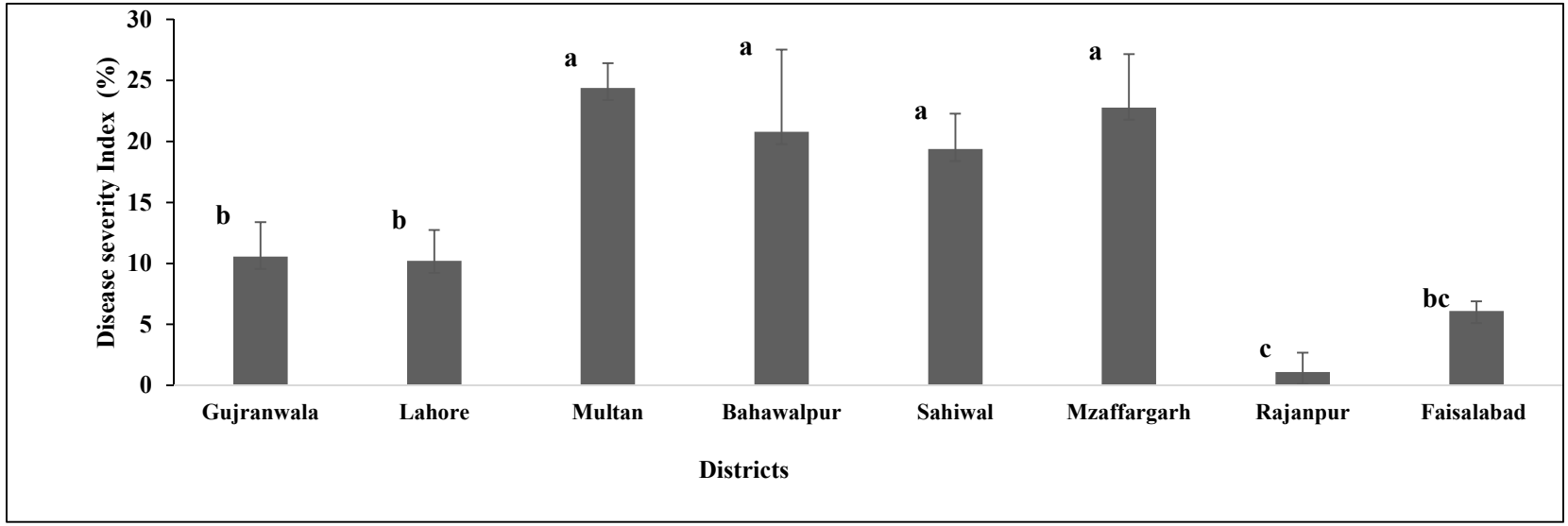

Fig. 4: Early blight disease severity index means in different districts of Pakistan during 2016-17. Error bars represent the standard deviation. The means with same letters are not statistically different according to LSD test at $p < 0.05$.

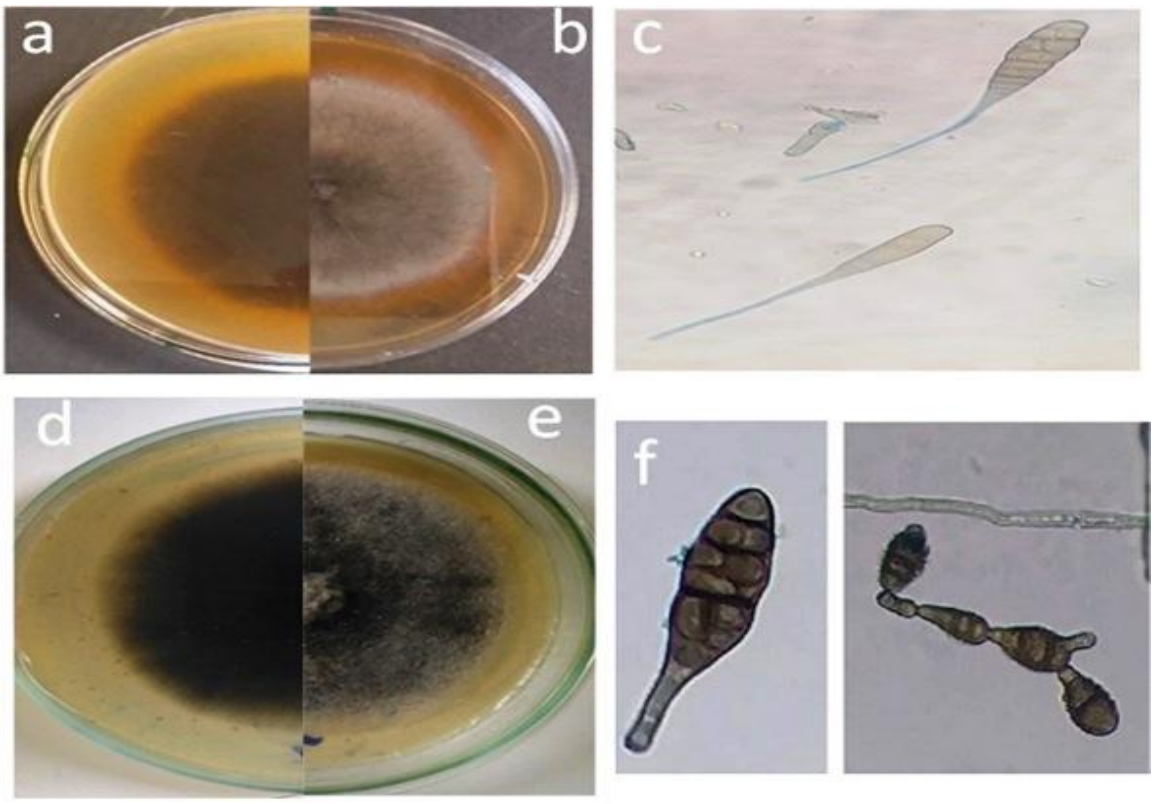

Fig. 5: (a) Colony growth of *A. solani* on PDA lower sides, (b) Colony growth of *A. solani* on PDA upper side (b), (c) Conidia of *A. solani*, (d) A. *Alternata* growth on PDA lower side, (e) *A. Alternata* growth on PDA upper side and (f) conidia of *A. Alternata* as single and chain form

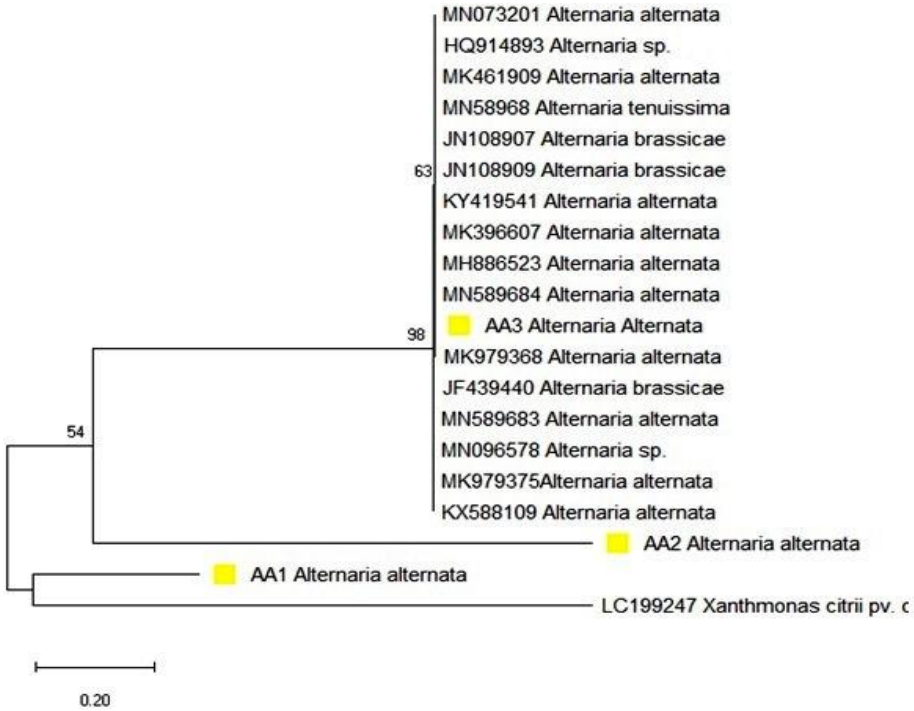

Fig. 6: Phylogenetic tree *of A. alternata* isolates. Statistics on the nodes denoted bootstrap percentage values based on 1,000 replicates. Samples with yellow labeled indicate those strains obtained in this work from Pakistan.

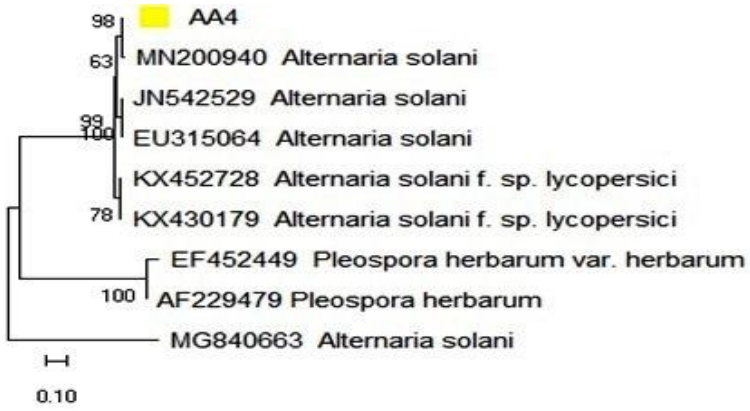

Fig. 7: Phylogenetic tree of *A, solani* isolate Statistics on the nodes denoted bootstrap percentage values based on 1,000 replicates. Sample with yellow labeled indicated the strain obtained in the present study.

Pathogenicity variability among *Alternaria* spp. isolates: All eight isolates caused necrotic lesion on the leaves but varied as to degree of pathogenicity. The isolate was categorized on a pathogenicity basis, a disease score over 3 was categorized as highly pathogenic. *Alternaria solani* strain AA4 was placed in this category as it caused whole necrosis or chlorotic on detached leaves. Isolates with a disease score between 1.5 and 3, were categorized as pathogenic. *Alternaria alternata* strains AA3, AA2 and AA1 were placed in this category as they caused necrosis or chlorotic covering to 1/3 and 2/3 of leaf based on disease scores. The *Alternaria* isolates showing a disease score below 1.5 were categorized as less pathogenic as they showed minor symptoms on detached leaves. This was the case for the *Alternaria* strains AB1, AB2, AB3 and AB4. Pathogenicity test results have been shown concisely in Fig. 8 and table 3. Pathogenicity of isolate AA4 was also checked on attached leaves and compared with detached leaf assay. Results indicated that isolate caused the same level of disease both on detached and attached leaf (Fig. 9) and there was no significant difference between both assays concerning the pathogenicity of the isolate (Fig. 10).

Fig. 8: Early blight symptoms on detached leaf rated using a 0–5 scale during pathogenicity. No necrosis (a), Initial necrosis (b) necrosis covering ½ (c), necrosis covering ¾ (d) and whole leaf and petiole necrosis (e)

Table 3: Pathogenicity and colony growth of different isolates of *Alternaria* genus according to their locations.

Isolates	Districts from where Isolates were collected	Pathogenicity reaction (Detached leaf Method)	Pathogenicity	Colony diameter after 10 days (mm)
AA1	Faisalabad	1.66cd	Moderate Pathogenic	51.33d
AA2	Multan	2.33bc	Moderate Pathogenic	79.33a
AA3	Bahawalpur	2.66ab	Moderate Pathogenic	42.00f
AA4	Muzaffargarh	3.33a	Highly pathogenic	46.00e
AB1	Sahiwal	1.33de	Less pathogenic	45.33b
AB2	Gujranwala	1.00de	Less pathogenic	67.00e
AB3	Lahore	0.66e	Less pathogenic	60.33c
AB4	Rajanpur	0.66e	Less pathogenic	20.00g

Data are means of three replicates. Values trailed by the same letters within columns do not significantly differ at P ≤0.05

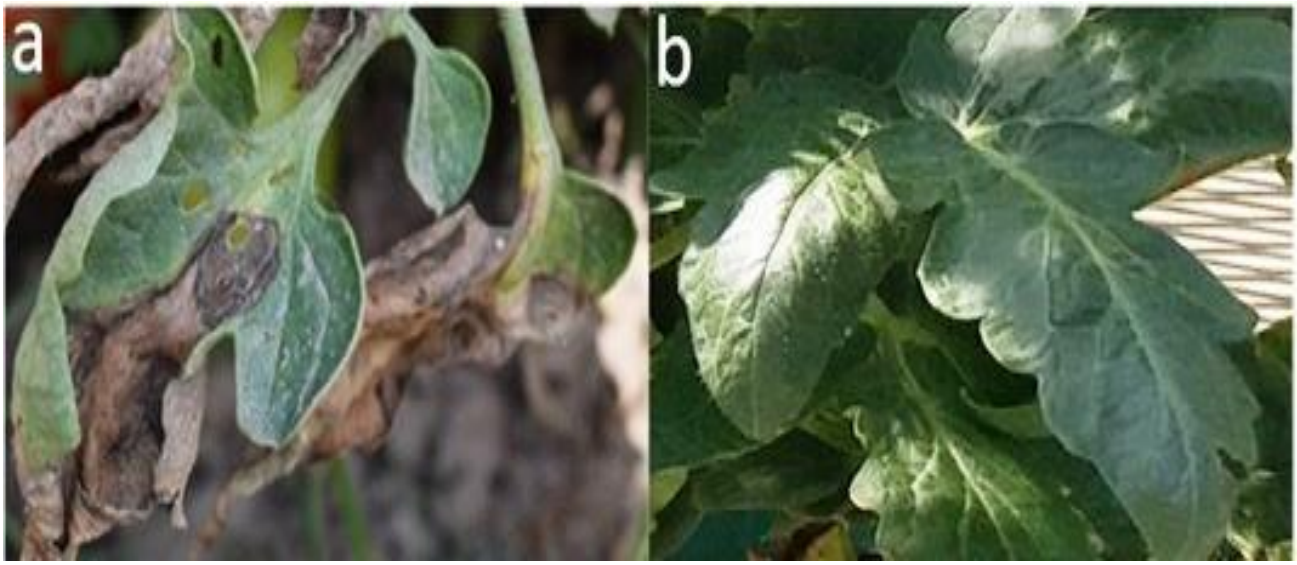

Fig. 9: The plant's leaf on the right part was inoculated with water (a) and used as control while on left side leaf was inoculated with spore suspension of pathogenic isolate (b). These pictures were taken 35 days after inoculation.

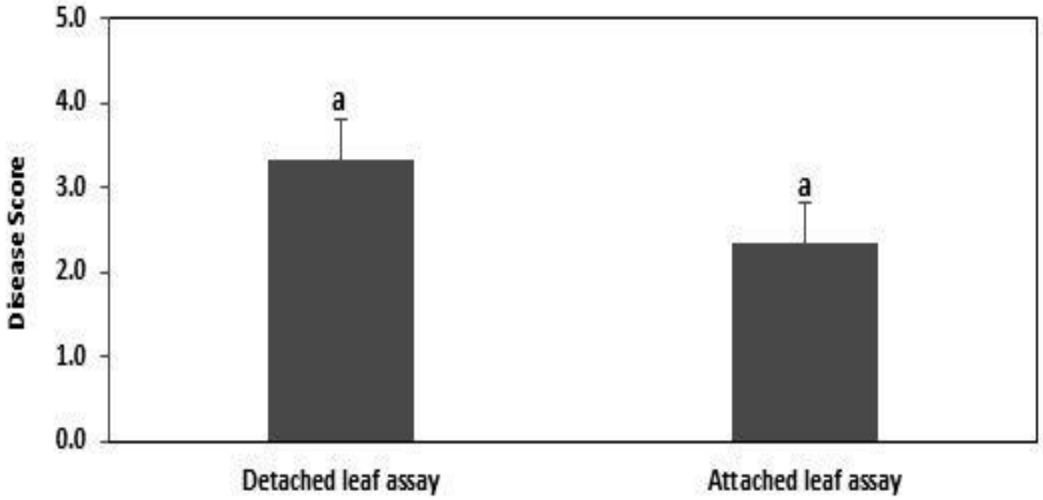

Fig 10: Pathogenicity of *A. solani* isolate (AA4) on attached and detached leaf. The values are the mean of three replicates. Error bars represent the standard deviation. The means with same letters are not statistically different according to LSD test at $p < 0.05$.

Correlation analysis between weather parameters and early blight prevalence: Correlation analysis of Spearman's rank was used, considering disease incidence, disease severity and weather parameters recorded in the same month (March) of the two years when diseases assessment was recorded. For the year 2016 and 2017, mean relative humidity was recorded at 61% and 56%, respectively. Disease incidence for the year 2016 and 2017 was noted as 60% and 44% and the disease severity index was recorded as 15% and 14 %, respectively. The combined data of years 2016-17 showed that the disease incidence has a significantly positive correlation with the mean relative humidity (r = 0.3209), while mean relative humidity also showed relation with disease severity (Table 4). The disease severity showed significantly negative correlation with mean temperature (r =-0.3336) and maximum temperature (r = -0.3033), while mean temperature and maximum temperature showed no relation with disease incidence. The highest disease incidence (75 %) was recorded in Bahawalpur at temperature 14-32°C and relative humidity 55-61%. Highest disease severity (24%) was estimated in Multan where temperature and relative humidity ranged as 24 -36°C and 57 to 57%, respectively. Lowest disease severity (1%) was recorded in Rajanpur where temperature and relative humidity ranged as 23- 46°C and 38-63%, respectively (Table 4).

Table 4: Correlation coefficient with significant levels between the disease and meteorological parameters applying Pearson's test 2016-17.

Variables	2016-17	
	Disease Incidence	Disease Severity
Mean Relative humidity (%)	0.3209*	0.2454
Mean Temperature (°C)	-0.2902	-0.3336*
Rain Fall (mm)	0.1797	-0.0795
Temperature Maximum (°C)	-0.2663	-0.3033*
Temperature Minimum (°C)	-0.2267	-0.2668
UV	0.1748	0.2155

*Correlation is significant at $p < 0.05$

DISCUSSION

This study reported a huge extent of disease in Punjab ranging from 9% to 74% incidence with 6.11% to 24% severity. This shows the massive disease incidence and severity along with a great variation depending on location. The disease incidence and severity in different areas might be due to uninterrupted and rigorous farming of tomato and potato crops (Ayad *et al.*, 2019). The genetic makeup of commercial hybrid and lack of awareness among farmers, non-adoption of disease management practice also lead the huge disease incidence (Akhtar *et al*. 2019; Ayad *et al.*, 2019; Hussain *et al.,* 2019). Solanaceous crops cultivation in the locality of tomato may be the reason of high inoculum like Sahiwal, which is the hub of potato cultivation (Raza *et al,.* 2019). This can also lead towards the epidemics as the same *Alternaria* species cause the early blight of potato (Gannibal *et al*., 2014). Therefore, disease incidence is higher in Sahiwal than in the other regions of Punjab. Early blight is higher in Sahiwal due to cultivation of tomato and potato in the same areas of Sahiwal. The disease causing *alternaria* spp. are same for both crops. Reason for high incidence in Bahawalpur and Multan might be due to the infection of tomato by virulent *Alternaria* spp. followed by wet or humid and dry weather conditions (Rotem *et al.,* 1978; Koike, 2007). Another reason is survival and spreading ability as the life cycle of *A. solani* includes soil as well as air borne stages, making the pathogen difficult to control by means of rotation and sanitation (Chaerani, 2006).

Interestingly, in the present study, *A. solani* was the most pathogenic species while *A. alternata* was the most commonly occurring *Alternaria* spp. in Punjab Province. Previously, the variation in *Alternaria* species causing early blight disease has been ignored and only *A. solani* has been associated with tomato early blight disease in Pakistan. Many studies have revealed that *A. alternata* and *A. solani* could be recovered at the same time from the early blight infected plants (Babler *et al.,* 2004). The coexistence of both species (*A. alternata* and *A. solani*) may lead to severe infections as described by Leiminger and Hausladen (2011), which may imply future serious epidemic events in Pakistan. Worldwide studies showed that *A. solani* is the leading early blight disease causing agent among other associated *Alternaria* species (Bessadat *et al*., 2017; Kokaeva *et al*., 2018; Ramezani *et al*., 2019). We found a great variability in pathogenicity among the eight representative *Alternaria* isolates on the host plant.

The variation of pathogenicity among these fungal isolates can be perceived as the severity of expression. (Belosokhov *et al.,* 2017; Ding *et al.,* 2019; Tymon *et al.,* 2015). Overall, *A. alternata* was found less pathogenic than *A. solani* but was involved in causing early blight of tomato. The scientific community has reported a great variation in pathogenicity among the *Alternaria* spp. isolates as we found in the present study. The confirmation of pathogenicity by attached leaf assay in current study was in line with literature (Özer *et al.,* 2018). The pathogenicity test was performed by detached leaf assay which is a rapid technique that requires little space and is not affected by other factors like in greenhouse or field (Özer *et al.,* 2018). Pathogenicity have been described in many studies using detached leaf and attached leaf assay (Stammler *et al.,* 2013; Tymon *et al*., 2015; Ramezani *et al.,* 2019). Same results were also attained by Gannibal *et al*. (2014) and Hubballi *et al* (2011), when the pathogenicity of *A. solani* isolates was observed in both potato and tomato using detached leaf assay. Some reports also showed that *Alternaria* spp. isolates vary in pathogenicity in stirring stem lesion and early blight diseases development (Chaerani *et al*., 2018). In this study, the slow growth of the most pathogenic fungi *A. solani* on PDA as compared to other *Alternaria* species (Table 3) but surprisingly its growth or establishment on detached leaves and later on plants (attached leaves) was higher than other fungi that may be due to the nutrition and quick establishment on host by breaking its resistance.

The districts of Punjab like Muzaffargarh Bahawalpur, Multan and Sahiwal have alternation of wet and dry conditions. Wet and dry conditions support the prevalence of disease therefore disease occurrence was higher in these districts. Environmental variables such as air temperature, wetness duration is prerequisite for early blight disease development on tomato crop (Bashir *et al.,* 2014; Bashi and Rotem, 1974). In this study, the correlation results show that disease incidence has significant positive

relationship with relative humidity (Table 4). These results are in line with Strandberg (1998) and Hong (1995) studies, who described the early blight disease increased with increase in relative humidity. Vloutoglou and Kalogerakis in 2000 described that tomato early blight met the potentially destructive epidemiological characteristics in Greece as short wetness requirements, short incubation period, high host susceptibility and increased inoculum pressure caused premature shedding of infected leaves. A positive correlation was determined by Hiremath *et al.* (1990) between disease severity and relative humidity. In this study a negative correlation of early blight with temperature was revealed which agree with the study conducted by Reddy (2013) who noticed positive correlation between disease incidence and high relative humidity and negative correlation with maximum temperature. It was described that the mean minimum temperature had no influence on the development of disease. A study conducted by Rani *et al*. (2015) demonstrated significant negative correlations between disease intensity and minimum and maximum temperature, and a significantly positive relationship between disease intensity and relative humidity.

Conclusion: This study showed the significance of early blight disease of tomato in Punjab province of Pakistan and the associated fungal species. Two *Alternaria* species including *A. alternata* and *A. solani*, were found as being the main cause of early blight disease. *A. alternata* was the most dominant, while *A. solani* is the most aggressive fungal pathogen. The disease incidence showed significantly positive correlation with relative humidity and negative correlation with increase in temperature. The study suggests that early blight disease can be ranked very high for its serious threat due to the existing conducive environment and variation in associated fungal pathogen in Pakistan. Therefore, it is recommended to use resistant cultivars, multiple cropping system, proper irrigation schedule, chemical and biological measures in districts of the Punjab to control early blight of tomato.

Conflict of interest: The authors declare that they have no conflict of interest.

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