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SURVEILLANCE OF BACTERIAL CANKER OF PEACH IN AZAD JAMMU AND KASHMIR, AND ITS BIO-MANAGEMENT

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ABSTRACT

Bacterial canker of peach caused by *Pseudomonas syringae* is an increasing problem in Azad Jammu & Kashmir with more than 50 pathovars on the basis of host. A detailed survey was conducted in peach growing areas of Azad Jammu and Kashmir for the determination of disease prevalence and incidence. Pathogen was isolated and characterized from the collected samples using LOPAT and GaTTA scheme. Furthermore the results of biochemical identification was confirmed using PCR followed by sequencing of obtained expected bands. For the bio-management of virulent pathogenic strains indigenous rhizobacterial isolates were obtained, characterized and evaluated using dual culture as well as in a greenhouse pot experiment against *P. syringae* pathovars. During a positive sampling method used for the surveillance it was found that maximum disease prevalence was 75% in district Bagh while there was 25% disease prevalence found in Haveli and Sudhanoti. Similarly maximum disease incidence was found in district Bagh that was 16%. A total of 32 isolates were recovered from the collected samples that were further screened on the basis of their virulence. It was found that the total of 18 isolates screened were similar to the already reported strains of *P. syringae* pv. *syringae* (Pss) on the basis of biochemical and molecular tools. From total 17 rhizobacterial isolates recovered were subjected to dual culture technique and was found that 06 isolates showed promising zone of inhibition upto 11.5 mm. The results of greenhouse evaluation revealed that the minimum disease incidence was recorded 3% using R-9 and R-17 in consortium against Pss as compared to the control treatment (39%). It was concluded that the ecofriendly management practices against this destructive disease must be adopted and appreciated.

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INTRODUCTION

Bacterial diseases of peach caused by *Pseudomonas syringae* is an increasing problem in Azad Jammu & Kashmir. No work has been done on so far on its

pathological aspect and there is a need of preliminary studies to investigate the pathovars and races of *P. syringae*. It has more than 50 pathovars (Ahmed, 2022) while only two pathovars i.e. *syringae* and *morsprunorum* are responsible for bacterial canker on peach (Ahmed et al., 2016). These pathovars cause death of buds, blossom blast and tree decline due to canker and ultimately reduce the yield. Geographically, both pathovars of are distributed mainly in the colder region of the world (Renick et al., 2008). Stone fruits in the colder areas of Pakistan are severely affected by this pathogen. Although a survey to report disease losses of up to 40-60% was conducted by Haque et al. (1996) however, no work has been done on the pathogen identification. Recently, Ahmed et al. (2016) reported this pathogen was prevalent in the all visited orchards of Khyber Pakhtunkhwa (KPK) province of Pakistan and found 21 virulent isolates confirmed as *P. syringae*. Ahmed et al. (2018) further reported *Pseudomonas syringae* pv. *morsprunorum* race 1 on peach from KPK.

Using the LOPAT scheme, 32 isolates that were identified as gram-negative, levan and tobacco hypersensitivity positive, and oxidase, potato soft rot, and arginine dihydrolase negative, were confirmed to be Pseudomonas syringae. Furthermore, using the GAATa scheme, 11 isolates were identified as pathovar morsprunorum race 1, as they were negative for gelatin and aesculin hydrolysis, but positive for tyrosinase and tartrate (Ahmed et al., 2018).

According to Walker et al. (2003) complex interactions occur in a narrow zone surrounding roots of plants called as rhizosphere that also contain microbes beneficial for plant growth. According to Verma et al. (2013) these rhizospheric bacteria directly involves in growth promotion of plant so called Plant growth-promoting rhizobacteria (PGPR). These PGPRs produce hormones like auxins (Cassan et al., 2009), solubilization phosphorus from soil (Krey et al., 2013), and also suppressing plant pathogens that may cause plant diseases (Wang et al., 2009). This indirect method of pathogen suppression may be through space and nutrition competition, production of antibiotics and hydrolytic enzymes, toxin inhibition that are produced by pathogens (Weyens et al., 2009).

According to Bibi et al. (2017) ten rhizobacterial isolates were subjected to evaluate their rhizobacterial potential against bacterial canker of stone fruits and were found eight rhizobacterial isolates possessed single or multiple growth promoting character and have potential to suppress bacterial growth using dual culture method in lab conditions. There is need to evaluate rhizobacterial isolates in green house and field conditions to develop biopesticide or biofertilizers for farmers community.

The pathogenicity of three Pss isolates (PS3, PS9, and PS17) was tested, and they were found to be highly virulent on peach, plum, and apricot. The resistance of different cultivars to Pss was then evaluated by foliar spraying leaves and shoots of five peach varieties (Early Grand, Florida King, 4 ½, 5 ½, 6 ½), four plum varieties (Red Beauty, Fazal-e-Manani, Stanley, Producer), and two apricot varieties (Chinese Apricot and Golden Amber) with a mixed culture of Pss at a concentration of 10-8 cfu ml^{-1}. The sprayed cultivars were covered with plastic bags for three days to maintain moisture and kept under close observation in a glasshouse for symptom appearance. Among the tested cultivars, 5 ½ peach, Fazal-e-Manani plum, and Chinese Apricot were found to be resistant to Pss, 6 ½ peach and Stanley plum were susceptible, while Golden Amber apricot was moderately susceptible to Pss (Bibi et al., 2022).

Out of 40 initial isolates of rod-shaped Gram-negative bacteria, 12 were confirmed to be *Pseudomonas syringae* pv. *syringae*, while the rest were identified as belonging to the *Stenotrophomonas*, *Xanthomonas*, and *Erwinia* genera. To combat the bacterial canker pathogen, potential biocontrol agents in the form of *Streptomyces* bacteria strains were screened and selected via in vitro experiments and tested on apricot seedlings under in vivo conditions (Doolotkeldieva and Bobusheva, 2020).

MATERIALS AND METHODS

Pathogen

An extensive survey of peach growing areas of AJK was done for determination of disease prevalence and incidence of bacterial canker of peach on the basis of symptomology. Collected infected samples were disinfected, washed, blot dried followed by direct plating and streaking methods, then incubated on nutrient agar media for 24-48h at 27±2 °C for isolation. King's B media was used for the purification by picking single colony from 24-48h old culture and was streaked on King's B media followed by incubation.

Pathogenicity test

Pathogenicity was done to check the virulence of recovered isolates on fresh peach fruits. Healthy fruits were disinfected using ethanol and were treated with 1ml

bacterial suspension of recovered isolates followed by incubation at 27±2 °C for 4-5 days (Ahmed et al., 2018). All the treatments were repeated thrice. Distilled water was used as control treatment while a positive control treatment (*P. syringae* pv. *syringae*) was also be used.

Identification of Pathogenic isolates

Identification of recovered isolates were done using LOPAT and GAATa tests performed to identify specie as well as pathovar as suggested by (Ahmed et al., 2018; Kaluzna et al., 2013). To reconfirm the results of biochemical tests and the identification of races, PCR was done for the amplification of 16SrRNA (Kaluzna et al., 2013).

Biocontrol agents

Soil samples were collected from the root zones of peach trees and were processed using serial dilution method for the isolation of rhizobacterial isolates on nutrient agar media followed by incubation at 25±2 °C for 24-48h. Again Purification was done by picking single colonies from 24-48h old cultures.

Rhizobacterial properties of recovered isolates

All the recovered rhizobacterial isolates were evaluated for their rhizobacterial properties i.e. HCN production, Siderophore production, Biofilm formation.

***In-vitro* evaluation of recovered rhizobacterial isolates**

Dual culture technique was used for the evaluation of recovered rhizobacterial isolates against *P. syringae* pathovars. Experiment was repeated thrice and distilled water was used as negative control while already reported *P. syringae* strain was used as positive control to compare the results.

Greenhouse evaluation of recovered rhizobacterial isolates

The efficacy of recovered rhizobacterial isolates were tested on most susceptible Peach germplasm collected from registered nursery in Swat. One-year old peach seedlings were grown in sterilized soil under greenhouse conditions and rhizobacterial isolates were inoculated using soil drenching method. Two-weeks after the inoculation of rhizobacterial isolates, foliar inoculation of pathogenic isolates was done using injection method and spry inoculation consecutively. All the experiments were repeated thrice. Again Distilled water was used as negative control while already reported *P. syringae* strain was used as positive control.

RESULTS AND DISCUSSION

A systematic survey of peach growing areas of four districts of Poonch division (Poonch, Haveli, Bagh and Sudhanoti) was done for the calculation of bacterial canker disease prevalence and incidence on peach trees. Positive sampling method was adopted in the areas where planting of peach trees were not uniform and scattered. It was observed that Maximum disease prevalence was 75% in district Bagh followed by 50 % in district Poonch while there was 25% disease prevalence found in Haveli and Sudhanoti (Figure 1).

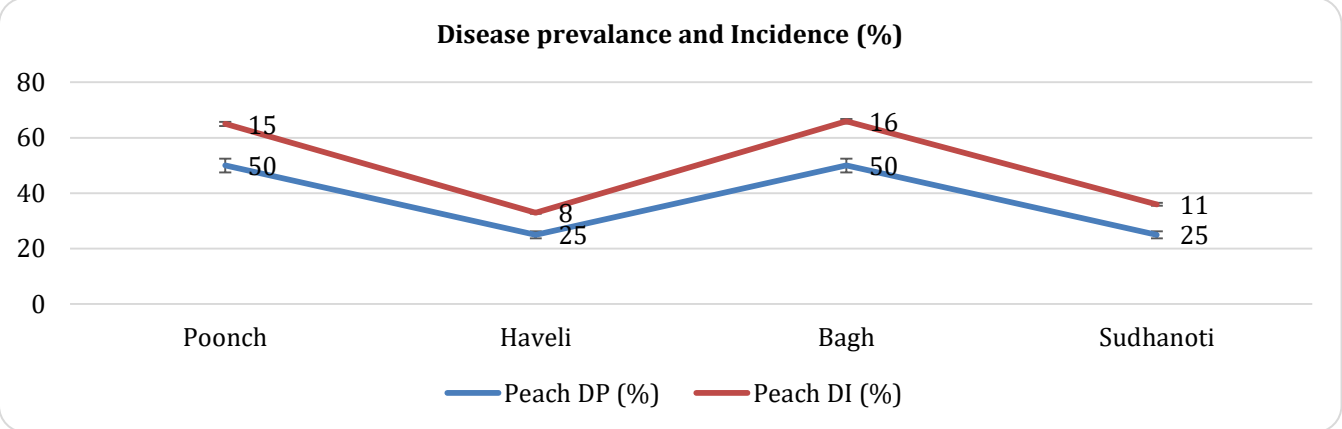

Figure 1. Disease prevalence and disease incidence of bacterial canker on peach in four districts of Poonch division.

For determination of bacterial canker disease incidence, 12 trees were selected in each orchard using X-plus sampling method. In contrary 10 trees were selected in each area selected during positive sampling where peach tree plantation is not uniform and scattered. Again it was observed that Maximum disease incidence was 16%, in

district Bagh followed by district Poonch (15%), Sudhanoti (11%) and Haveli (8%) (Figure 1). All these results were on the basis of symptoms observed in the field and were compared with the symptoms of bacterial canker that were already reported and available in the literature.

Pathogenicity test

A pathogenicity trial was conducted on all 32 bacterial isolates using fresh and healthy peach fruit. After incubation at 27 ± 2 ºC for 3-4 days, it was observed that 19 of the isolates displayed clear canker necrotic symptoms on the fruit, indicating a positive result for pathogenicity (Table 1). Our research aligns with the findings of Kałużna and Sobiczewski (2009), which demonstrated that fruitlets of cherry can be utilized for testing the pathogenicity of bacteria. This testing method not only enables the assessment of virulence through canker development, but also facilitates pathovar differentiation.

Table 1. Pathogenicity of bacterial isolates on fresh peach fruits using pin prick method.

S. No.	Isolates	Response	S. No.	Isolates	Response
1	AJKP-1	+	17	AJKP-17	+
2	AJKP-2	-	18	AJKP-18	+
3	AJKP-3	-	19	AJKP-19	-
4	AJKP-4	-	20	AJKP-20	-
5	AJKP-5	+	21	AJKP-21	+
6	AJKP-6	+	22	AJKP-22	+
7	AJKP-7	-	23	AJKP-23	-
8	AJKP-8	-	24	AJKP-24	+
9	AJKP-9	+	25	AJKP-25	-
10	AJKP-10	+	26	AJKP-26	+
11	AJKP-11	-	27	AJKP-27	-
12	AJKP-12	+	28	AJKP-28	+
13	AJKP-13	-	29	AJKP-29	+
14	AJKP-14	+	30	AJKP-30	-
15	AJKP-15	+	31	AJKP-31	+
16	AJKP-16	+	32	AJKP-32	+

Biochemical identification

All the 19 virulent isolates shown positive levan formation test while inoculated on sucrose supplemented nutrient agar media develop mucoid, dome like colonies on sucrose media. Also reaction with oxidase, rottening of potato slice and Arginine dihydolysis was negative (Table 2). They were not able to produce pectolytic ability so potato slices were not rotten when isolates were applied and incubated confirmed all the isolates were *P. syringae*. The results were similar with the previous findings of El-Siesy (2007), reported that three isolates shown positively with hypersensitivity test, levan formation while negative in case of potato soft rot and oxidase activity were characterized as *P. syringae* isolates.

Also the findings were very close to the previous reports that to identify the specie level of *Pseudomonas* set of five tests named LOPAT test are very useful tool that proves positive results for Levan production and hypersensitive response while negative for oxidase test and arginine test but variable results with soft rot of potato (Kałużna et al., 2010). *P. syringae* have more than 50 pathovars on the basis of their hosts, two pathovars *syringae* and *morsprunorum* of *P. syringae* bacterium are responsible to cause bacterial canker disease on stone fruits (Ahmed, 2022). After LOPAT tests it was confirmed that all the 19 virulent isolates were *P. syringae* (Table 3) and the pathovars confirmation was done using GATTa test developed by Lelliot and Stead (1987). GATTa scheme confirmed that all the 19 isolates were *P. syringae* pv. *syringae* shown positive Gelatin and aesculin hydrolysis while negative tyrosinase activity and tartrate utilization. According to Schaad et al. (1980) it was reported that GATTa scheme was negative for Gelatin and aesculin hydrolysis while positive for tyrosinase and tartrate utilization and also negative for L-lactate utilization test, that was (--++-) in case of pathovar *morsprunorum* race 1 while in case of

morsprunorum race 2 it may me positive or negative (Kaluzna et al., 2013).

Table 2. LOPAT scheme for the identification of *P. syringae* according to Lelliott and Stead (1987).

S. No.	Isolates	Levan Production	Oxidase Test	Potato Rot	Arginine hydrolysis	Tobacco hypersensitivity
1	AJKP-1	+	-	-	-	+
2	AJKP-5	+	-	-	-	+
3	AJKP-6	+	-	-	-	+
4	AJKP-9	+	-	-	-	+
5	AJKP-10	+	-	-	-	+
6	AJKP-12	+	-	-	-	+
7	AJKP-14	+	-	-	-	+
8	AJKP-15	+	-	-	-	+
9	AJKP-16	+	-	-	-	+
10	AJKP-17	+	-	-	-	+
11	AJKP-18	+	-	-	-	+
12	AJKP-21	+	-	-	-	+
13	AJKP-22	+	-	-	-	+
14	AJKP-24	+	-	-	-	+
15	AJKP-26	+	-	-	-	+
16	AJKP-28	+	-	-	-	+
17	AJKP-29	+	-	-	-	+
18	AJKP-31	+	-	-	-	+
19	AJKP-32	+	-	-	-	+

Table 3. GATTa scheme for the confirmation of pathovars in *P. syringae* according to Lelliott and Stead (1987).

S. No.	Isolates	Gelatin Hydrolysis	Aesculin Hydrolysis	Tyrosinase Activity	Tartrate Utilization
1	AJKP-1	+	+	-	-
2	AJKP-5	+	+	-	-
3	AJKP-6	+	+	-	-
4	AJKP-9	+	+	-	-
5	AJKP-10	+	+	-	-
6	AJKP-12	+	+	-	-
7	AJKP-14	+	+	-	-
8	AJKP-15	+	+	-	-
9	AJKP-16	+	+	-	-
10	AJKP-17	+	+	-	-
11	AJKP-18	+	+	-	-
12	AJKP-21	+	+	-	-
13	AJKP-22	+	+	-	-
14	AJKP-24	+	+	-	-
15	AJKP-26	+	+	-	-
16	AJKP-28	+	+	-	-
17	AJKP-29	+	+	-	-
18	AJKP-31	+	+	-	-
19	AJKP-32	+	+	-	-

Molecular identification

On the basis of PCR results with universal primer (16s rRNA) isolates with approximate 1400 bp were purified (Figure 2) and were send to Macrogen Korea for sequencing. All the obtained sequences were aligned using Bio-Edit software followed by BLAST on NCBI web and then final sequences were submitted to NCBI GenBank database. MEGA 7.0 was used for phylogenetic analysis of all the sequences obtained with 16s RNA. Phylogenetic analysis revealed that the isolates were 97-100% similar with the previously submitted isolates of *P. syringae* pv. *syringae*. Tree was constructed by using maximum likelihood method in MEGA version 7 and on the basis of genetic similarity tree was divided into two clusters (Figure 3).

The results were similar to the previous studies that amplification of 16s rRNA gene with PCR and then sequencing determines 97% or more similarity while comparison of 21 *Pseudomonas* species with already published strain of *P. aeruginosa* (Toschka et al., 1988).

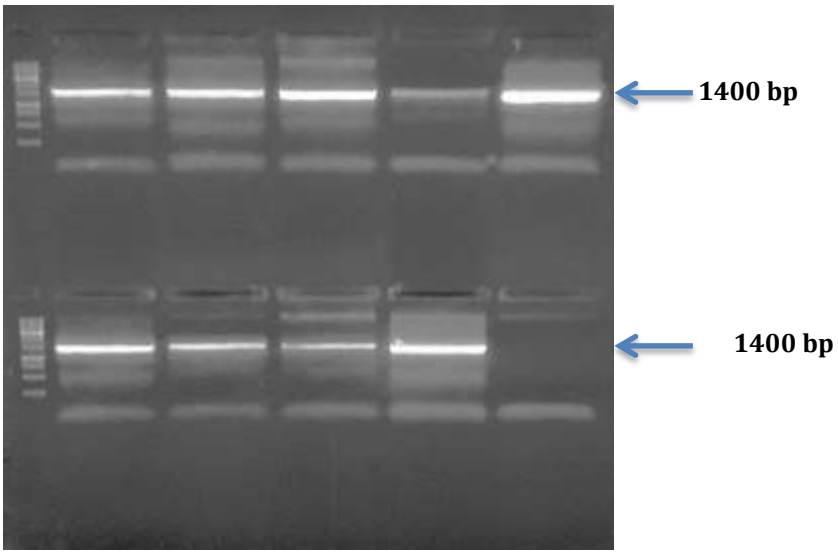

Figure 2. Amplified PCR product with approximately 1400 bp fragments using 16sRNA primer.

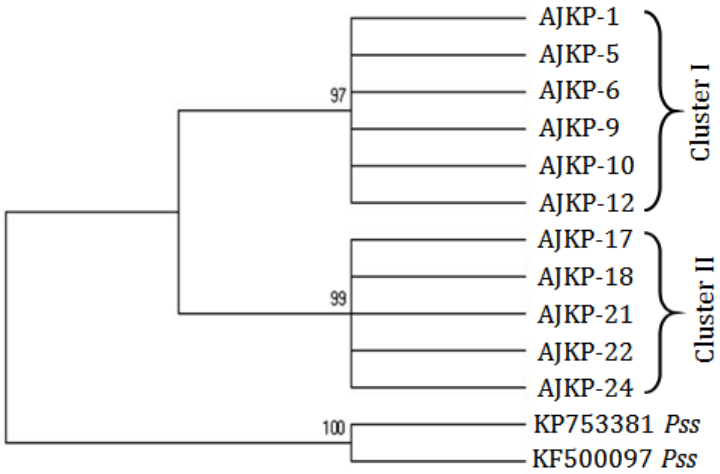

Figure 3. Phylogenetic tree constructed using maximum likelihood method *Pseudomonas syringae* pv. *syringae* isolates using 16S rRNA primers.

Isolation and *in vitro* evaluation of rhizobacterial isolates

Total 17 isolates were recovered from the soil samples using serial dilution technique followed by streaking on nutrient agar media for the growth of bacteria colonies and were subjected to dual culture technique for

evaluation of its inhibition potential against *P. syringae* pathovars. Results revealed that 06 isolates shown maximum zone of inhibition that suppressed the growth of pathogenic bacteria and were subjected for further studies. Maximum zone of inhibition was recorded for isolate R-17 (11.5 mm), while 11 isolates showed minimum or no zone of inhibition and were considered negative and discarded. Isolates having average zone of inhibition ranged from 9-11.5 mm in diameter were selected for further studies (Figure 4).

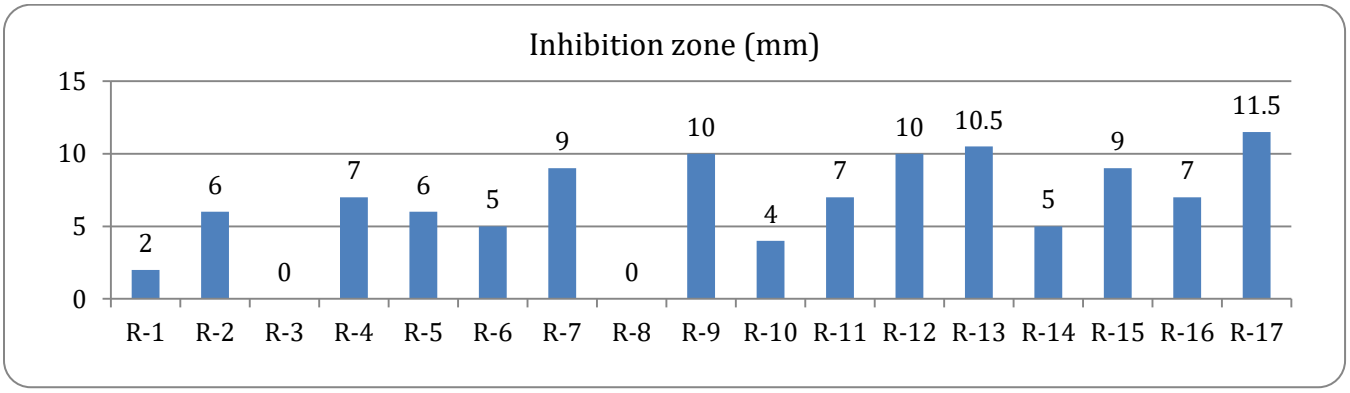

Figure 4. Evaluation of isolated rhizobacterial isolates against *P. syringae* pv. *syringae.*

Rhizobacterial properties

Rhizobacterial properties of selected isolates were tested and were found that all the isolates produce siderophore positive results while in case of HCN production two isolates showed negative results for HCN production. Biofilm formation was also studies and the results showed that all the isolates changed light to dark yellow color indicated that bacterium produce biofilm (Table 4).

Table 4. Study of rhizobacterial properties of selected isolates.

S. No	Isolates	Gram Test	KOH Test	Siderophore production	HCN Production	Biofilm Formation
1	R-7	-	+	+	+	+
2	R-9	+	-	+	+	+
3	R-12	-	+	+	-	+
4	R-13	-	+	+	+	+
5	R-15	+	-	-	+	+
6	R-17	+	-	+	+	+

Greenhouse evaluation of rhizobacterial isolates

The selected isolates showed maximum results in *in-vitro* conditions, also having rhizobacterial properties were subjected for green house evaluation alone and in consortium. A total of 14 treatments were applied in the green house experiment (Table 5). One week prior to the application of inoculum, all the rhizobacterial treatments were prepared in distilled water maintaining 1×10^8 CFU/mL bacterial concentration and were applied using soil drenching method in the pots. A set of non-inoculated plants were considered as control. Plants are maintained with proper watering and fertilizer requirements. After each 7 days data was recorded.

The results revealed that the minimum disease incidence was recorded 3% and 4% in T13 and T9 respectively while using the rhizobacteria in consortium as compared to the control treatment showing disease incidence 39% (Figure 5). While disease incidence was recorded 7% when all the rhizobacterial isolates were used in a single treatment this may be due to the incompatibility of one or more bacteria with each other.

The result of the current study was found similar with the previous findings that one year old seedlings of peach were maintained in pots under greenhouse conditions. *P. syringae* inoculum was prepared by harvesting 24 h old bacterial cultures on nutrient agar media in double distilled water and were homogenized and found promising results of disease suppression with

minimum disease incidence percentage as compared with control (Boukaew et al., 2017).

Table 5. Treatments used in greenhouse evaluation of rhizobacterial isolates against *P. syringae* pv. *syringae.*

Treatments	Isolates	Treatments	Isolates
T0	Control	T8	R-9 + R-13 + *Pss*
T1	R-7+ *Pss*	T9	R-9 + R-17 + *Pss*
T2	R-9 + *Pss*	T10	R-13 + R-17 + *Pss*
T3	R-13 + *Pss*	T11	R-7 + R-9 + R-13+ *Pss*
T4	R-17 + *Pss*	T12	R-7 + R-9 + R-17 + *Pss*
T5	R-7 + RH-9 + *Pss*	T13	R-9 + R-13 + R-17 + *Pss*
T6	R-7 + RH-13 + *Pss*	T14	R-7 + R-9 + R-13 + R-17 + *Pss*
T7	R-7 + R-17 + *Pss*		

Pss = P. syringae pv. syringae

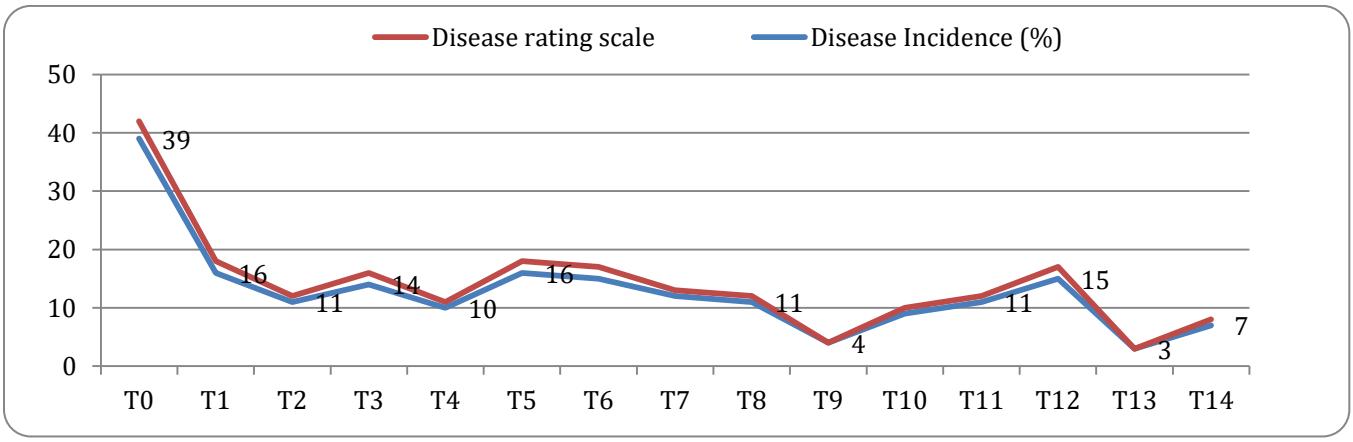

Figure 5. Evaluation of different treatments of rhizobacteria alone and in consortium against *P. syringae* pv. *syringae*.

CONCLUSION

It was concluded that bacterial canker disease caused by *p. syringae pv. syringae and morspnorum* was prevailed in almost all the peach growing areas of AJK, Pakistan. Ecofriendly management practices against this destructive disease must be adopted and appreciated. Bio-control agents should be used as biological control agents against this disease. Quarantine practices must be adopted to limit the disease in specified areas. Results of the research project will be helpful in breeding programs to develop resistant varieties against bacterial canker.

AUTHORS' CONTRIBUTIONS

All the authors contributed equally in the manuscript write up, editing and trail.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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