Pak. J. Bot., 56(2): 711-718, 2024. DOI: http://dx.doi.org/10.30848/PJB2024-2(30)

PSEUDOMONAS SYRINGAE INDUCED PATHOGENICITY IN *MANGIFERA INDICA* LEADS TO SEVERE APICAL NECROSIS

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Abstract

Mango apical necrosis caused by *Pseudomonas syringae* has been documented by the current inquiry. Punjab, Pakistan's healthy mango output is being threatened by bacterial apical necrosis. Thirty-eight orchards were chosen in three districts (Multan, Khanewal, and Muzaffargarh) with the aim of evaluating the prevalence, incidence, and severity of disease in order to validate the status of mango apical necrosis. According to survey, disorder was widely distributed with 100% prevalence in Multan, Khanewal and 70% in Muzaffargarh. Bacterial apical necrosis highest (0.9%) incidence was recorded in Multan, Khanewal and lowest (0.5%) in Muzaffargarh. Pathogens were identified based on 16S rRNA gene analysis as *Pseudomonas syringae* (strain ICMP 3023, NCPPB 281, ATCC 19310, tomato strain DC3000), *Pseudomonas putida* (strain ATCC 12633), *Pseudomonas savastanoi* (ATCC 13522) and *Pseudomonas syringae* pv. *phaseolicola* (1448A). After the first, second, and third weeks of artificial inoculation, results for pathogenicity were obtained. Tobacco, potato tubers, mango leaves, tomatoes, and lemon fruit samples were tested against all the identified strains and lemon was found vulnerable among them. The host least prone to *P. syringae* infection was the potato. Toxin was extracted by methanol: chloroform from the cell-free culture of *P. syringae* for toxin analysis. P.S. ICMP 3023, one of the four *P. syringae* pv isolates, was the most virulent and caused illness in all the host plants. In the future, TLC analysis of the mango apical necrosis toxins will be necessary, and research will be beneficial for management techniques.

Key words: Aggressiveness, Bacteria, Toxin, Pathovar, Mango.

Introduction

Due to nutritive qualities, organoleptic value, and economic potential, the mango, *Mangifera indica* L. (Anacardiaceae), is a fruit tree species of enormous commercial importance (Matheyambath *et al*., 2016). It is indigenous in south Asia and Indo-Pak subcontinent is famous for its cultivation (Young, 2008). Mangoes make up around half of all tropical fruits produced globally. More than 80 countries commercially grow mangoes. In 2014, there were more than 46 million tonnes of mangoes produced worldwide (Anon., 2016). India, Brazil, and China are the three main mango-producing nations in the world. Thailand, Egypt, Indonesia, Mexico, Pakistan, Philippines, and Vietnam are among the list. Asia produces the vast majority of the world's mangoes, by far. With more than 40% of the world's production, India is by far the largest producer. India is the world's largest mango-producing country with a share of 38% of the global mango production. Asian countries, especially India, Pakistan, Bangladesh, China, Indonesia and Thailand, account for 67% of the mango production (Wardhan *et al.,* 2022). Because of its adaptation and growth in various edaphoclimatic environments and ecological niches, primarily in warmer temperatures, crops are widely dispersed and grown in the majority of tropical and subtropical locations (Rodrigo-Comino *et al*., 2014; Guerra *et al*., 2018). The United Nations Food and Agricultural Organization estimated the production for 2017 at 40 million tonnes (t), with 74% of the production coming from Asia, 15% from Africa, and 11% from Latin America and the Caribbean. Demand for this tropical fruit has grown day-to-day as a result of the increasing population, necessitating improved production and yield to meet this demand. Though, as stated by United Nations Conference on Trade and Development (Anon., 2015), to accomplishing this, producers face numerous obstacles such as variations in precipitation levels, climatic unpredictability, distraction of rainy and dry seasons, and the prevalence of insecticides, pests, and diseases. The last could result in major production disruptions and economic losses (Gil- Vallejo *et al*., 2013).

Fungal infections are the main ailments impacting the quality of mango fruit and foliage. In Colombia the most imperative are anthracnose disease (*Colletotrichum gloeosporioides* Penz.), the desiccating or deteriorating death of branches and stems (*Lasiodiplodia* sp.), and deformity in vegetation caused by *Fusarium subglutinans* (García *et al*., 2017). In the meantime, among bacterial infections, most prevalent disease is black spot which is triggered by *Xanthomonas campestris* Pammel's. There are also significant bacterial illnesses, though, such as bacterial apical necrosis, previously linked to *Pantoea* spp. (Lee & Tzeng, 2006; Gutiérrez-Barranquero *et al*., 2019a, 2019b) and is now caused by *Pseudomonas syringae* Van Hall. The majority of the countries where this disease has been documented are Spain, Portugal, Italy, Israel, and Australia, where it causes yield losses of up to 50% for the mango crop (Gutiérrez-Barranquero *et al*., 2012; Gómez-Ramírez *et al*., 2022).

Mango is the most produced and valuable fruit in Pakistan, noted for its vibrant colors, enticing aroma, soothing taste, and high nutritional value. Thus, it is cultivated on an area of 158,659 hectares with a total production of 1.8 million tons in 2019 with an export estimate figures of around 120,000 tons (Anon., 2020). Furthermore, Pakistan is the world's fifth major producer of mango just after India, China, Thailand and Indonesia. In Pakistan, from the last several years, the yield of mango is facing decline that is because mango trees are vulnerable to many diseases (Ullah *et al.,* 2017). Among all these diseases, apical necrosis is one of the most novel ones and considered to be the most dangerous for mango production (Gutiérrez- Barranquero *et al*., 2012).

Necrotic lesions on the petioles, panicles, and floral buds are among the disease's typical signs. The fruit, if developed, is unaffected. Water-soaked inter-veinal angular spots initially appear as necrotic lesions on leaves that consolidate, turn black, and have small elevated areas (Trantas *et al*., 2017). Mango crops develop best at temperatures 25-27°C, and below 15°C, the plant enters a dormant state (Galán-Saúco, 2015). Due to the plant's orientation in relation to the temperature, bacteria have a chance to attack during the dormant season. The illness outbreak is greatly influenced by wetter, colder winters and springtime temperatures since this bacterium thrives in low temperature and highly humid environments.

The panicles undergo necrosis and blighting, resulting in reduction of flowering which results in reduced and in severe cases absent of fruit set (Naqvi *et al*., 2016). The disease was discovered by Dr. Steve Akiew in Spain in September 1999 in Bundaberg, Queensland (Young, 2008). The disease is identified in Israel (Manicom, 1986) and named as 'bacterial black blight' while in Spain and Portugal it is known as 'Bacterial Apical Necrosis'. In Punjab Pakistan, the disease was identified on the basis of symptoms and pathogen was isolated from the infected buds which were identified as *Pseudomonas syringae*. This disease is the most novel one and has been reported for the first time in 2016 so there is a need of further investigations and more work is required to be done.

The objective of this study was to examine the incidence of disease apical necrosis and its causal agent in Multan, Khanewal and Muzaffargarh. It was verified that bacterial strain, *Pseudomonas syringae* was the main causal agent of disease apical necrosis in all study areas. The present research explores whether symptoms of this disease may be found in a mango under cold conditions of selected areas. Inferences were supported by morphological examination, basic biochemical tests, pathogenicity tests, and molecular identification. The study hypothesis is that *Pseudomonas syringae* may be a causative agent of mango tree disease. This finding is highly relevant since it will certify a route of solutions and preventions to achieve integrated disease management.

Material and Methods

Epidemiological survey, characterization and preparation of inoculum: From Multan, Muzaffargarh and Khanewal (Punjab districts) nearly 10-15 mango orchards were surveyed to record the apical necrosis prevalence, incidence and severity. Small pieces of affected buds were cut from the edge of a necrotic lesion, placed in sterile plastic bags, labeled and transported to Fatima Jinnah Women University, Rawalpindi for isolation and identification. Evaluation of buds with disease symptoms was done based on visual observation and infected buds were washed thrice with distilled water. The buds were then cut into small pieces of 3-4mm from the edge of necrotic lesion and immersed in sterile aqueous solution of 0.1% (wt/vol) $HgCl_2$ and plated on the autoclaved King's medium B plates and Nutrient agar plates. The plates were incubated at 22°C for two days for pathogen to ooze out and daily observation of plates was done for colony development. In another method, buds were surface disinfected by using 70% ethanol and left to dry. A drop of 0.8% NaCl was introduced on the surface and after 3 to 4 minutes the oozed pathogen was picked by using sterile platinum wire. The platinum wires were streaked on the nutrient agar and King's B medium plate and incubated for 2 days at 22°C. An isolated colony was picked and re streaked to get pure colonies. For chemical characterization of the pathogen, gram staining of the pure colony of the pathogen was done (Table 1).The pure colonies of the pathogen were isolated and a single colony was picked and mixed in already prepared 3 mL of nutrient broth in a test tube (Shila *et al*., 2013).The test tubes were incubated at 22°C for 2 days with continuous agitation. Bacterial suspension of 10^6cells/mL was prepared by using serial dilution method.

Identification of *Pseudomonas syringae* by DNA sequencing: Based on16S rRNA gene sequence analysis the strains were identified. For this reason, DNA of the strain (previously grown on NA and KB) was extracted by the methodology of (Ahmed *et al*. 2007). Amplification of 16S rRNA gene was carried out in polymerase chain reaction (thermocycler) using forward primer **27F (5'-AGA GTT TGA TCM TGG CTCAGA-3') and reverse primer 1492R (5'- TAC GGY TAC CTT ACG ACTT-3').** The reaction mixture (50 µl) for PCR was prepared by adding Template DNA (02 µL), dNTPs (01 µL), NH_4 $(SO_2)_2$ (05 µL), $MgCl_2$ (05 µL), 27F (2.5 µL), 1492 R (2.5 µL), Taq DNA polymerase (01 µL), DEPC water (31 µL)**.** For 16S rRNA gene amplification following profile was used for PCR programmed: Cycle 1(x) for initial denaturation at 94ºC for 2 minutes, Cycle 2 (29 x) for denaturation at 94°C for 1 minutes, primers annealing at 50ºC for 1 minutes and extension at 72°C for 1:30 minutes, Cycle 3 (1 x) for final extension at 72°C for 5 min, Cycle 4 (x) for final storing at 4°C. The amplified DNA of 16S rRNA gene was confirmed on agarose gel (1%, w/v) run for 160 min at of 20 V. With reference to manufacturer's guidelines the amplified PCR products were purified by using purification Silica Bead Gel Extraction Kit (Thermo Scientific). The purified PCR product was sequenced from Macrogen, Korea using forward primer 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse primer 1492R (5'-TAC GGY TAC CTT ACG ACTT-3'). Obtained nucleotide sequences were read and manually edited by using DNA dragon software. For phylogenetic analysis of isolated strains, the assembled nucleotide sequences of 16S rRNA gene of closely related validly published species were retrieved from the BLAST (Basic Local Alignment Search Tool) to compare similarity in NCBI (*National Center for Biotechnology Information).* Sequences of isolated strains and closely related species were aligned using CLUSTAL W alignment (Dinesh *et al*., 2015) and to determine the evolutionary relationship of the strains with other validly published strains the phylogenetic analysis was performed. Using Neighbor-Joining algorithms contained in MEGA-5 software package phylogenetic tree was constructed (Tamura *et al*., 2011).

Aggressiveness analysis of *Pseudomonas syringae*: Aggressiveness of isolated strains was tested by two methods.

Direct injection of bacterial inoculums into host plant: Aggressiveness tests were applied on potato tubers (Choiseul *et al*., 2006), lemon and tomato (Arrebola *et al*., 2003) using? detached mango leaves. The aggressiveness was attested by inoculating the hosts with bacterial inoculum. Lesions were recorded longitudinally and vertically after one, two and three weeks respectively. For comparison, necrotic area in [controls] was also measured. Five-point rating disease severity scale (Iram *et al*., 2019) was used to assess disease severity (Table 2). The lesion area of the inoculated plant after showing disease symptoms was measured by using the following formula:

$$Area = \pi lw$$

where π = 3.14, l = length of necrotic lesion, w = Width of necrotic lesion

To confirm the Koch's postulate diseased parts were cut and placed on nutrient agar and King's B medium plates. After 2 days of incubation, the colonies were picked and streaked on nutrient agar and King's B medium plates.

Injection of toxin into host plant: Four strains of *P. syringae* were used for the production of toxin (Arrebola *et al*., 2003). *P. syringae* strains were mixed in 3 mL nutrient broth to be grown for at 22℃ with continuous agitation. The mixture was observed 5 days for growth and centrifuged at 4000 g for 10 min. Once the phase separation was achieved the supernatant was filtered through 0.2µm nitrocellulose membrane followed by the addition of 2:1 methanol: Chloroform (v/v). After that 2:1 methanol chloroform (v/v) was added in the cell free culture, again centrifuged at 2500g for 10 mins, and then left at room temperature to get concentrated mixture through evaporation. To test the toxin extracted from the pathogen, same method used for bacterial culture was applied on the host plant parts and results were observed after the interval of 1 week, 2nd and 3rd week and then compared with the results obtained by bacterial suspension.

Results and Discussion

Disease assessment: Severity of mango apical necrosis was significant among assessed districts of the Punjab. Maximum severity was observed in Multan and Khanewal ranging from 4-5%. Least severity was observed in orchards of Muzaffargarh (3-4%) (Table 2). Maximum disease index of apical necrosis was observed in Multan (31%) followed by Khanewal (12%), least was observed in Muzaffargarh (8%). In the current study, maximum disease incidence was investigated on mango trees having thick covering compared to the prune trees. Increased rainfall and decreased temperature significantly affected the increase mango apical necrosis because at low temperature the distribution of the bacterium was mostly by splash dispersion. When the temperature falls below 15°C, the mango crop enters a dormant period and grows well between 20 and 25°C (Galán-Saúco, 2015). Kennelly *et al*., (2007) also stated that chilly temperatures and rainy times were crucial for the emergence of BAN symptoms, which had also been linked to other *P. syringae* infections in other woody hosts. To articulate the symptoms winter rain is critical. In our survey we also noticed disease symptoms of apical necrosis on mango bud suddenly appeared after the rainfall. Therefore, it was found that disease was more prominent in colder days as soon as temperature drops below 15°C.

Abdullah *et al*., (2021) studied infection frequency (%) of the prevalent pathogen at the eleven (11) phases of mango flowering and the relationship between infection frequency (%) and other environmental parameters. Environmental parameters were observed to be associated with the frequency of infection of pathogens, particularly *Pseudomonas syringae* pv. *syringae*. Also, it was discovered that this disease only affected mango plants under stress, such as dormancy or bud break. As environmental factors have a strong correlation with pathogen infection, it is very likely that, once the pathogen is established, infection of this entity will be seen at later stages of flowering if the environmental conditions more or less remain favorable. The investigations revealed a strong negative association between *Pseudomonas syringae* pv. *syringae* and temperature. The unfavorable symptom indicates that the illness will spread more readily when the temperature drops under humid conditions and in present study more than 80% of the relative humidity was very hospitable to the growth of bacteria. Because of this, there was a strong positive correlation between infection and relative humidity. The frequency of pathogen infections was significantly impacted by the rainfall as well. The field circumstances were favorable for the development of the disease. While the conditions were extremely favorable for the disease, infection was most frequently seen at "stage A" (dormant bud) by 93.33%. The average recorded temperature and relative humidity at stage A were 16ºC and 100%, respectively. Stage B (bud swell) saw a 90% infection frequency due to an increase in average temperature, while 100% relative humidity was present.

This demonstrated that a set of favorable environmental factors is required for the disease to develop. Similarly, at stage C (bud break), the infection rate was 90.67%, indicating a slight increase in infection. Despite the fact that the relative humidity was 93% and the temperature was 15 degrees Celsius the increase in infection was solely due to rain. (2.55mm).

Biochemical characterization and species identification of isolated bacteria: Sequence analysis of 16R rRNA discovered that, detected specie belonged to genus *Pseudomonas*. Ccomparison of sequence of isolated strain with other authentically published strains revealed that isolated strain showed 100% sequence identity with *Pseudomonas syringae* strain ICMP 3023, *Pseudomonas syringae* strain NCPPB 281, *Pseudomonas syringae* strain ATCC 19310, *Pseudomonas syringae* pv. *tomato* str. DC3000, *Pseudomonas putida* strain ATCC 12633, *Pseudomonas savastanoi* strain ATCC 13522, *Pseudomonas syringae* pv. *Phaseolicola*1448A strain respectively. Under the current research bacterial pathogen was also characterized at genetic level by sequencing of the PCR products to develop phylogenetic relationship among detected strains and already published species. Sequences so obtained on comparison showed diverse degree of resemblance with accessible

sequences on NCBI. The 16S rRNA sequences for each isolate using the Bio Edit were trimmed both at the beginning and at the end, aligned and consensus created. The aligned sequences by Clustal W alignment using MEGA 5 software demonstrated that the sequence variation between species was due to deletions and insertions in the ITS1 and ITS2 regions. Analysis of these sequences against the NCBI GenBank database using BLAST program determined the taxonomy of the isolates (Fig. 1).

Diseased buds collected during the survey were used to isolate the casual pathogen on Nutrient Agar (NA) and Kings B (KB) media and obtained cream color colonies (Cazorla *et al*., 1998). Preliminary investigation revealed that the bacterium responsible for the apical necrosis disease of mango in Pakistan is *Pseudomonas syringae* pv. *syringaeas*, which was isolated by cultivating the affected tissue on Nutrient agar and Kings B media. The results of our study are in harmony with the results of previous workers (Young, 2008). Ivanović *et al*., (2017) reported white to cream colored (off-white) and fluorescent bacterial colonies with circular shape on NA and KB media. After performing gram staining procedure pink color was observed when counter stained with safranin under the microscope at 100X magnification confirming that bacterial cultures were gram negatives, a phytopathogenic bacteria which could cause disease in mango trees as bacterial apical necrosis. The different groups of isolates of *Pseudomonas syringae* pv. *syringae* causing bacterial leaf blight of litchi were identified and differentiated into gram negative by KOH solubility test and gram staining test (Afrose *et al*., 2014).

These results are in parallel with Carrión *et al*., (2012) who performed an experiment to characterize *mgo* operon in *Pseudomonas syringae* strain required for mangotoxin production. They also found an operon-like organization which was parallel with other *P. syringae* pathovars for which complete genomes were available (*P*. syringae pv. *syringae* B728a, *P*. syringae pv. tomatoDC3000 and *P*. syringae pv. *phaseolicola* 1448A), as these mentioned strains were not able to produce mangotoxin. Our findings are further corroborated by Martnez-Garca *et al*., (2015), who examined the chromosome sequence of the mango tree pathogen UMAF0158 with other *P. syringae* pathovars that aren't actually mango pathovars but had 80–90% of the same coding DNA sequences (CDs) as UMAF0158. Our research revealed the pathogenicity of bacteria on mango trees that produce mangotoxin and identified the bacteria by 16S rRNA sequencing as the cause of mango apical necrosis in Punjab, Pakistan orchards. The present study is the first information to characterize the disease-causing agent by genetic identification using molecular technique in Pakistan. Previously considerable work has not been performed on genetic identification and detail investigation is required to find more reliable solutions for control of this disease. This clustering in one group means they share high level of similarity in their genomes and behavior. Taking into consideration the argument above it is concluded that the pathogen is *Pseudomonas.*

Aggressiveness analysis of *Pseudomonas syringae* by direct injection of bacterial inoculums into host plant

Potato tubers: Four isolates of *Pseudomonas syringae* were applied on different potato tubers and first symptom appeared after 1 week of the inoculation. Lesion appearing time for all the isolates was different. Isolate P.S ICMP 3023 was highly aggressive isolate on potato tubers.

Detached mango leaves: Inoculum of bacteria was injected in the midrib of the leave and lesions were observed and data was collected for consecutive 3 weeks. According to the results, P.S ATCC 19310 was the most aggressive isolate. The first lesion appeared on the host after 2nd day of inoculation. P.S ATCC 13522and P.S ICMP 3023showed diseased symptoms after 4th day and P.S NCPPB 281 showed the symptom after 3rd day.

Tomato: Tomato fruit is very susceptible to the pathogen causing apical necrosis because most of the isolates have caused significant disease. P.S ATCC 13522 can be said the most aggressive for tomato while P.S NCPPB 281 has also caused severe damage to the fruit. The lesion appeared soon after the 2nd day of inoculum on all the fruits with each isolate. Black spots were observed as a result of aggressiveness tests on the surface of the fruits.

Lemon: Lemon fruit is said to be the most susceptible host for the pathogen as all the isolates have succeeded in causing severe disease to it. The first lesion appearing on all the host was recorded after the 3rd day when the inoculum was injected. It was recorded that P.S NCPPB 281 was the most aggressive isolate as it had caused larger lesions than others (Fig. 2 and Table 3).

The role of *Pseudomonas syringae* pv. *syringae* in causing of apical necrosis of mango was exhibited by the appearance of the same symptoms as of the pathogen on the host plants (Cazorla *et al*., 1998). In this study, partial purification of the phytotoxin was done in order to check the aggressiveness and compare it with the results of inoculum of bacterial culture made in nutrient broth. All the strains used in the study were able to produce phytotoxins and it was confirmed by applying pathogenicity tests. When the results of the toxin inoculated hosts and pathogen inoculated host were compared, it was concluded that the toxin has done a more damage to them. As previous study of Spain (Carrión *et al*., 2010)and the present study reveals the presence of toxin in the pathogen.

Injection of toxin into host plant

Potato tubers: Toxin extracted from the pathogen was applied on the potato tubers. All the 4 isolates were tested and all of them caused disease. The first lesion for all the isolates was observed on the 2nd day of application of toxin. P.S ICMP 3023was the most aggressive isolate among all the isolates.

Mango leaves: Four Isolates were injected in the midrib of the detached mango leaves. The first lesion for P.S ATCC 19310 appeared after 1st day, for P.S NCPPB 281 it appeared after 2nd day and for P.S ATCC 13522 and P.S ICMP 3023, the first lesion was observed after 3rd day of testing. The most aggressive isolate among all was isolate P.S ATCC 19310which caused significant damage to the leaves.

Table 1. Isolation of strains of *Pseudomonas syringae* (PS)from the infected buds of mango.

S. No.	Strain	Plant part	Location	Biochemical characterization
1.	P.S ATCC 19310	Mango Buds	Multan, Pakistan	+
2.	P.S ATCC 13522	Mango Buds	Multan, Pakistan	+
3.	P.S ICMP 3023	Mango Buds	Multan, Pakistan	+
4.	P.S NCPPB 281	Mango Buds	Multan, Pakistan	+

Note: + = 95% strains were gram negative

Table 2. Mean value of incidence, severity, and prevalence of apical necrosis in three different mango-growing districts of Punjab.

Survey area	No of tree assessed	Incidence (%) (mean)	Severity (%) (mean)	Prevalence (%)	Disease index %
Multan	65	0.9	4-5	100%	31%
Khanewal	50	0.9	4-5	100%	12%
Muzaffargarh	75	0.5	3-4	70%	8%

Table 3. Aggressiveness and toxin analysis of isolates on different hosts by disease severity scale.

Host	Isolates for aggressiveness analysis	Location	Source	Disease severity scale grading (0-5)			Ranking on severity scale		
				Week1	Week 2	Week 3	Week 1	Week 2	Week 3
Mango	PS1	Multan	Buds	1	2	3	Trace	Mild	Moderate
	PS2	Multan	Buds	1	2	2	Trace	Mild	Mild
	PS3	Multan	Buds	0	1	1	No Disease	Mild	Mild
	PS4	Multan	Buds	1	2	3	Trace	Mild	Mild
Potato	PS1	Multan	Buds	1	1	2	Trace	Trace	Mild
	PS2	Multan	Buds	1	1	1	Trace	Trace	Trace
	PS3	Multan	Buds	2	2	2	Mild	Mild	Mild
	PS4	Multan	Buds	0	1	1	No disease	Trace	Trace
Lemon	PS1	Multan	Buds	1	2	3	Trace	Mild	Moderate
	PS2	Multan	Buds	0	2	4	No disease	Mild	Severe
	PS3	Multan	Buds	2	3	4	Mild	Moderate	Severe
	PS4	Multan	Buds	1	3	4	Trace	Moderate	Severe
Tomato	PS1	Multan	Buds	1	1	1	Trace	Trace	Mild
	PS2	Multan	Buds	2	3	4	Mild	Moderate	Severe
	PS3	Multan	Buds	1	2	2	Trace	Mild	Mild
	PS4	Multan	Buds	1	2	4	Trace	Mild	Severe
	Isolates for toxin analysis								
Mango 1	PS1	Multan	Buds	1	2	4	Trace	Mild	Severe
	PS2	Multan	Buds	0	0	1	No Disease	No disease	Mild
	PS3	Multan	Buds	1	1	1	Mild	Mild	Mild
	PS4	Multan	Buds	1	1	3	Mild	Mild	Moderate
Potato 1	PS1	Multan	Buds	0	1	1	No Disease	Trace	Trace
	PS2	Multan	Buds	1	2	2	Trace	Mild	Mild
	PS3	Multan	Buds	2	3	4	Mild	Moderate	Severe
	PS4	Multan	Buds	1	1	2	Trace	Trace	Mild
Lemon 1	PS1	Multan	Buds	1	1	2	Mild	Mild	Moderate
	PS2	Multan	Buds	1	1	2	Mild	Mild	Moderate
	PS3	Multan	Buds	1	2	3	Trace	Mild	Moderate
	PS4	Multan	Buds	2	3	4	Mild	Moderate	Severe
Tomato 1	PS1	Multan	Buds	1	1	1	Trace	Trace	Trace
	PS2	Multan	Buds	1	1	3	Trace	Trace	Moderate
	PS3	Multan	Buds	0	2	3	No disease	Mild	Moderate
	PS4	Multan	Buds	1	4	5	Trace	Severe	Very severe

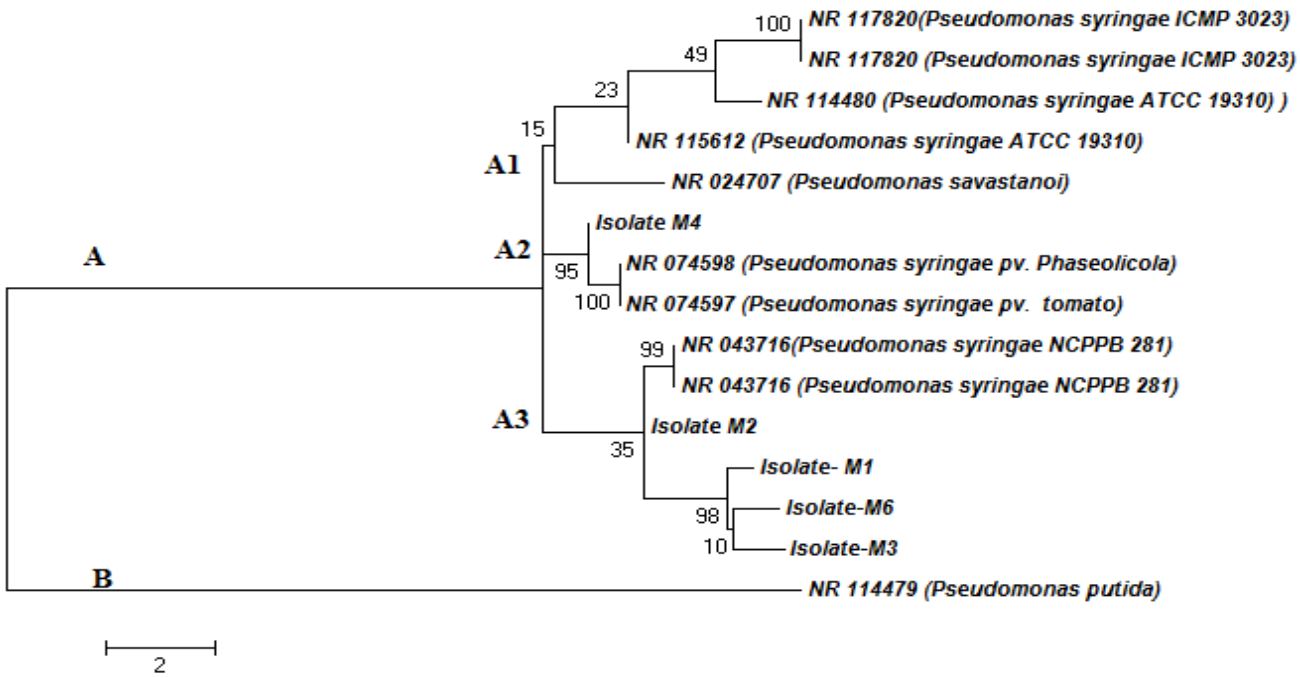

Fig. 1. Neighbor joining phylogenetic cluster of *Pseudomonas* specie obtained after 16S rRNA sequencing data.

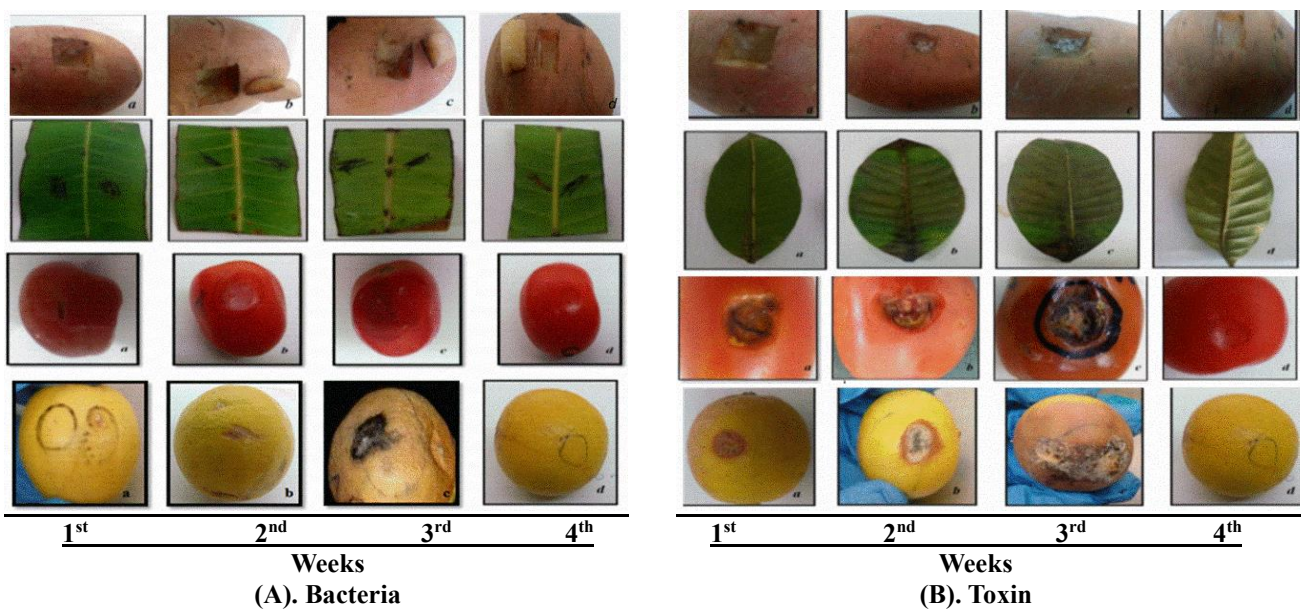

Fig. 2. Disease symptoms on potato tuber, mango leaf, tomato and lemon during 1st, 2nd, 3rd and 4th week of inoculation, by isolated bacteria (a) and toxin(b).

Tomato: Almost all the toxins have caused grave damage to the host. The first lesion appeared after the 1st day of inoculation. The most aggressive isolate was isolate 4 which caused disease so rapidly and spread to almost whole fruit. Other isolates such as 2 and 3 have also caused major damage to the fruit. From the results, it can be concluded that tomato is the most susceptible host for pathogen *Pseudomonas syrinae*.

Lemon: Inoculum made from all the four toxins was injected in the lemon fruit. The first lesion appeared after 2nd day of the inoculation. It was concluded that Isolate 4 was the most aggressive isolate among all as it damaged the whole lemon. Isolate 3 also caused grave damage along with isolate 1 and 2 (Fig. 2 and Table 3). The causal bacterium, *Pseudomonas syringae* pv. *syringae* Van Hall, affects many perennial fruit crops (Hirano & Upper 1990). The strains from mango produce an antimetabolite toxin, mangotoxin, which plays a role in pathogen virulence and symptoms development (Carrión *et al*., 2012). However, it was usually possible to make successful identifications of isolates because the host is known and therefore identification requires only the differentiation of a few pathogenic species or pathovars (Young, 2008). This identification would help in management of the disease as the cure would be in synchronization with the toxin type. The findings of the present study would be useful to design a comprehensive molecular based study of biodiversity of

the pathogen *Pseudomonas syringae* pv. *syringae* and to adopt a proper management strategy suitable for the integrated disease management programs. It is also concluded that identification of pathotype will be helpful for future studies to plan the management measures of apical necrosis disease in Punjab. Various new strains of *Pseudomonas syringae* were also detected in the present research which demonstrated that change in climatic conditions might also be the main source to initiate the disease in most of mango growing areas of Pakistan. In future, thin layer chromatography (TLC) analysis is needed to find out about the type of the toxin present in the pathogen that is causing the disease. A TLC analysis will give information about the toxin name and identification of the toxin produced by different strains would be done. In order to control the disease spreading, it is important to select healthy plants from nurseries that follow strict phytosanitary practice and tolerant/resistant cultivars to bacterial apical necrosis should be identified and be given to the orchard owners. As bio control agents, which have been effectively employed to diminish the population of numerous bacterial infections, could be an alternative technique usable in the near future, it is best to avoid using copper compounds exclusively in control strategies.

Conclusion

The apical necrosis disease was predominant in Multan and Khanewal compared to Muzaffargarh. On the other hand disease incidence in Muzaffargarh was also somewhat less than Multan and Khanewal. Morphological studies confirmed that *Pseudomonas syringae* was the causative agent of mango apical necrosis and its identity was reconfirmed by 16S-rRNA sequencing. Pathotype identification will be helpful for future studies planning the management measures of apical necrosis disease. According to study, the pathogen *Pseudomonas syringae* can only cause disease in low temperature i.e., less than 25°C. The study suggests that all the isolates have different effect on different hosts in term of severity; some of isolates have shown aggressiveness while some had mild effect. On severity scale, most of the isolates when applied on lemon, showed severe results, while on tomato most of the isolates have shown severe to mild results. While on mango leaves and potato results were mild.

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(Received for publication 20 September 2022)