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# Pathogenesis and molecular confirmation of *Pseudomonas Syringae PV. Syrinage* Isolates from peach and plum in Pakistan

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## Abstract

Detailed survey of Peach and Plum orchards was done in 2014-2015 for the confirmation of bacterial canker disease in Pakistan and the isolates were isolated from diseased stone fruit tissue samples from 16 Orchard sites in Khyber Pakhtunkhwa (KPK), Pakistan. These isolates were tested for pathogenicity and the confirmation of *Pseudomonas syringae* pv. *syringae* was done using *virD4* primer for the amplification of *virD4* gene. All the 21 isolates from 24 isolates tested were moderately to highly pathogenic on peach seedlings while other 3 isolates showed no pathogenicity. During PCR assay of all the 24 isolates to amplify *virD4* gene, the predicted 1453bp PCR products were obtained by 20 isolates and the 4 isolates were considered negative. The results highlighted a new threat in Pakistan, which must be further studied for its characterization and epidemiology for better management of bacterial canker of stone fruits in local environmental conditions.

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## Introduction

Stone fruits, including peach, apricot, plum and cherries are very important in the economy of the Pakistan that earns a lot of foreign exchange but unfortunately their yield is not according to the potential. The most characteristic symptoms of bacterial canker are generally cankers and necrosis developing on branches and trunk, often located around spurs, wounds and branch junctions. In early infections on branches, the tissue is sunken, water-soaked and slightly brown discolored. Later, it becomes darker and finally reddish-brown black. Cankers and necrosis can be associated with orange-brown gummosis (Agrios, 2005).

According to lvanova (2007) bacterial canker caused by *P. syringae* pv. *syringae* is the most important disease in apricot decline which is the biggest phytopathological problem of stone fruit species and limiting factor for the successful apricot cultivating in many countries of the world. In the Northern Areas of Pakistan there is abundant of fruit trees scattered all over Northern Areas including cherry, apricot, apple, peach, plums etc. The climate of Northern Areas (severe in winter, cool spring and hot and dry summer) is ideally suited for cultivation of deciduous fruit trees. Severe winter fulfills the chilling requirement, cool spring for fruiting and hot and dry summer for complete ripening (Lvanova, 2007).

Bacterial canker of stone fruits caused by *Pseudomonas syringae* has become a serious problem in many parts of the world (Cameron, 1962; Mohammadi *et al.,* 2010).

The disease occurs on the aboveground parts of the trees, and may result in localized canker or death of entire limbs or trees. Symptoms of bacterial canker appear on branches, twigs, buds, leaves, and fruits. The most conspicuous symptoms are the cankers that exude gum during late spring and summer (Severin *et al.,* 1986; Hetherington and Wales, 2005). Bacterial canker stone fruit trees, caused by *Pseudomonas syringae* pv. *syringae*, affects all commercially grown *Prunus* species including peach (*Prunus persica*), Plum (*P. domestica*), cherry (*P. avium*) and apricot (*P. armeniaca*).

Khan *et al.* (2000) studied the differentiation of pathovars of *Pseudomonas syringae* using polymerase chain reaction (PCR) to classify phytopathogenic *P. syringae* at the pathovar level, and it may be a useful diagnostic tool for these important plant pathogens. The current study is focused on the symptomatology of bacterial canker disease and the etiology of the bacteria associated with bacterial canker disease of stone fruits from Pakistan. Basis molecular tools were also used to confirm the presence of virulence gene using specific primers.

## Materials and methods

*Bacterial isolation*

The bacterial isolates used in this study were isolated from healthy and infected peach tissues (leaf, stem and gum) collected from peach orchards in KPK Pakistan. Isolation was done on nutrient agar and then subcultured by picking a single colony on King's medium B (KB).

*Pathogenicity tests*

Bacterial suspension was prepared by harvesting 24 hr old bacterial cells grown on solid KB at 27°C and a concentration of ~5 × $10^7$ CFU/ml was maintained. Then 1 ml bacterial suspension was injected into the stems of 10 – 12 week old peach seedlings already grown in controlled conditions by using a needle inserted tangentially under the cambium. One tester isolate of *Pseudomonas syringae* pv. *syringae* and water was injected as a positive control and negative control respectively. The plants were maintained in a greenhouse at 27°C and symptoms were observed after 10 days for disease development on a disease rating scale of 0 to 3 (Table 1).

Each seedling was inoculated in three places with same isolate as repetitions and an average pathogenicity rating for each isolate was used to determine the mean of the pathogenicity for all

isolates isolated from a particular host.

*DNA preparation and PCR assay*

A single colony per isolate was grown in 3ml Nutrient broth medium and kept over night in shaker at 28°C then cells were collected in 1.5ml eppendorf tube and total nucleic acid of bacterial isolates was isolated with the standard protocol of Genomic DNA purification Kit (#K0721 Thermo scientific). Conditions for 50 µL PCR reaction mixture was comprised of 25 µL master mixture (Thermo scientific), 1µL of each primer DR (5'-CACTTCAGACGCTTTGTC-3'), DF (5'- -CAGCTACGGGCAACAG-3') designed on the *virD4* sequence of pPSR1(Sundin *et al.,* 2004), 2 µL of template DNA and 21 µL of DEPEC water. The reaction conditions were initial denaturation at 94°C for 5 min, followed by 30 thresh hold cycles of denature (94°C for 1 min), annealing (56°C for 30 second) and extension (72°C for 1 mint) and final extension was performed at 72°C for 10 mint (Karimi-Kurdistani and Harighi, 2008). The PCR amplified products were run on 1% Agrose gel (w/v) and size of amplified fragment was measured with 1kb DNA ladder.

**Results and discussion**

Disease samples (Fig. 1) were collected from 12 peach orchards located in KPK province, Pakistan. Total 24 isolates (Fig. 2) were isolated from diseased samples collected (Table 2). All the isolates were tested for pathogenesis to confirm the virulence of the isolates.

A total of 24 isolates isolated in 2014 and 2015 from peach hosts were tested for pathogenicity on 12 week old peach seedlings.

**Table 1.** Disease rating scale for pathogenicity test.

| Rating Scale | Description |
|---|---|
| 0 | Light necrosis associated with wounding at the area of inoculation. |
| 1.0 | Dark, water-soaked necrosis, with some streaking in the cambium. |
| 2.0 | Streaking in the cambium extending away from the site of inoculation, necrosis around the wound up to 2 mm above and below the wound with gumming. |
| 3.0 | Necrotic lesion and streaking involving the entire stem, often with girdling and death of distal portions and extensive gumming. |

In addition, one tester isolate of *P. syringae* pv. *syringae* obtained on request from culture bank Punjab University Lahore was tested. 21 out of 24 isolates and a tester isolate was moderately to highly pathogenic on peach, as evidenced by a pathogenicity rating of 2.0 to 3.0 (Table 3), except for 3 isolates (P-7, P-13 and P-21), which had a disease rating 1.0 represents very low or no virulence. While negative control show no symptom with disease rating 0 and positive control shown severe symptoms with disease rating 3.0 (Table 3). *P. syringae* is a phytopathogenic bacterial species currently divided into more than fifty pathovars and nine genomospecies (Bradbury, 1986; Young *et al.,* 1996; Gardan *et al.,* 1999). *P. syringae* is a polyphagous phytopathogenic bacterium associated with more than 180 species of both annual and perennial crops, including vegetables, fruits and ornamental plants (Agrios, 2005).

For molecular confirmation of isolates, PCR assay was optimized and D4 primer of *virD4* gene was successfully amplified approximately 1453 bp fragments (Fig. 3a, 3b). Total of 24 isolates were used, from which 22 isolates show satisfactory results and amplified specific bands.

So, the results are similar with results already reported by (Karimi-Kurdistani and Harighi, 2008) who also used the polymerase chain reaction (PCR) technique for differentiating the pathovars of *Pseudomonas syringae* by detecting *vir-D4* and *vir-B1* genes by amplifying band size of 513 bp and 1453bp respectively that shown PCR is rapid and reproductive technique to identify pathogen at pathovar level and it may be a useful diagnostic tool for these important plant pathogens. According to (Kotan and Sahin, 2002) 20 isolates of *P. syringae*

were isolated from apricot tress showing typical bacterial canker symptoms in Turkey and confirmed using pathogenicity test. Again, (Vicente and Roberts, 2007) identified 54 *Pseudomonas syringae* isolates from cherry and using biochemical, serological and pathogenicity tests.

**Table 2.** Bacterial isolates collected from different localities during 2014 – 15.

| S/No. | Host | Location | Isolate Code |
|---|---|---|---|
| 1 | Peach | Madrota Orchard-1 | P-1 |
| 2 | Plum | Madrota Orchard -1 | P-2 |
| 3 | Peach | Madrota Orchard -2 | P-3 |
| 4 | Plum | Madrota Orchard -2 | P-4 |
| 5 | Peach | Madrota Orchard -3 | P-5 |
| 6 | Peach | Madrota Orchard -4 | P-6 |
| 7 | Plum | Madrota Orchard -4 | P-7 |
| 8 | Peach | Madrota Orchard -5 | P-8 |
| 9 | Peach | Madrota Orchard -6 | P-9 |
| 10 | Peach | Madrota Orchard -7 | P-10 |
| 11 | Peach | Madrota Orchard -8 | P-11 |
| 12 | Plum | Madrota Orchard -8 | P-12 |
| 13 | Peach | Fate jang Orchard-1 | P-13 |
| 14 | Peach | Fate jang Orchard-2 | P-14 |
| 15 | Plum | Fate jang Orchard-2 | P-15 |
| 16 | Peach | Fate jang Orchard-3 | P-16 |
| 17 | Plum | Fate jang Orchard-3 | P-17 |
| 18 | Peach | Fate jang Orchard-4 | P-18 |
| 19 | Plum | Fate jang Orchard-4 | P-19 |
| 20 | Peach | Kamra Orchard -1 | P-20 |
| 21 | Peach | Kamra Orchard -2 | P-21 |
| 22 | Peach | Kamra Orchard -3 | P-22 |
| 23 | Plum | Kamra Orchard -3 | P-23 |
| 24 | Peach | Kamra Orchard -4 | P-24 |
| 25 | Peach | Tester Isolate (Panjab University Lahore) | P-T |

**Table 3.** Pathogenesis of isolates isolated from peach and plum hosts.

| S/No. | Isolate | Disease Rating |
|---|---|---|
| 1 | P-1 | 2.0 |
| 2 | P-2 | 2.0 |
| 3 | P-3 | 2.0 |
| **4** | **P-4** | **3.0** |
| 5 | P-5 | 2.0 |
| 6 | P-6 | 2.0 |
| 7 | P-7 | 1.0 |
| **8** | **P-8** | **3.0** |
| 9 | P-9 | 2.0 |
| 10 | P-10 | 2.0 |
| 11 | P-11 | 2.0 |
| **12** | **P-12** | **3.0** |
| 13 | P-13 | 1.0 |
| **14** | **P-14** | **3.0** |
| 15 | P-15 | 2.0 |
| 16 | P-16 | 2.0 |
| 17 | P-17 | 2.0 |
| 18 | P-18 | 2.0 |
| **19** | **P-19** | **3.0** |
| **20** | **P-20** | **3.0** |
| 21 | P-21 | 1.0 |
| 22 | P-22 | 3.0 |
| 23 | P-23 | 2.0 |
| 24 | P-24 | 2.0 |
| **25** | **P-T** | **3.0** |
| 26 | Control | 0 |

Fiori *et al.* (2003) used the biochemical and physiological tests as well as the repetitive PCR method with BOX primers to compare between *P. syringae* pv. *syringae* isolated from the severe die-back in hazelnut orchards in Italy (Scortichini *et al.,* 2003) used the repetitive PCR using the BOX primer to compare between 101 *P. syringae* pv. *syringae* strains, from international culture collections or isolated from diseased tissues of herbaceous and woody plant species.

**Fig. 1.** Infected leaf samples collected from Peach.


**Fig. 2.** Isolation of bacteria on nutrient agar.

So, based on the results of current study, maximum isolates are observed as *P. syringae* pv. *syringae* causal agent of bacterial canker of stone fruits in Pakistan. Results suggest that the ability to differentiate between individual isolates may be of potential use in the studies determining the epidemiology of bacteria canker of stone fruit and host pathogen interactions relating to stone fruit cultivar infection as well as developing improved strategies for breeding for resistance to *P. syringae* pv. *syringae*.

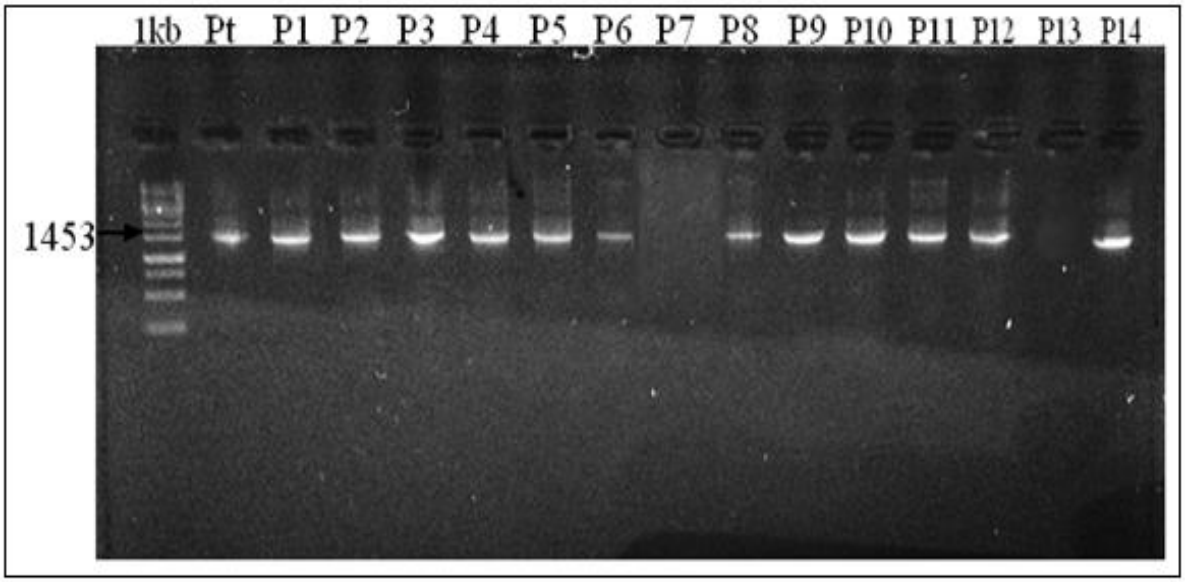


**Fig. 3a.** PCR product approximately 1453 bp fragments using *virD4* primers.

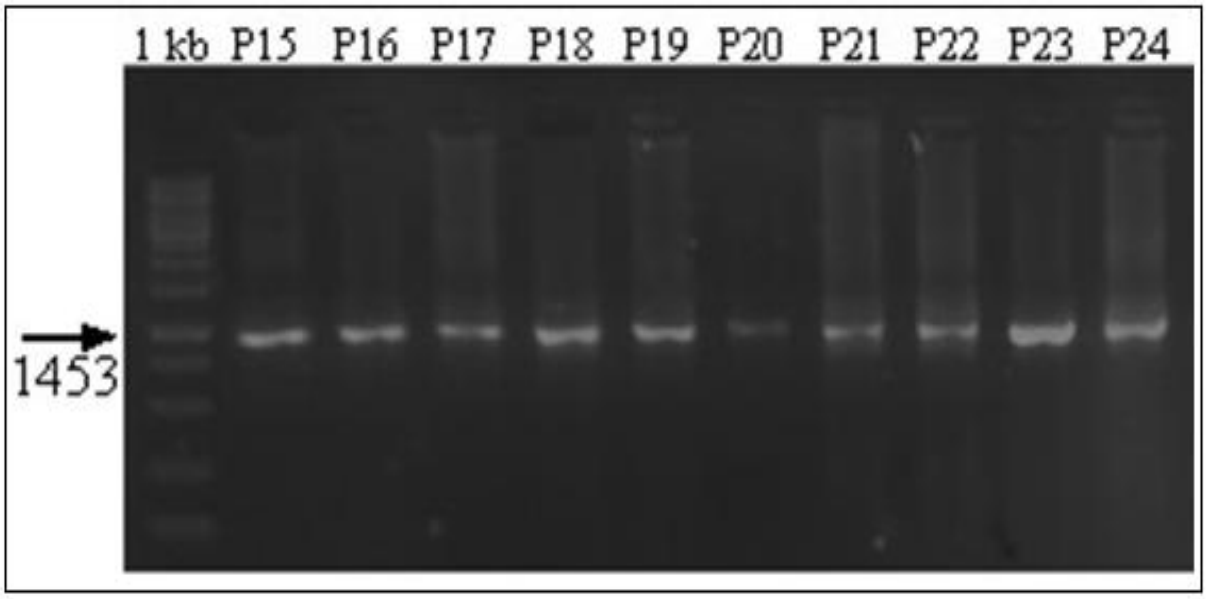


**Fig. 3b.** PCR product approximately 1453 bp fragments using *virD4* primers.

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