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BACTERIAL SPOT CAUSED BY *XANTHOMONAS VESICATORIA* DEVASTATED TOMATO PRODUCTION OF KHYBER PAKHTUNKHWA-PAKISTAN

Ijaz Ahmad, Musharaf Ahmad

Department of Plant Pathology, The University of Agriculture, Peshawar, Pakistan.

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ABSTRACT

The bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) has devastated commercial tomato output in Pakistan's especially in Khyber Pakhtunkhwa (KP) province. Field surveys were conducted in 2017 and 2018 to analyze the effects of the bacterial spot in tomato producing areas of KP, including Swat, Lower Dir, and Mansehra. During the growing seasons, diseased samples were collected from tomato fields, and disease incidence and severity were documented. The pathogen was isolated, purified, and morphologically and biochemically characterized. Most of the morphological and biochemical characteristics such as yellow colonies were like those of *Xanthomonas* spp. Further, the pathogen was confirmed using XV1F/XV1R pair of species-specific primers, which amplify the *atpD* gene sequence solely in *Xanthomonas vesicatoria*. Out of 30 isolates, 16 isolates were confirmed to be *Xcv*. In these agro-ecological zones, this was the first report on the presence of *Xcv*. Furthermore, humidity and temperature were found to impact the incidence and severity of bacterial spot substantially. Most isolates grew at high temperatures and low humidity; however, some preferred high-temperature and low-humidity environments. In conclusion, bacterial spot disease caused by *Xcv* has now spread throughout all tomato-producing areas of KP province. To avert future plant epidemics, ongoing climate and pest monitoring is required to build an effective disease warning system for producers and other agriculture-related agencies.

Corresponding Author: Ijaz Ahmad

Email: ijazahmad155@aup.edu.pk

INTRODUCTION

Tomato (*Lycopersicon esculentum* L.) is one of the most common and economically important vegetables worldwide. China, India, USA, and Turkey are global leaders contributing 70% of global tomato production, whereas Pakistan ranks 36th in the production list and 52nd in the export list (FAOSTAT, 2021). Pakistan contributes 1.3% to the total world area for tomato growing, 0.33% to the world tomato production, and 0.06% to the world tomato export. In Khyber Pakhtunkhwa (KP) province, tomato is grown over 13.4 thousand hectares with 0.128 million tons production (Fatah *et al.*, 2020). It is a high-earning crop for small-scale farmers in KP especially in frost-free zones of Malakand division, where off-season winter tomatoes are grown. However, continuous mono-cropping has led to various plant diseases that limit tomato production, resulting in huge losses in the field, transit, and storage (Fatah *et al.*, 2020).

Bacterial leaf spot, caused by *Xanthomonas vesicatoria*, is a severe disease and is a constant hazard to the commercial production of tomatoes worldwide (Sundin *et al.*, 2016; Rodriguez-R *et al.*, 2012; Ryan *et al.*, 2011; Leyns *et al.*, 1984). The pathogen is a gram-negative, rod-shaped bacterium, motile with a single polar flagellum, strictly aerobic, and 0.7 to 1.0 µm by 2.0-2.4

µm in diameter. Other characteristics include producing a yellow, water-insoluble pigment, xanthomonadin and an extracellular polysaccharide (EPS) named xanthan (Dai *et al.*, 2019; Yang *et al.*, 2005). Because of its rapid spread and destructive nature, the European Plant Protection Organization (EPPO) designated the pathogen as an important quarantine organism. The pathogen is retained in infested tomato seed for an extended period (Kizheva *et al.*, 2013).

Pathogen-infested seed is one of the major factors in long-distance movement into pathogen-free zones. Infested seed is the primary source of inoculum in the field. The secondary infection occurs through rain splashes, the inoculum is carried through wind and aerosols, and infection occurs through wounds caused during transplantation and weeding (Stall et al., 2012; Zhao et al., 2002; Barak et al., 2001). Hot and humid conditions and dense cropping patterns favor disease development (Velásquez et al., 2018). In tropical and sub-tropical climates, pathogen survives in crop residue for a few months (Stall et al., 2012). However, after decomposition of crop residue, the survival chances of the pathogen decline to a couple of days. On the other hand, alternative hosts, bridging crops and volunteer plants can play a vital role in the survival of bacteria (Workayehu et al., 2021; Gangwar et al., 2018).

The pathogen enters tomato plants through small wounds or stomata and produces small, water-soaked lesions. These lesions become necrotic later on (Morales *et al.*, 2005). The disease damages tomato leaves, stems, flower parts, and fruits. Small, irregular, black and greasy lesions appear as initial leaf symptoms. The loss in the green leaf area hinders photosynthesis. Leaves with many lesions may turn yellow and fall off. The greasy lesions on flowers cause serious blossom drops. Small, water-soaked lesions appear on green fruits, rapidly enlarging in diameter. Later, these spots become dark brown and slightly sunken, with a scabby surface (Agrios, 2005).

Besides, pathovars of the pathogen have been declared as one the most important diseases of tomato, with losses reaching 10% to 60% (Rashid *et al.*, 2016; Mitrev *et al.*, 2013; Mitrev, 2001) (Burlakoti *et al.*, 2018; Stall *et al.*, 2009; Jones *et al.*, 1998). Furthermore, direct fruit yield losses were recorded up to 23-44% when severe leaf infection occurred at an early stage of plant growth (Singh *et al.*, 2017). The qualitative loss due to blemishes and spots on fresh-market tomato skin, especially when the crop is grown under warmer and humid conditions, has also caused more losses to small-scale local growers (Wen *et al.*, 2009). As much as 95% of tomato fruit loses its commercial market value (Emana *et al.*, 2017; Bashan *et al.*, 1985).

In Pakistan, the quality of tomato seed tomato producing areas of Khyber Pakhtunkhwa (KP), province has remained questionable for decades. Besides poverty and lack of access to agriculture credit, most small-scale tomato-growers still use uncertified tomato seeds, severely aggravating the disease. However, no report on bacterial spots caused by *X. vesicatoria* is available in commercial tomato-producing areas of KP, Pakistan. Hence, the present studies were taken up with the following objectives: (1) Field surveys in major tomato growing areas of KP, Pakistan for bacterial spot disease incidence and severity; (2) Morphological, biochemical and molecular characterization of isolates of *X. vesicatoria* associated with bacterial spot.

MATERIALS AND METHODS

Survey for Assessment of Bacterial spot

Prompted by the observations of bacterial spot-like symptoms on tomato fruits being sold in different local vegetable markets and subsequent disease complaints coming from commercial tomato-growers of different areas of the province, we conducted extensive surveys of the tomato-growing districts of KP, Pakistan, to assess the situation. The surveys were conducted in tomato growing seasons of 2017 and 2018, and disease incidence and severity were recorded, and diseased tomato plants showing bacterial spot-like symptoms were collected. Chitral, Upper Dir, Lower Dir, Swat, Malakand, Shangla, Bunir, Mardan, Swabi, Charsadda, Nowshera, Peshawar, Haripur, Abbot Abad and Mansehra districts were surveyed. During surveys, ten fields per district were visited. Data were recorded in five spots (each spot being a 10-feet long row of tomato plants) per field. The total number of plants per spot and number of plants showing disease symptoms were counted (Figure 1). Disease incidence was calculated as the number of plants with disease symptoms divided by total number of plants per spot multiplied by 100. The values of all spots per field were averaged together to get disease incidence per field. Diseased plant specimens were collected to isolate the causal organisms using the two-stage clustered sampling

technique described previously (Nafiu, 2012). Samples were put in paper bags, kept cool and *Xanthomonas* sp. were isolated on media plates in Plant Pathology Lab, The University of Agriculture Peshawar.

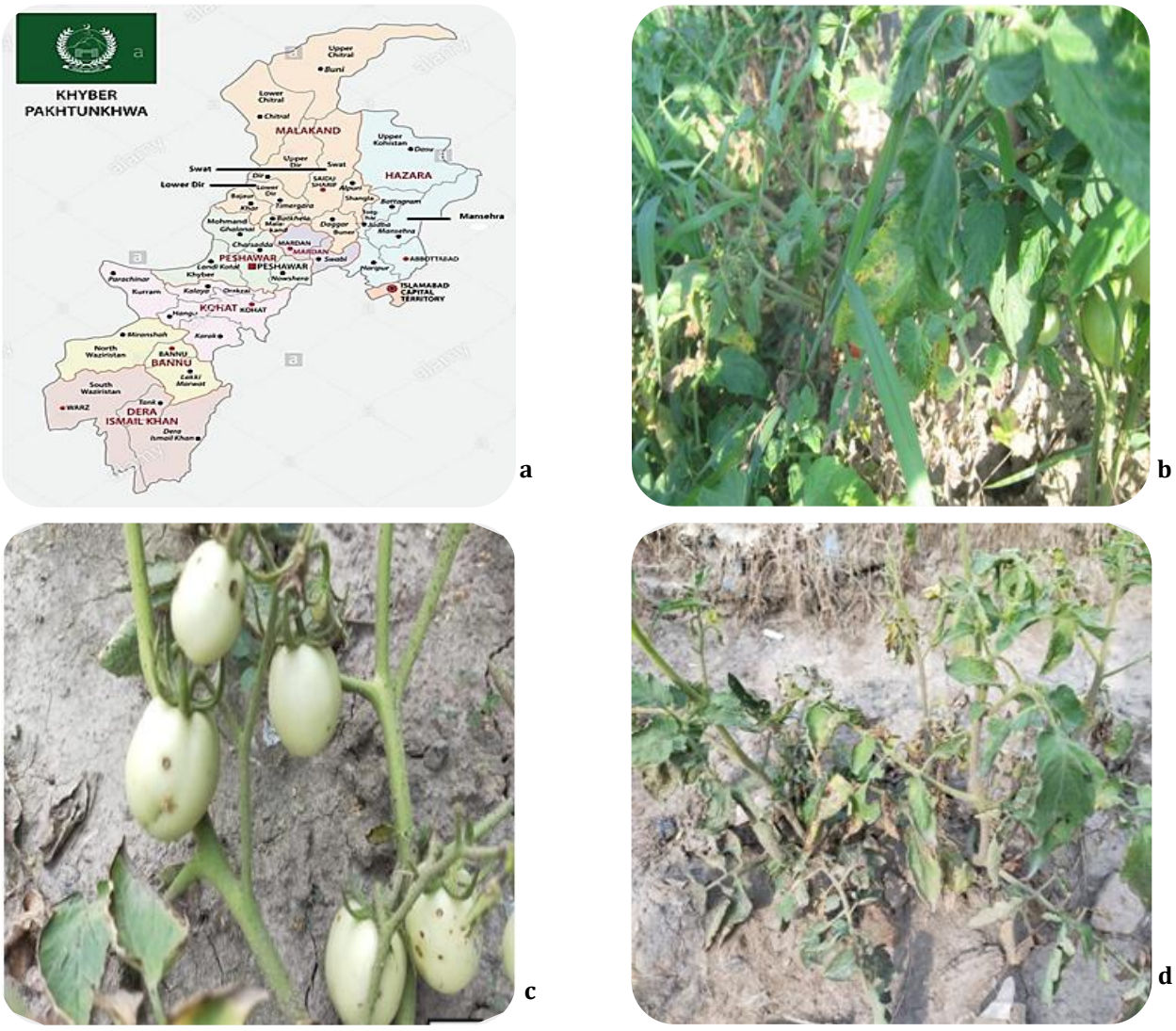


Figure 1. Survey for tomato spot disease in the targeted districts of Khyber Pakhtunkhwa (KP). a) Map of KP with specified district boundaries and targeted districts namely; Swat, Lower Dir and Mansehra. Plants and tomato fruit showing typical symptoms of tomato spot diseases in the fields b) Mansehra, c) Swat and d) Lower Dir.

Assessment of Disease Incidence and Disease severity in tomato fields

Data on disease severity were recorded using the four categories (1-4) disease rating scale (Vallejos *et al.*, 2010): where 1 = symptomless; 2 = slight to moderate yellowing and slight necrosis; 3 = extensive yellowing and moderate necrosis; and 4 = complete necrosis (plant dead). Disease severity index (DS %) values were calculated for each replicate as per Abdel-Monaaim *et al.* (2011):

$$DS\% = \frac{\Sigma n}{4N} \times 100$$

∑n = sum of the ratings of all plants, i.e., ∑ (1A+2B+3C+4D) where A, B, C, and D are the number of plants of categories 1, 2, 3, and 4, respectively; N = the total number of plants used for disease rating, and 4 = biggest category of the disease rating scale.

Isolation and purification of *X. vesicatoria* from diseased samples

The bacteria were isolated from infected tissue by streaking onto nutrient agar (NA), yeast dextrose calcium carbonate (YDC) agar (Potnis *et al.*, 2015a), sucrose peptone agar (SPA) amended with 50 ppm streptomycin sulfate (Louws *et al.*, 2001). Well-isolated colonies were re-streaked to purify the pathogen.

Characterization of *X. vesicatoria* isolates

Colony morphology

Colony morphology of *X. vesicatoria* on NA, SPA and YDC media were studied for color, shape, growth rate, texture and margins to make initial inference about the identity of the pathogen.

Biochemical Characterization

Different biochemical tests done included: Gram staining, KOH solubility, catalase test, growth at 36 ℃, 2% NaCl tolerance, mucoid growth on SPA medium etc. Standard protocols for all these tests were followed as described in the previous study (Louws *et al.*, 2001).

Hypersensitive response (HR)

Isolates were grown overnight on YDA and LB medium at 28℃. The colonies were harvested using 0.85% normal saline solution, and suspensions were adjusted to 10^8 CFU/ ml. HR was observed on tobacco plants (*Nicotiana benthamiana*) (Li *et al.*, 2015a; Bocsanczy *et al.*, 2012) and infiltrated on the abaxial side of the nonhost plants with the help of a hypodermic syringe. After inoculation, infiltrated zones were observed for tissue collapse and necrotic symptoms within 48 h.

Molecular Characterization of *X. vesicatoria* isolates

Extraction of genomic DNA from *X. vesicatoria* isolates

The bacterial isolates were cultured on LB agar (Difco, Detroit, MI, USA) and incubated at 28°C for 48 h. To get pure culture, yellow colonies of *Xanthomonas* were sub-cultured until pure colonies were obtained. Genomic DNA was extracted from bacterial cells using CTAB method (Jaufeerally-Fakim and Dookun, 2000). The quality and quantity of extracted DNA were confirmed by agarose gel (1%) electrophoresis and NanoDrop™ (ThermoScienific, USA), respectively.

Specie specific PCR for confirmation of *X. vesicatoria* isolates

The identity of the *X. vesicatoria* isolates (identified via colony morphology, pathogenicity and biochemical tests) was confirmed by species-specific PCR. Standard protocols were used to isolate template DNA, run PCR, doing gel electrophoresis and gel staining. The primers Xv1F (5-CAGTCCTCCAGCACCGAAC-3) and Xv1R (5-TCTCGTCGCGGAAGTACTCA-3), designed by the alignment of *atpD* sequences (Beran and Mráz, 2013), were used. Standard PCR conditions (30 cycles, each consisting of 45 s at 94 ℃, 45 s at 58 ℃, and 40 s at 72 ℃, with initial 5 min denaturation at 94 ℃ and final extension of 10 min at 72 ℃) were not discriminatory enough; so, we had to optimize the PCR conditions (30 cycles, each consisting of 45 s at 94 ℃, 45 s at 60.5 ℃, and 40 s at 72 ℃, with initial 5 min denaturation at 94 ℃ and final extension of 10 min at 72 ℃). After optimization of PCR conditions, however, only *X. vesicatoria* samples produced the expected band of 365 bp.

RESULTS

Seasonal surveys (spring and fall) were conducted at the crop maturity stage in major tomato growing areas during 2017 to understand the incidence and severity of the newly emerging diseases in KP. There were black spots on leaves, fruits, and stems, and `symptoms on leaves surrounded by yellow halos were observed. Based on these characteristic symptom manifestations, the bacterial spot was identified as the newly emerging disease. The disease significantly affected tomato crops of district Swat, Lower Dir and Mansehra of KP during 2017 and 2018. These districts are major producers of commercial tomato and supply tomatoes to all markets of Pakistan till mid-winter. However, the emergence of bacterial leaf spots affected the quantity of the produce and negatively impacted the socioeconomic status of small-scale farmers in these districts. No plantation of tomato was noticed from September to October in the rest of the districts in KP (Data not shown). The remaining districts, i.e., Malakand, Charsadda, Chitral, Peshawar, Mardan, Swabi, Bunir, Nowshera and Haripur, supply fresh tomatoes from February to August and were free from the bacterial spot. Among the commercial tomato growing districts, Swat, Lower Dir, and Mansehra were surveyed for two consecutive years (2017 and 2018) during July, August and September.

Disease incidence

In 2017, maximum percent disease incidence (%DI) was calculated for district Swat (64.42%) at the end of August followed by district Mansehra. District Lower Dir ranked lowest in terms of %DI (46.42%) among the affected districts (Figure 2 A). In 2018, maximum %DI was calculated for district Mansehra (65.78%) at the end of August followed by district Lower Dir (%DI= 53.28%). District Swat accounts for the lowest %DI (43.78%) during 2018 (Figure 2 B).

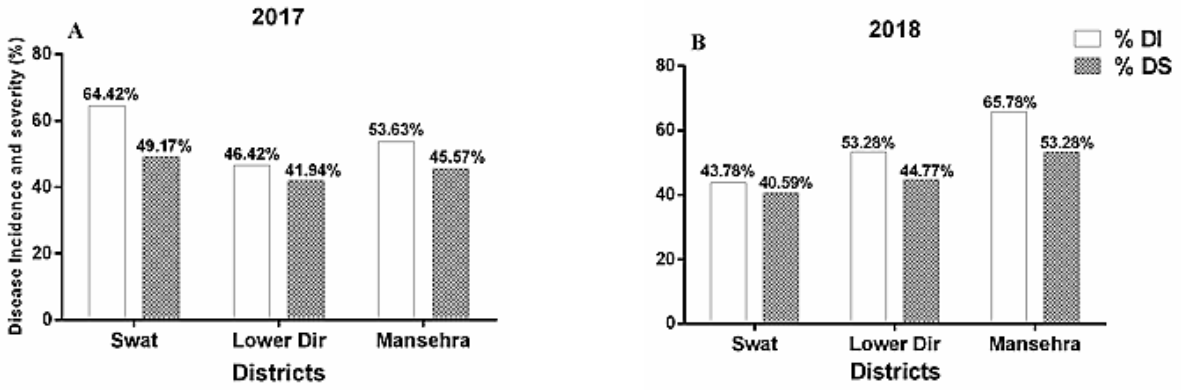


Figure 2. Mean disease incidence (%) and disease severity (%) of districts Swat, Lower Dir and Mansehra. A) 2017 and B) 2018.

In district Swat, BS infestation was 100% in the fields surveyed with varying degrees of %DI during 2017 and 2018 (Figure 3). In fields surveyed during 2017 (Figure 3A and 3B), the maximum mean %DI (72.13%) was observed in Ghalegay followed by Guljaba (70.45%). Comparatively, lower mean for %DI (57.24%) was noted in the fields surveyed at Tindo Dag. Surprisingly, %DI during 2018 was much lower than previously observed (2017). The report documented in 2018 revealed that maximum mean %DI (49.004%) was observed in Manyar, followed by %DI of 46.56% (Takhta Band) (Figure 3A and 3B). Comparatively, a minimum mean for %DI was observed in Kabal (38.73%). Individual field inspection data is also enlisted in S-1. Fluctuation of BS during 2017 and 2018 in terms of %DI and %DS were monitored in district Lower Dir (Figure 4). After calculating the means for 2017 (Figure 4A and 4B), Chakdara ranked on top in terms of mean %DI (58.88%), followed by Dherai Talash in terms of mean %DI (57.14%). Minimum mean %DI was noticed in the fields of Tekni Bala (48.00%) during 2017. In the following year (2018), maximum %DI (58.52%) was observed in Chakdara followed by Inzir Dara, %DI (56.35%) (Figure 4A and 4B). Comparatively, lower means %DI (48.19%) was observed in Tekni Bala. Individual field inspection data are illustrated in S-2. BS infestations with different magnitudes were observed for %DI in district Mansehra during 2017 and 2018 (Figure 5). Among the surveyed locations (2017) (Figure 5A and 5B), maximum mean %DI (73.26%) was noted in the fields of Bagriyan-Dilbori followed by fields at Baffa (%DI=70.11%). While the minimum mean %DI spotted for Khaki were 57.14%. Second survey was conducted in the following year (2018) to observe the disease. Maximum mean %DI (73%) was recorded in Bagriyan-Dilbori, which was followed by Baffa (%DI= 71.21%). Upon comparison, the lowest mean %DI (59.84%) was observed in Khaki (S-3) (Figure 5A and 5B).

Disease severity

In 2017, maximum percent disease severity (%DS) was calculated for district Swat at the end of August (49.17%) followed by district Mansehra (%DS=45.57%). District Lower Dir ranked lowest in terms of %DS (41.94%) among the affected districts (Figure 2 A). In 2018, maximum %DS was calculated for district Mansehra at the end of August (53.28%) followed by district Lower Dir (44.77%). District Swat accounts for the lowest %DS (40.59%) among the affected districts during 2018 (Figure 2 B). In district Swat, the maximum mean %DS (52.40%) was observed in Ghalegay followed by Guljaba (51.98%). Comparatively, lower mean for %DS (46.29%) was noted in the fields surveyed at Tindo Dag. Surprisingly, %DS during 2018 was much lower than previously observed (2017). The report documented in 2018 revealed that maximum mean %DS (42.97%) was observed in Manyar, followed by Kanjo 41.61% (Figure 3A and 3B). Comparatively, a minimum mean for %DS was observed in Kabal (37.5%). Individual field inspection data is also enlisted in S-1. Fluctuation of BS during 2017 and 2018 in terms of %DS were monitored in district Lower Dir (Figure 4). After calculating the means for 2017 (Figure 4A and 4B), Chakdara ranked on top in terms of mean %DS (46.96%), followed by Talash (46.57%). Minimum mean %DS was calculated for the fields in Khan Pur (41.99%). In the following year (2018) same pattern was repeated

as previous one, %DS (46.86%) was observed in Chakdara followed by Talash (46.52%) (Figure 4A and 4B). Comparatively, lower means %DS (41.93%) was observed in Khan Pur. Individual field inspection data are illustrated in S-2. BS infestations with different magnitudes were observed for %DS in district Mansehra during 2017 and 2018 (Figure 5). Among the surveyed locations (2017) (Figure 5A and 5B), maximum mean %DS (56.98%) was noted in the fields of Bagriyan-Dilbori followed by fields at Shinkiyari Tando (%DS= 56.40%), while the minimum mean %DS spotted for Khaki was 47.62%. Second survey was conducted in the following year (2018) to observe the disease. Maximum mean %DS (56.7%) were recorded in Bagriyan-Dilbori, which was followed by Sher Garh (56.17%). Upon comparison, the lowest mean %DS (48.9%) was observed in Khaki (S-3) (Figure 5A and 5B).

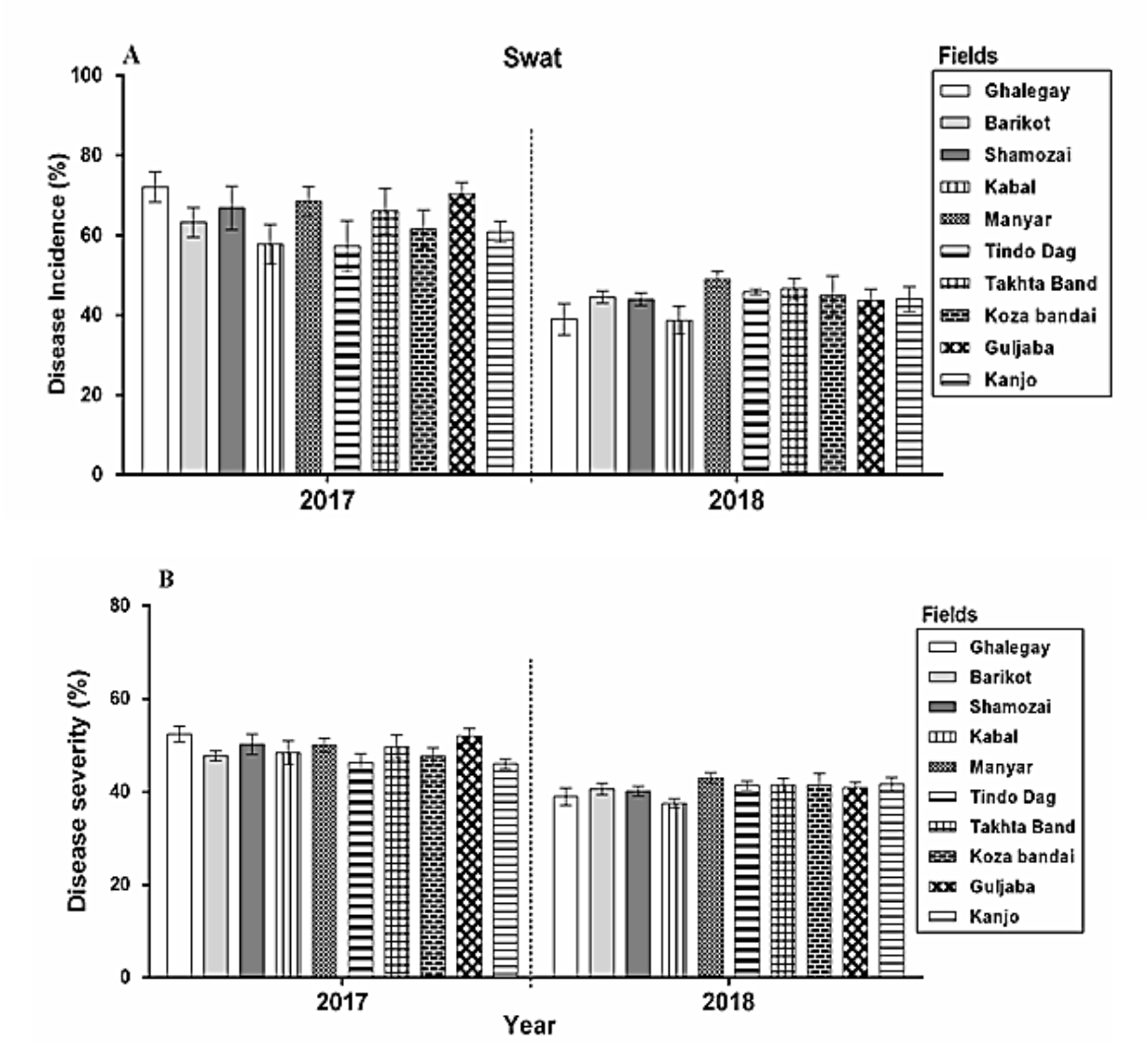


Figure 3 Mean disease incidence (%) and disease severity (%) of bacterial spot at different locations of District Swat during 2017-18. A) Disease Incidence (%) and B) Disease Severity (%) recorded in the fields. Each location indicates mean data of five fields surveyed.

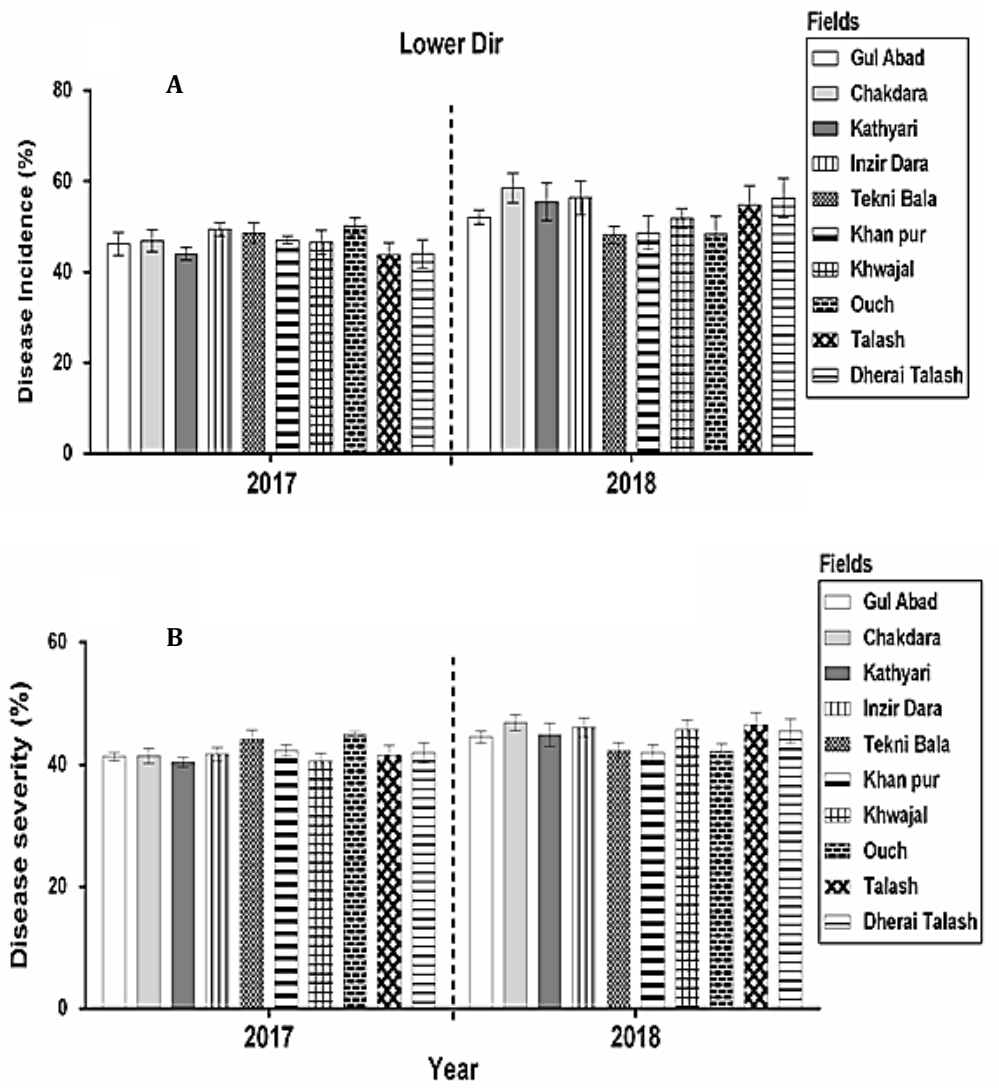
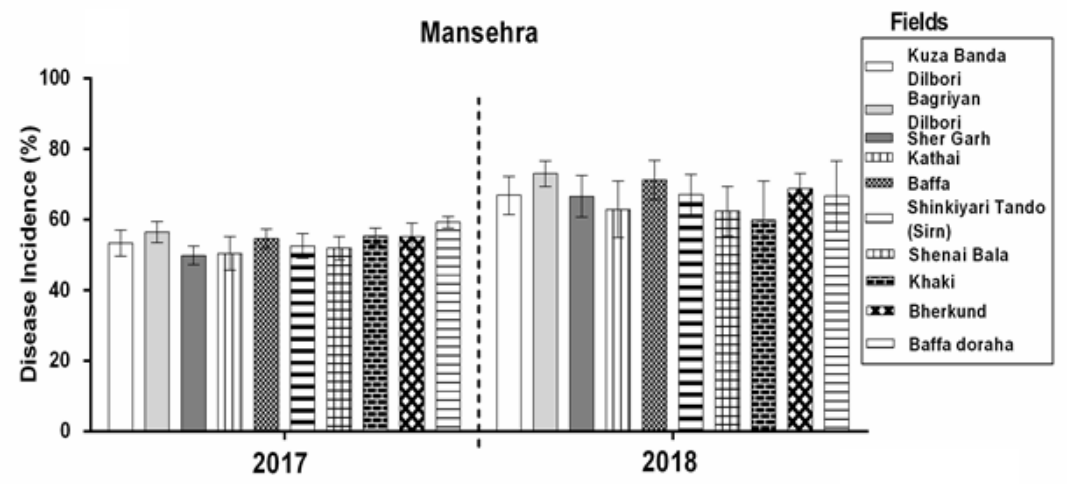


Figure 4. Mean disease incidence (%) and disease severity (%) of bacterial spot at different locations of District Lower Dir during 2017-18. A) Disease incidence (%) and B) Disease severity (%) recorded in the fields. Each location indicates mean data of five fields surveyed.



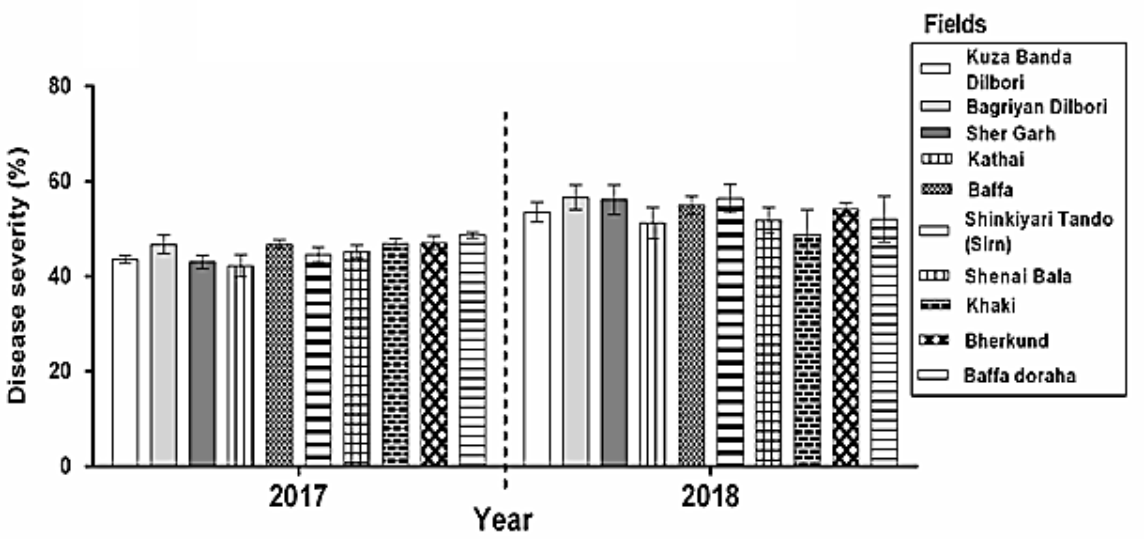


Figure 5. Mean disease incidence (%) and disease severity (%) of bacterial spot at different locations of District Mansehra during 2017-18. A) Disease incidence (%) and B) Disease severity (%) recorded in the fields. Each location indicates mean data of five fields surveyed.

Identification of the *Xanthomonas vesicatoria*

Biochemical Identification

Isolation of bacterium

Inoculated Nutrient agar media (NA) plates were checked for the presence of the bacterium**.** Colony characteristics, i.e., yellow-colored, shiny, dome-shaped colonies, were tentatively designated as *X. vesicatoria* (Figure 6a). A few of these colonies (for each isolate) were re-streaked on NA plates for culture purification purposes. Several dark yellow and flattened colonies were also observed; however, they were not characterized due to limitations of time and resources. Colony characteristics for isolates are mentioned in Table 1.

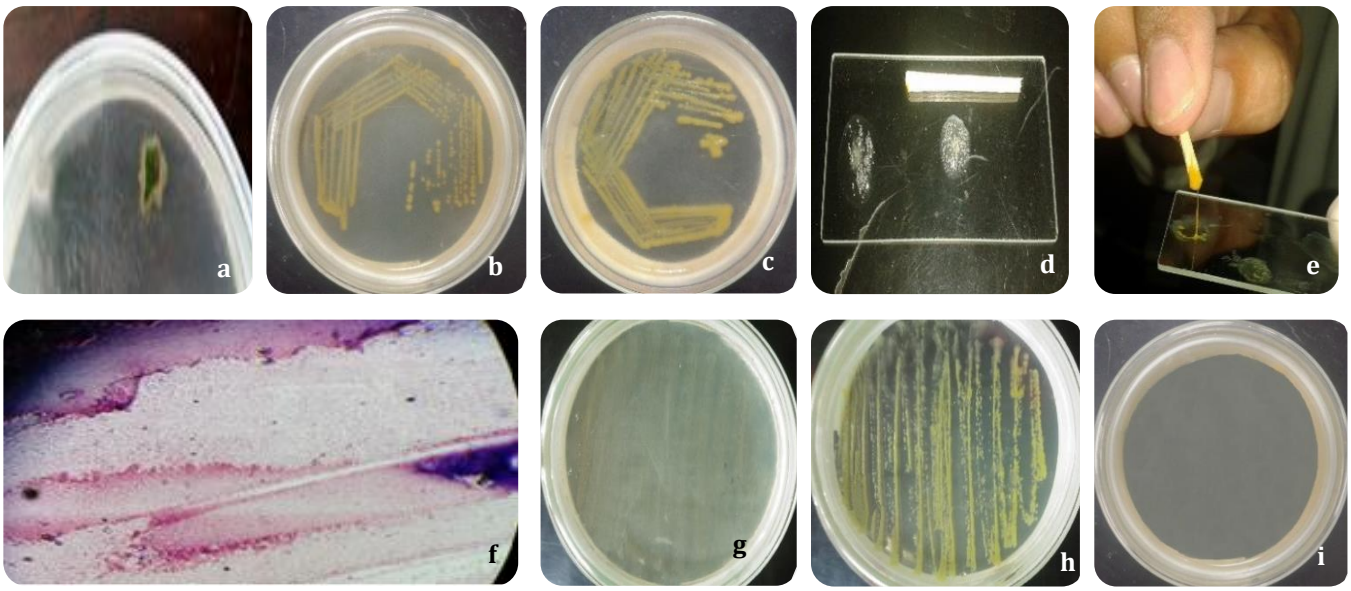


Figure 6. Morphological and biochemical characterization of *X. vesicatoria* isolates collected from diseased tomato crops grown during 2017 and 2018. a) Isolation of X. vesicatoria from infected tissues of tomato, b) Growth on YDC media, c) Growth on SPA media, d) KOH test, formation of threads indicating G-ive bacterium, e) Catalase test, formation of bubbles, f) Gram staining, red color, g) Growth on 1% NaCl amended NA media, h) Growth on 0.5% amended NA media and i) Growth at 36 ℃.

Table 1. Preliminary characterization of *Xanthomonas vesicatoria* isolates.

Districts	Isolates	Locations	Gram staining	3% KOH Test	Colony morphology	Growth at 36 °C	PCR	YDC	SPA	Growth on NaCl amended NA media			
										0.5%	1%	2%	3%
Swat	S1	Tindo Dag 34°43'52.9"N 72°16'43.9"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	S2	Barikot 34°41'18.9"N 72°12'32.7"E	- ive	Yes	Dark Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	S3	Kanjo 34°48'16.5"N 72°20'00.8"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	S4	Shamozai 34°40'55.5"N 72°09'08.1"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	S5	Ghalegay 34°42'04.1"N 72°15'16.3"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	S6	Manyar 34°43'01.2"N 72°16'17.2"E	- ive	Yes	Dark Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	S7	Koza Bandai 34°49'16.7"N 72°22'35.5"E	- ive	Yes	Yellow	No	+ ive	No	Yes	Yes	No	No	No
	S8	Guljaba 34°47'39.8"N 72°17'51.0"E	- ive	Yes	Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	S9	Kabal 34°46'20.5"N 72°15'54.4"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	S10	Takhta Band 34°46'53.7"N 72°19'31.8"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
Lower Dir	D1	Gul Abad 34°42'02.7"N 72°02'07.9"E	- ive	Yes	Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	D2	Kathyari 34°45'06.6"N 72°04'10.0"E	- ive	Yes	Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	D3	Chakdara 34°40'39.0"N 72°02'55.4"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	D4	Tekni Bala 34°47'23.8"N 72°04'16.8"E	- ive	Yes	Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	D5	Khwajal 34°45'58.6"N 72°01'35.3"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No

Mansehra	D6	Khan pur 34°46'24.6"N 72°04'47.2"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	D7	Talash 34°43'36.8"N 71°52'15.3"E	- ive	Yes	Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	D8	Inzir Dara 34°48'04.9"N 72°04'02.5"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	D9	Dherai Talash 34°46'10.1"N 71°51'13.5"E	- ive	Yes	Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	D10	Ouch 34°44'07.6"N 72°01'29.9"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	M1	Kuza Banda Dilbori 34°33'15.6"N 73°00'26.4"E	- ive	Yes	Dark Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	M2	Kathai 34°31'10.6"N 73°05'04.2"E	- ive	Yes	Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	M3	Sher Garh 34°26'51.9"N 72°59'20.6"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	M4	Bagriyan Dilbori 34°33'16.7"N 73°00'01.2"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	M5	Shinkiyari Tando 34°28'32.2"N 73°16'02.5"E	- ive	Yes	Dark Yellow Round	No	- ive	No	Yes	Yes	No	No	No
	M6	Baffa 34°26'03.2"N 73°13'26.4"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	M7	Shenai Bala 34°26'32.1"N 73°15'41.0"E	- ive	Yes	Brownish Round	No	- ive	No	Yes	Yes	No	No	No
	M8	Khaki 34°24'23.8"N 73°08'13.2"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	M9	Bherkund 34°23'34.6"N 73°08'59.8"E	- ive	Yes	Yellow Round	No	+ ive	Yes	Yes	Yes	No	No	No
	M10	Baffa doraha 34°25'10.0"N 73°13'43.9"E	- ive	Yes	Brownish Round	No	- ive	No	Yes	Yes	No	No	No

KOH and catalase test

The test indicated that the candidate isolates were Gram-negative bacteria as they produced thread-like structures when stirred on a glass slide and then picked with a toothpick (Table 1) (Figure 6d). Meanwhile, a catalase test was also performed, and the isolates produced bubbles, which demonstrated catalase enzymes produced by bacterium breakdown $2H_2O_2$ in to O_2 and H_2O molecules (Figure 6e).

Gram staining test

To validate observations made in KOH test, gram staining of the selected bacterial colonies was carried out and observed under the light microscope at 100X using oil immersion. Gram staining confirmed the results of KOH test. The isolates were rod-shaped and pinkish when stained with counter strain safranin and observed under the microscope (Table 1).

Influence of temperature on the growth of *Xanthomonas* isolates

The inoculated plates of the bacterium were exposed to thermal stress at 36 ℃. No growth was observed at 36 ℃ for any isolate. Touching the surface of the 36 ℃-exposed inoculated plates with a sterilized loop and streaking on NA plates and incubating at normal temperature also did not yield bacterial colonies (Figure 6i).

Influence of growth media on the growth of *Xanthomonas* isolates

The isolates were grown on Yeast-Extract-Calcium Carbonate (YDC) agar medium. Most of these isolates produced characteristics like yellow, mucoid, and doom-shaped colonies with entire margins (Figure 6c). In contrast, few isolates (such as S2, S6 S7, S8, D1, D2, D4, D7, D9, M1, M2, M5, M7and M10) didn't show any of these characters on the YDC medium (Table 1). NA-grown isolates were also sub-cultured on Sucrose Peptone Agar (SPA) medium, a semi-sensitive medium for *Xanthomonas* species. All isolates showed yellow pigmentation and stickiness in SPA inoculated plates, indicating the *Xanthomonas* species (Figure 6b). These isolates were further validated by morphological and biochemical tests (Table 1).

Influence of salts on the growth of *Xanthomonas* isolates

Xanthomonades mostly prefer saline media for the growth, and therefore, the growth was assessed at different NaCl concentrations in the media. Sensitivity of the isolates on NaCl amended NA media with different concentrations, i.e., 0.5 (Figure 6h), 1 (Figure 6g), 2 and 3%, revealed that the isolates could successfully grow on media containing 0.5% salt. The increase in salt concentration (above 0.5% NaCl) and the Xanthomonades showed higher sensitivity and restricted growth (Table 1).

Correlation of disease incidence with rainfall and temperature

Environmental factors such as temperature and rainfall (humidity) play an important role in establishing and spreading bacterial spots in tomato plants. Sometimes these factors highly favor the disease and help it to prevail in every field where the host is available. A similar impact was observed in district Lower Dir, Swat, and Mansehra during surveys (2017-2018). During 2017, the disease was highly influenced (83.46%) by August rainfall and 65.00% by September rainfall, while a weak (6.4%) correlation was observed between rainfall and disease incidence in July. Regarding high temperature and disease incidence, the disease is negatively correlated with the high temperatures in July, August and September (-66.96%, -65.22% and -75.65%, respectively). The r-square value shows a positive correlation between disease and rainfall (69.66%) in August while 42.25% with September rainfall (Figure 7A). Similarly, in 2018, a positive correlation was observed between rainfall and diseases incidence, i.e., September (96.28%), July (95.72%) and August (95.15%). Like the previous year, a negative correlation was observed between high temperate and diseases incidence during July, August and September (-23.26%, -49.25% and -7.85%, respectively). Here too, the r-square value shows that BS disease is strongly correlated with rainfall; September (92.7%), July (91.62%) and August (90.54%) (Figure 7B).

Correlation of disease severity with rainfall and temperature

The disease severity is also influenced by environmental factors. Based on a correlation study, rainfall of August (68.42%) and September (45.61%) contributed to disease severity. Based on July rainfall (16.9%), contribution to disease severity was recorded minimum during 2017. Temperature also plays an important role in establishing the disease; at optimum temperature, the disease severity remains highest. The maximum temperature observed in these months negatively correlates with the disease severity. The r-square value shows the impact of disease over rainfall which was 46.81% by August rainfall while 34.14% by

September rainfall (Figure 8A). In 2018, the disease severity was highly influenced (98.76%) by September rainfall, 98.42% by July rainfall, 98.07% by August rainfall and a positive correlation was observed between rainfall during these months and disease severity. In terms of maximum temperature and disease severity, the disease is negatively correlated with the maximum temperatures in July, August and September (-34.35%, -58.98% and -19.33%, respectively). The disease severity mostly depends on rainfall but is slightly enhanced by optimum temperature. The r-square value disease severity and September rainfall is 97.54%, July rainfall 96.87% and August rainfall 96.18% (Figure 8B).

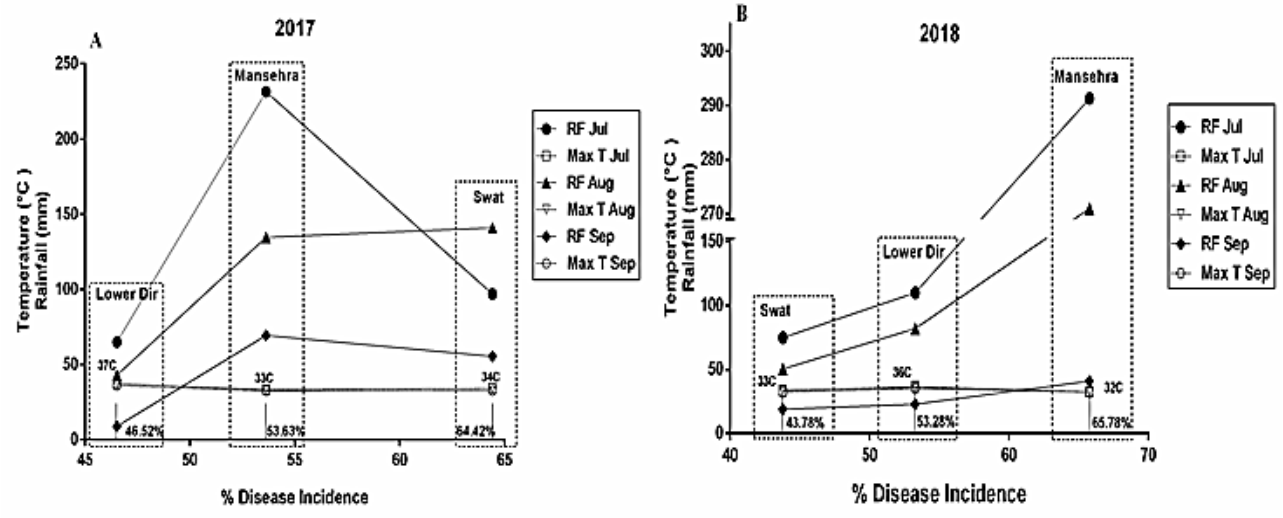


Figure 7. Correlation of disease incidence (%) with rainfall (RF) and maximum temperature (Max T) in district Lower Dir, Swat and Mansehra, in the months of (Jul) July, (Aug) August and (Sep) September A) 2017 and B) 2018.

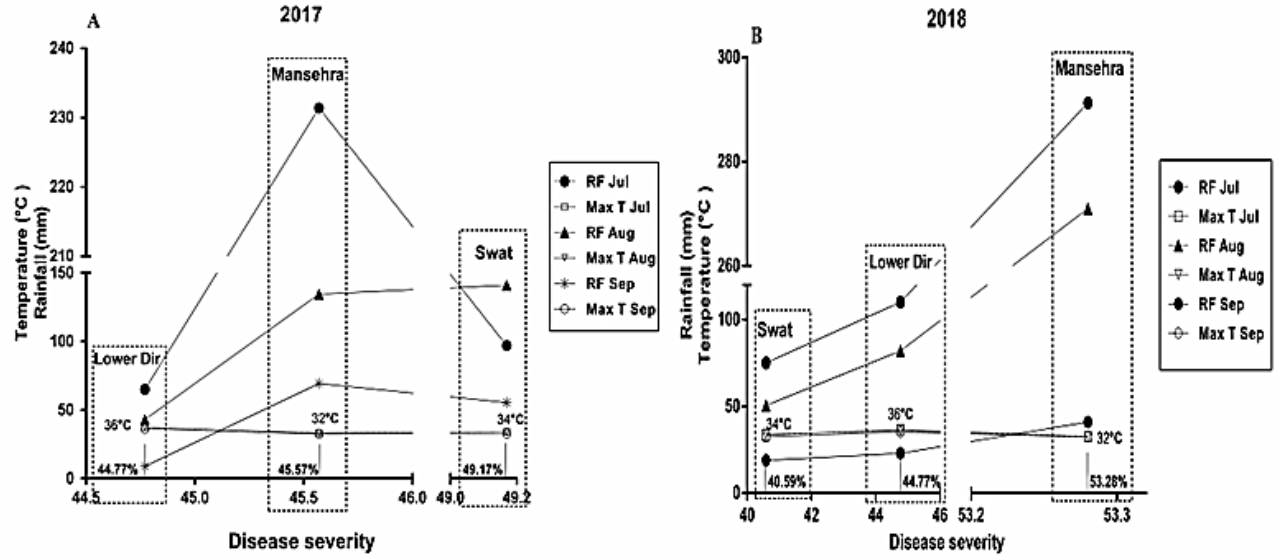


Figure 8. Correlation of disease severity (%) with rainfall (RF) and maximum temperature (Max T) in district Lower Dir, Swat and Mansehra, in the months of (Jul) July, (Aug) August and (Sep) September A) 2017 and B) 2018.

Molecular Identification of *X. vesicatoria*

All the isolates were tested using conventional biochemical tests as the initial requirements to narrow down the list to isolates of interest. Initially two specific

primers were selected for detection of *X. vesicatoria* namely; XCVF (5′-AGAAGCAGTCCTTGAAGGCA-3′) and XCVR (5′-AATGACCTCGCCAGTTGAGT-3′) (517bp) (Park *et al.*, 2009) and XV1F (5′-CAGTCCTCCAGCACCGAAC-3′) and XV1R (5-TCTCGTCGCGGAAGTACTCA-3) (365bp) (Beran and Mráz, 2013) (Figure 9). Both primers amplified band from pool sample of *X. vesicatoria*. Most clear bands were obtained using XV1F/XV1R primer, yielding 365 bp product size. The selected isolates were further confirmed using XV1F/XV1R specific primer based on their targets in *X. vesicatoria* genome (Table 1). The primer pair XV1F/ XV1R is an amplified region that lies on the *atpD* gene of *X. vesicatoria* with a product size of 365bp (Beran and Mráz, 2013). The primers amplified band of 365bp from six samples of district Swat, i.e., Tindo Dag (S1), Kanjo (S3), Shamozai (S4), Ghalagay (S5), Kabal (S9) and Takhtaband (S10). Meanwhile, samples from Barikot (S2), Manyar (S6), Koza Banda (S7) and Guljaba (S8) failed to produce the target region (i.e., 365bp band). Similarly, the DNA isolated from samples of district Dir, the same primer amplified a band of 365bp for Chakdara (D3), Khwajal (D5), Khanpur (D6), Inzar Dara (D8) and Ouch (D10). However, samples collected from Gul Abad (D1), Kathyari (D2), Tekni Bala (D4), Talash (D7) and Dherai Talash (D9), were found negative for *X. vesicatoria.* Likewise, among the samples from Mansehera samples from Sher Garh (M3), Bagriyan (M4), Baffa (M6), Khaki (M8), and Bharkund (M9) tested positive for *X. vesicatoria*. In contrast, the remaining samples did not amplify the target region (Figure 10).

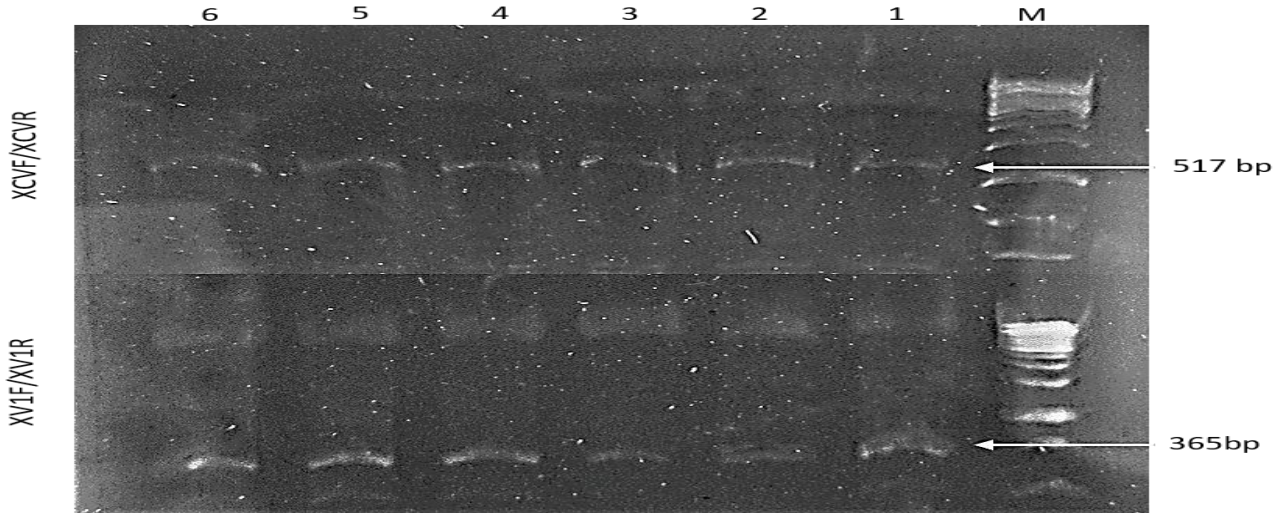


Figure 9. Specific PCR amplification of the *atpD* gene from *X. campestris* pv. *vesicatoria* with specific primers XCVF and XCVR and XV1F and XVIR . Lane M, Size marker (1Kb DNA plus ladder; Gibco BRLTM); lanes 1–6 corresponding to bands of *X. campestris* pv. *vesicatoria*

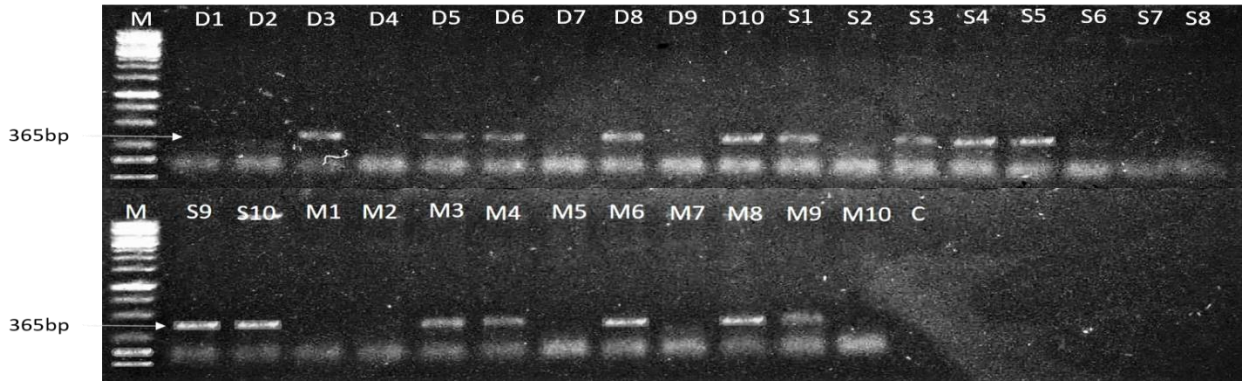


Figure 10. PCR-amplification of the *X. vesicatoria*-specific 365 bp DNA band. The primer pair Xv1F/ Xv1R, amplified 365bp band from six samples of Swat i.e. Tindo Dag (S1), Kanjo (S3), Shamozai (S4), Ghalagay (S5), Kabal (S9) and Takhtaband (S10); some samples of Lower Dir i.e., Chakdara (D3), Khwajal (D5), Khanpur (D6), Inzar Dara (D8) and Ouch (D10) and some samples of Mansehera i.e., Sher Garh (M3), Bagriyan (M4), Baffa (M6), Khaki (M8) and Bhakund (M9).

Hypersensitivity response test

The hypersensitivity response of *X. vesicatoria* was evaluated on potted, green-house-grown tobacco plants (*Nicotiana benthamiana*). Isolates collapsing the infiltrated area within 24 h were considered as HR-positive. (Figure 11).

DISCUSSION

Bacterial spot pathogen which has been extensively studied and enlisted among the quarantine pathogens, needs comprehensive management strategies (Costa *et al.*, 2021). In the summer of 2015-16, our observation of novel symptoms characterized by brownish to blackish spots with yellow halos on tomatoes in commercial vegetable markets and subsequent comprehensive farmer's fields surveys indicated that the presence of bacterial spot disease (previously unreported from Pakistan). This disease prevailed in almost all fields growing fresh market tomatoes in the temperate but frost-free zone of KP of North-Western Pakistan. Since tomato is one of the most popular cash crops grown in most of KP region and is one of the major sources of income for small-scale farmers. Therefore, any loss in quality and quantity due to biotic or abiotic factors, especially the newly emerging diseases, would be devastating for these farmers. Investigations (cultural, biochemical and molecular approaches) confirmed the new disease to be bacterial spot caused by *X. vesicatoria*. BS of tomato causes heavy yield losses and threatens the productivity of tomato around the world (Vallad *et al.*, 2013; Horvath *et al.*, 2012; Ma *et al.*, 2011). The pathogen is distributed globally (Potnis *et al.*, 2015b), and therefore, it leads to major outbreaks (Abrahamian *et al.*, 2019) under favorable climatic conditions. The presence and distribution of bacterial spot in the temperate zone and other major tomato growing areas are documented.

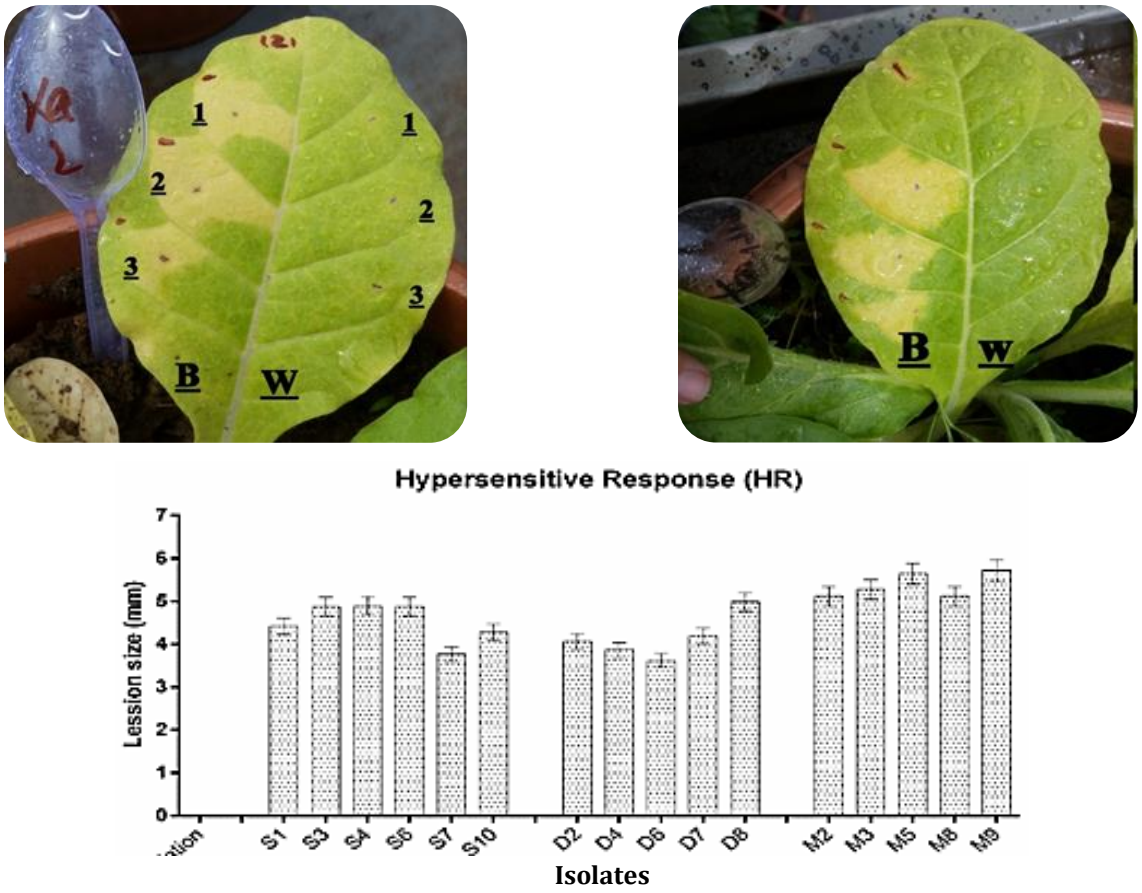


Figure 11 Hypersensitive response (HR) of X. vesicatoria isolates collected from district Swat (S1, S3, S4, S6, S7 and S10), Lower Dir (D2, D4, D6, D7 and D8) and Mansehra (M2, M3, M5, M8 and M9). Isolates collapsing the infiltrated area within 24 h were considered as HR-positive.

We found that during the autumns of 2017 and 2018, the disease was prevalent in tomato fields in the temperate high-altitude districts of KP, i.e., district Swat, Lower Dir, and Mansehra. These districts are major producers of commercial tomatoes and cater to fresh-market tomatoes all over Pakistan till mid-winter. The average temperature ranges from 18-28℃, while relative humidity remains between 60-90% throughout the cropping season. Such factors favor disease development and lead to exotic strains (Panno *et al.*, 2021; Potnis *et al.*, 2015a; Pohronezny *et al.*, 1992). Furthermore, tomato crop grown in low-altitude and high-temperature districts of KP was free of disease. These districts supply fresh tomatoes to vegetable markets from February to July. Reportedly, (Potnis *et al.*, 2015a; Jones *et al.*, 1986), showed that the bacterium can survive in crop residue for more than a year in temperate climate while it hardly survives for a few months in tropical and subtropical regions. High temperatures coupled with the inability of the pathogen to survive in the diseased-crop residue (main over-seasoning source) for more than a few months explains why low-altitude tomato-growing areas were free of disease. Furthermore, the farmers in the temperate regions don't practice crop rotation and often grow spring and summer tomatoes; therefore, the back-to-back cultivation results in high inoculum build-up that contributes to high disease incidence. In summer 2017, maximum %DI (64.42%) and disease severity %DS (49.17%) were recorded in district Swat, followed by district Mansehra (%DI= 53.63%) and %DS of 45.57%. Disease incidence for district Lower Dir was 46.42%, while %DS was 41.94%. In summer 2018, %DI and %DS in district Mansehra were recorded to be the highest which were 65.78% and 53.28%, respectively. Lower Dir (%DI= 53.28%) with %DS of 44.77% and district Swat 43.78% with %DS up to 40.59%. The reduction in disease incidence and severity in district Swat during summer 2018 could be mainly attributed to the occurrence of lower rain fall compared to that in summer 2017.

Similarly, the increase in %DI and %DS in Lower Dir and Mansehra districts during 2018 could be explained based on increased rainfall as compared to that in summer 2017. Climatic conditions, including temperature and relative humidity favor BS incidence and accelerate disease severity during the growing season (Araújo *et al.*, 2010; Abbasi *et al.*, 2002). The surveyed regions lie in temperate zones that usually have higher rainfall and higher percent humidity with mean low temperatures compared to tropical and subtropical regions of KP (Ali *et al.*, 2018).

Our studies found a good correlation between BS and weather components. Maximum BS infection (Swat; %DI=64.42% and %DS = 49.17%) showed high specificity with August 2017, when mean temperature was low (19-32°C) followed by a high rainfall pattern (r = 0.8346). Similarly, in 2018, correlation of maximum infection (Mansehra; %DI = 65.42% and %DS = 53.28%) with rainfall in July, August, and September (r=0.9162, 0.9054 and 0.9270 respectively) with varying specificity. Similar findings were reported by (Jarial *et al.*, 2022; Ravikumar and Khan, 2001). The occurrence, prevalence, and severity of plant diseases are affected by climate change in many parts of the world. Climate factors, such as humidity and temperature, are also main components of the disease triangle i.e., environment, host, pathogen; and play a key role in the occurrence and spread of most foliar diseases (Jarial *et al.*, 2022; Amanda *et al.*, 2019; Harvell *et al.*, 2002). In developing countries like Pakistan (which ranks 7th most vulnerable countries to climate change), micro-farmers' food security and socioeconomic status can be seriously impacted by climate change. Climate change may result in changes in the synchrony in crop phenology and pathogens that lead to susceptibility/resistance responses to pathogens. In such cases, ignorance may lead to huge losses (van Maanen and Xu, 2003).

Almost all infected samples (showing typical bacterial spot symptoms) yielded bacterial colonies on a nutrient agar (NA) medium. These colonies (isolates), when purified, showed the characteristic colony morphology of *Xanthomonas vesicatoria* as reported by Horvath *et al.* (2012) and Ogolla and Neema (2019). However, some isolates produced whitish-yellow, less bright and less dense colonies on NA. Similar differences regarding colony morphology on NA medium have been previously reported by other researchers (Ryan *et al.*, 2011; El-Ariqi *et al.*, 2010). Such minor differences could be attributed to slight genetic differences among different strains/races or differences in growth conditions (Ogolla and Neema, 2019). These isolates also produced *Xanthomonas*-typical yellow, mucoid colonies on YDC and SPA media (Potnis *et al.*, 2015a; Al-Dahmani *et al.*, 2003; Louws *et al.*, 2001) and were Gram-negative, catalase-positive, sensitive to a higher temperature (36°C) and higher NaCl salt concentration (higher than 0.5%) (Morales *et al.*, 2017; Büttner and Bonas, 2010; Bouzar *et al.*, 1994). Catalase enzymes neutralize the toxicity of H_2O_2 in microbes and

are useful for bacterial activity (Abdo-Hasan *et al.*, 2008). Moreover, most of the isolates (S1, S3, S4, S6, S7 and S10 from district Swat; D2, D4, D6, D7 and D8 from district Lower Dir; and M2, M3, M5, M8 and M9 from district Mansehra) produced a hypersensitive response (HR) on *Nicotiana benthamina* (Li *et al.*, 2015b) resulting in cell death within 24 hrs of inoculation.

Sometimes the colony morphology-based and cultural/biochemical tests-based identity of bacterial isolates are insufficient to reach a conclusive decision in microbial taxonomy. Therefore, confirming the identity of isolates through the use of PCR or other molecular identification techniques becomes necessary. To confirm the identity of our isolates, before using them for further studies, we initially used two specific primers, Xv1F and Xv1R that amplified a fragment of 365 bp-size (Beran and Mráz, 2013). This primer pair amplifies the specific band (i.e., 365 bp) using *aptD* gene of the bacterium as a template. Out of 30 isolates, 16 isolates (six from district Swat; 5 each from districts Lower Dir and Mansehra) yielded the fragment of the expected size, indicating they were *X. vesicatoria*. A total of 14 isolates did not produce the expected 365 bp band. As this gene is a house-keeping conserved gene of *X. vesicatoria*, so its non-amplification would most probably mean that these isolates could belong to other species of the genus Xanthomonas but not “vesicatoria:.

Climate change affects the occurrence, prevalence and severity of plant diseases in many parts of the world. Climate factors, such as humidity and temperature, are also main components of the disease triangle, i.e., environment, host, pathogen; and play a key role in the occurrence and spread of most foliar diseases (Harvell *et al.*, 2002). Like other nations, climate change has considerably affected small-scale farmers' food security and socioeconomic status in Pakistan. Pakistan is prone to severe blows of global climate change due to lack of resources, scarcity of professionals in various sectors, and lack of advanced technologies to mitigate climate change and food insecurities. Research, if any, is mainly focused on etiology and management but very little is being done to evaluate the epidemiology in relation to climate change (Raja *et al.*, 2018).

Moreover, the lack of coordination between the Agriculture Research System (ARS), Agriculture Extension (AE), and communication of current findings to the farmers through technology transfer projects is scarce. Furthermore, an efficient and effective quarantine system can further exacerbate the situation. One of many risks associated with economic opportunities like the China Pakistan Economic Corridor (CPEC), includes the unchecked entry of exotic plant pathogens into Pakistan. Besides, plant disease forecasting in advance remains a paradox due to plant diseases cryptic and complex nature. It is inconsistent; as some pathogens only grow well when the temperature is high and humidity is low. On the other hand, low temperature and high humidity are pre-requisite for most pathogens. Therefore, continuous climate change and pest monitoring are needed to develop an efficient disease warning system for growers and other agriculture-related agencies to avoid future plant epidemics.

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NOVELTY STATEMENT

Bacterial spot caused by *X. vesicatoria* pv. *vesicatoria* was reported for the first time in commercial tomato growing areas of Khyber Pakhtunkhwa in this study. The pathogen was initially characterized using morphological and biochemical tests. Further molecular characterization confirmed the pathogen.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

Ijaz Ahmad conducted research as a part of PhD dissertation and Musharaf Ahmad supervised this research.