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Original paper

Diversity, pathogenicity and biocontrol efficacy of Pseudomonas syringae isolated from plants in northern Jordan

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Abstract Diversity, pathogenicity, and biocontrol efficacy of *Pseudomonas syringae* strains isolated from a wide range of habitats in Jordan were investigated. Thirty-five infected plant samples (vegetable leaves and woody plant twig) were randomly collected. King's B agar medium was implemented and 124 colonies of *P. syringae* candidates were selected. Phenotypic, biochemical and pathogenicity tests were further carried out to confirm the identity of *P. syringae* isolates. The frequency of fluorescent isolates varied between 11- 50 %. Even though all isolates were shown to induce chlorosis of tobacco leaves, only two were able to macerate potato slices causing their rotting. This may be due to variation in secretion of cellulase and pectinase enzymes involve in pathogenicity as clearly discussed in this study. Interestingly, 55% of isolates were found to clearly inhibit growth of *E. coli*. Exceptional inhibition was noticed in *P. syringae* isolated from tomato and a possible effect of syringomycin was suggested. Therefore, *P. syringae* obtained in this study was recommended to have a pivotal role in the biological control system. Results obtained from the experiment of ice nucleation activity revealed that this phenotypic feature dominates woody plants.

Keywords *Pseudomonas syringae,* cellulase, pectinase, ice nucleation activity, biocontrol efficacy, and King's B medium.

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Introduction

Since *Pseudomonas syringae* is first described as a plant pathogen in 1902, outbreaks of infections around the world have been documented and greater economic loss in a wide variety of crops has been reported (Cunty *et al.,* 2015, Berge *et al.,* 2014). Pathogenicity of some *P. syringae* pathovars is associated with the secretion of extracellular enzymes namely cellulase, pectinase and gelatinase cause tissue maceration or soft rot disease (Skerman *et al*., 1980; Andro *et al.,* 1984). *P. syringae* has two interconnected growth phases; epiphytic and endophytic phase. One of the most important features of epiphytic phase is the ice nucleation activity that known to produce ice from water supercooled at a temperature above normal freezing condition (Lindow *et al.,* 1982, Bultreys and Kaluzna, 2010). This activity creates openings in the hos plant releasing water and nutrients and leads to the frost injury which subsequently facilitation of infection by other pathogens (Xiu-Fang *et al.,* 2018).

Isolation and identification of this deleterious bacteria are laborious due to the considerable phenotypic and genotypic diversity. Additionally, an outgrowing list of newly isolated strains further complicate the scene. Phenotypic tests including production of levan, cytochrome c oxidase, arginine dihydrolase, as well as tobacco pathogenicity, potato rotting, ice nucleation activity and cross pathogenicity tests are routinely used to identify *P. syringae* isolates (Young *et al.,* 1996, Berge *et al.,* 2014). *P. syringae, a* fluorescent Pseudomonas, fluoresces under ultraviolet radiation due to the production of extracellular yellow green pigment (Nobutaka *et al.*, 2020) when cultivated on King's B agar selective medium (King *et al*., 1954).

Several studies reported the isolation of fluorescent pseudomonads showing high biocontrol efficacy. Effective strains were reported to protect host plants from across the plant kingdom in a wide range of environments (Takeuchi *et al.,* 2015; Ma *et al.,* 2016; Nandi *et al.,* 2017; Nobutaka *et al.,* 2020). As such, investigating the diversity of florescent pseudomonads from various habitats and plant species is a priority. In this study isolation, identification and characterization of the fluorescent *Pseudomonas syringae* in a wide spectrum of geographical locations and agronomical crops in Jordan is presented. This includes regions located 200 m below sea level to regions located 1100 m above sea level. In addition to discrepancy in altitudes, examined regions differ largely in temperature, humidity, as well as soil physical and chemical properties. This piece of work is therefore expected to fill a gap in this field in Jordan. Candidate isolates that could exhibit biocontrol activities and protect host plants was presented.

Materials and Methods

Sample collection and bacterial isolation

Thirty-five samples were collected from a wide array of infected plants, showing symptoms of *Pseudomonas* infection, including: tomato, green pepper, squash, almond, and olive. Plant samples were collected from different locations of the north part of Jordan. Samples were initially surface sterilized and then macerated in sterilized distilled water. After that, serial dilutions in distilled water were prepared and 0.1 ml from the appropriate dilution was spread on King's B agar selective medium (King *et al*., 1954). All plates were incubated at 27 °C for 72 h and randomly selected colonies were picked for further analysis.

Identification and characterization

Colony morphology of bacterial isolates was initially used to identify *Pseudomonas* isolates. White, convex, mucous and glistening colonies on King's B medium were selected for further identification. Gram staining and basic biochemical testes routinely used to discriminate *P. syringae* including: cytochrome c oxidase, levan production, arginine hydrolysis were performed (Cindy *et al.,* 2007; Berge *et al.,* 2014).

Tobacco Pathogenicity & Potato rotting tests

Tobacco pathogenicity test was performed according to Klement method (1964). Briefly, tobacco young leaves (20-day old) were wounded with sterilized needles, and inoculated with a loopful of 48 hour-old active *Pseudomonas* cultures obtained from the collected infected plant samples. Wounded leaf inoculated with a sterilized distilled water was used as a negative control. Pathogenicity was confirmed by observing symptoms of chlorosis which generally appear after 4 days of infection.

For potato rotting test, tubers were surface sterilized, peeled and sliced (1 mL thickness each). 100 µL of 48 hour-old active *Pseudomonas* cultures were spread on the surface of these slices in sterilized Petri plates. To keep slices moist, sterilized filters papers were plated in each plate before inoculation. As a negative control 10 mL of sterilized nutrient medium were added to the potato slices under the same conditions. Soft texture of the slice surface is a direct indicator of rotting which was appeared in average after seven days of infection.

Production of extracellular enzymes

Cellulase production was examined by inoculating a loopful of 48-hour old bacterial culture on 15% (w/v) agar medium containing the following components per 1L: yeast extract (1g), sodium carboxymethylcellulose (10g), KH_2PO_4 (4g), $FeSO_4.7H_2O$ (0.5g), as well as two g each of NaCl, $CaCl_2.2H_2O$ and NH_4Cl. The final pH was adjusted to 7.0. After 3days of incubation at 27°C, plates were flooded with 1% (w/v) aqueous congo red solution for 30 minutes at room temperature. Excess stain was poured off plates, destined with 1M NaCl, and kept at 4°C overnight. Colorless zones around inoculums indicate positive results (Mushtaq *et al*., 2019).

To test pectinase activity, 48-hour old active *Pseudomonas* was inoculated on a 15% pectin agar medium containing the following ingredients/1L: pectin (5g), KH_2PO_4 (4g), NH_4Cl (2g), $CaCl_2.2H_2O$ (2g), NaCl (2g), $MgSO_4.7H_2O$ (1g), $MnSO_4$ (0.05g), $FeSO_4.7H_2O$ (0.05 g),

as well and 0.1%. yeast extract. The final pH was adjusted to 7.0 and cultures were incubated at 27°C for 3 days. Plates were then flooded with 1% (w/v) acetyltrimethylammonium bromide and destined with 15% alcohol solution. Colorless zones around the inoculums, generally appear after 30 minutes, indicate positive results (Mushtaq *et al.,* 2019).

Ice nucleation activity and Induction of biotoxins against *E. coli*

To perform ice nucleation activity, freshly grown bacterial cultures were inoculated on a 15% agar medium containing per one L five g each of yeast extract, glucose, and peptone. Plates were incubated at -4.0°C. Crystal formation, usually appears after five minutes, is an indication of positive results (Berge *et al.,* 2014).

Induction of biotoxins against *E. coli* B strain was performed following Gasson method (1980). In this assay, sterilized cotton swabs were used to add 10^8 colony-forming cells of *E. coli* to Mueller Hinton agar medium. Cork borer was then used to create 5 mm diameter wells. Broth filtrate (50 µl) of freshly grown *Pseudomonas* cultures obtained from infected plants were placed in wells. Plates were then incubated at 27°C for 24h and clear zones around wells indicate positive biotoxin production against *E. coli*.

Results and discussion

This study was intended to characterize pathogenic *Pseudomonas* bacteria isolated from a wide taxonomic group of infected plants in different places of Jordan. Out of 35 samples obtained from infected tomato, green pepper, squash, almond, and olive, 124 colonies were isolated using King's B agar selective medium (King *et al*., 1954). The percentage of fluorescent inducing colonies was found to be 24% and varied from 11 % in olive and green pepper to 50 % in apple (Figure 1). Even in the same plant, percent of fluorescent colonies was varied from one place to the other.).

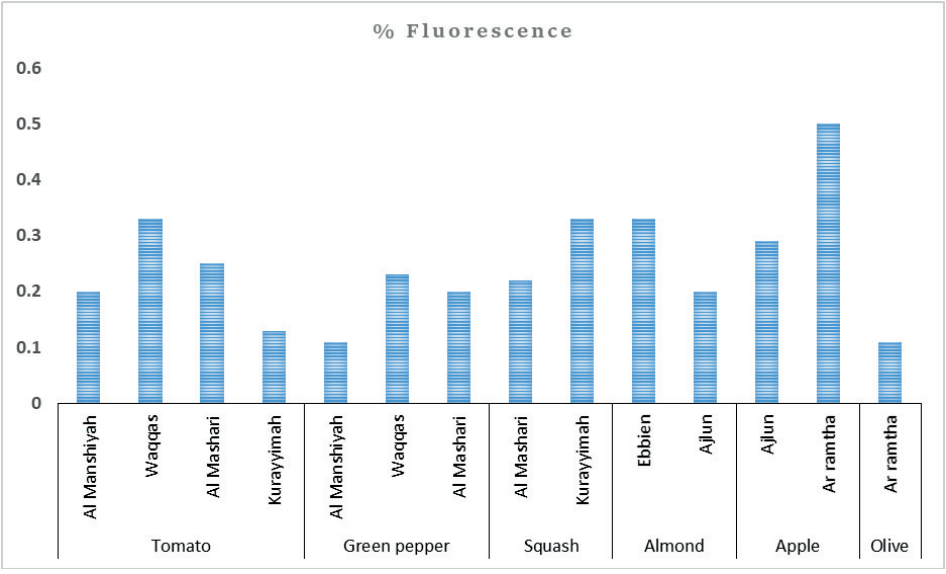

Figure 1. Percentage of fluorescent *Pseudomonas syringae* cultures (A) and the number of pathogenic isolate on tobacco leaves (B).

Pathogenicity tests by both induction of chlorosis of tobacco leaves and rotting of potato slices revealed that 18 out of 124 isolates (15%) were pathogenic. As shown in Figure 2, the highest percentage of pathogenic isolates was recovered from almond and the lowest one in green pepper.

Biological activity of pathogenic *Pseudomonas* isolates is indicated in Table 1. All isolates showed chlorosis or yellowish spots of the tested tobacco leaves whereas only two isolates (Gm1 and Ala2) were able to induce rotting on potato slices. Most bacterial isolates were able to hydrolyze cellulose, whereas only four *Pseudomonas* isolates (Tm, Gm1, Sal2, Ala2) isolated from tomato, green pepper, squash and almond respectively showed pectinase activity. The ability to hydrolyze gelatin is restricted to seven bacterial isolates including Tk, Gm2, Sal1, Sal2, Ale, Apa2, and Or collected from tomato, green pepper, squash, almond, apple and olive respectively. Toxicity or antibiotic activity for investigated isolates was tested against *E. coli* B strain (table 1).

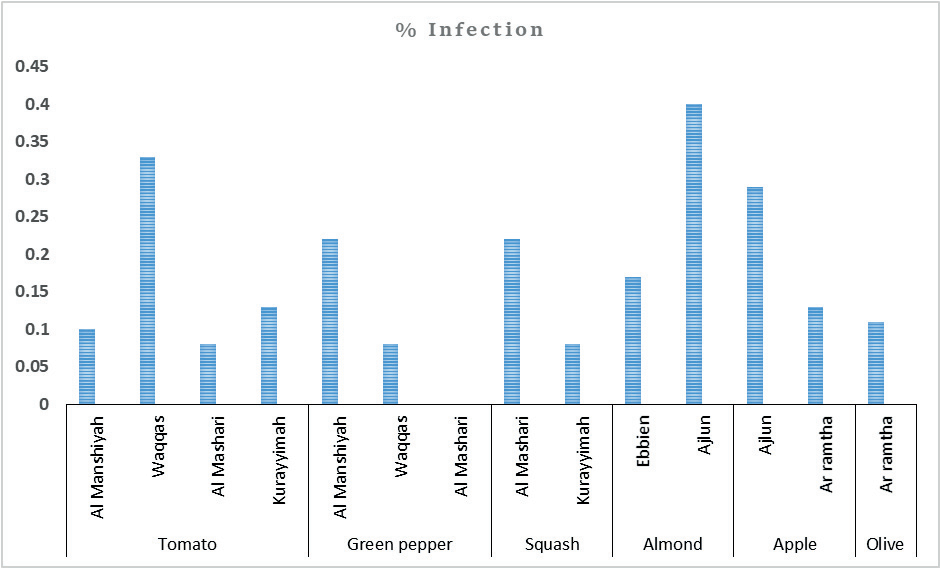

Figure 2. Number of pathogenic *Pseudomonas syringae* isolates on tobacco leaves.

Table 1. Biological activity of pathogenic isolates of fluorescent *Pseudomonas syringae.* INA= ice nucleation activity.[a] Symptoms of chlorosis as observed in the tobacco pathogenicity test. [b] Rotting as observed in the potato rotting test.

Plant material	Isolate number	Collection site	Hydrolytic Enzyme			*E.coli* inhibition	INA	Pathogenicity	
			Cellulase	Pectinase	Gelatinase			Chlrosis[a]	Rotting[b]
Tomato	Tm	Al Manshiyah	-	+	-	-	+	+	-
	Tw1	Waqqas	+	-	-	+	-	++	-
	Tw2		+	-	-	-	-	+	-
	Tk	Kurayyimah	+	-	+	+	-	++	-
	Tal	Al Mashari	++	-	-	+	+	+	-
Green Pepper	Gm1	Al Manshiyah	++	+	-	-	-	+	+
	Gm2		+	-	+	+	-	++	-
	Gw	Waqqas	+	-	-	+	-	+	-
Squash	Sal1	Al Mashari	-	-	+	+	-	++	-
	Sal2		-	+	+	+	-	+	-
	Sk	Kurayyimah	++	-	-	-	+	+	-
Almond	Ala1	Ajlun	+	-	-	-	-	+	-
	Ala2		+	+	-	-	-	++	+
	Ale	Ebbien	+	-	+	+	+	+	-
Apple	Apr	Ar ramtha	+	-	-	-	+	+	-
	Apa1	Ajlun	-	-	-	+	-	+	-
	Apa2		+	-	+	+	+	+	-
Olive	Or	Ar ramtha	+	-	+	-	+	+	-

Most of the phytopathogenic isolates were able to inhibit the growth of E. coli indicating their ability to induce biotoxin that could be used for biological control. For ice nucleation test, isolates numbers; Tm, Ta1, Sk, Ale, Apr, Apa2, and Or collected from tomato, squash, almond, apple and olive showed positive results.

Pseudomonas syringae is a common pathogen in diverse crops. The current study aimed to get a clear picture about the diversity, pathogenicity and biocontrol efficacy of P. syringae isolated from across a wide array of habitats in Jordan. This included regions located 200 m below sea level to 1100 m above sea level. The percentage of fluorescent Pseudomonas was found to be ranged from 11-50 % among the total Pseudomonas isolates collected from different habitats in Jordan. This frequency is in agreement with what was reported by Lindow et al. (1981), and Margaret & Hagedorn (1981).

Tobacco and potato show varying susceptibility to P. syringae isolates. All isolates were found to induce chlorosis of tobacco leaves. The high percentage of pathogenicity to tobacco is in agreement with Cindy et al. (2007). This could be due to host range specificity as reported by Gross et al. (1984) or cytotoxic production by the pathogenic isolates as reported by Fahy and Hayward (1983). Another possibility is that cellulase activity may be enough to induce pathogenicity to tobacco and other plants (He, 1996; Cindy et al., 2007). On the other hand, only two isolates were able to induce rotting to potato slices. This could be due to inability of isolates to secret pectinase enzyme as shown in table 1, a similar finding was obtained by Isabel et al. (1987). The scarcity of affecting potato may refer to the fact that P. syringae causes necrosis to aerial parts of plants rather than parts below the ground as the case with potato.

Among all tested bacterial isolates, 55% of them were found to inversely affect growth of E. coli (table 1). Three bacterial isolates out of five obtained from tomato were found to produce toxins and therefore inhibit growth of E. coli. Cindy et al., (2007) investigated the possibility of syringomycin production by 31 strains of P. syringae. They found that among seven host plants studied, syringomycin is almost exclusively produced by P. syringae infects tomato with a 13 mm zone of inhibition. Antimicrobial activity of the examined isolates could be due to the production of syringomycin and related biotoxins known to widely affect pathogenic bacteria. This activity makes P. syringae isolates as a possible strong candidate in the biological control system (Bender et al., 1999). On the other hand, yellowish symptoms or necrosis appears on tested plants could be due to the biotoxicity of these isolates as reported by Turner (1984) and Kang et al. (2015).

Data from the ice nucleation activity experiment revealed that unlike vegetables, ice nucleation substance is dominant in woody plants as four out of 7 isolates were found to have this phenotypic feature. Only 3 isolates out of 11 isolated from vegetables including tomato and squash were found to be ice nucleation active. Similar results were obtained for tomato by Cindy et al., (2007) who reported that a limited percent (22%) of tomato plants infected by P. syringae were found have this phenotype at an average temperature of -3 °C.

of the pathogenic isolates proved that 39% of them were with ice nucleation activity. Two of the pathogenic isolates from vegetables were with ice nucleation activity, this finding was in disagreement with Wolber et al. (1988) finding, they found that ice nucleation members are recovered from fruit pathogens especially stone fruit, this could be duo to genetic modification as the result of environmental conditions variation. Fahy and Hayward (1983) reported that 87% of their isolates were ice nucleation inducer and they were from stone fruit trees. This is an indication that there is no inconsistency of ice nucleation among phytopathogenic Fluorescent Pseudomonas.

References

1. Andro T, Chambost J, Kotoujauskya J, Cattaneo Y et al.. Mutants of *E. chrysanthemi* defective in secretion of pectinase and cellulose. *J Bact.* 1984; 160: 1199-1203.
2. Berge O, Monteil CL, Bartoli C, Chandeysson C et al. A user's guide to a data base of the diversity of *Pseudomonas syringae* and its application to classifying strains in this phylogenetic complex. *PLoS One*. 2014; 9(9): e105547. doi: 10.1371.
3. Bender CL, Alarcon-Chaidez F, Gross DC. *Pseudomonas syringae* phytotoxins: Mode of action, regulation and biosynthesis by peptide and polyketide synthetases. *Microbiol Molec Biol Rev*. 1999; 63: 266–292.
4. Bultreys A, and Kaluzna M. Bacterial cankers caused by *Pseudomonas syringae* on stone fruit species with special emphasis on the pathovars syringae and morsprunorum race 1 and race 2. *J. Plant Pathol.* 2010: S21-S33.
5. Cunty A, Poliakoff F, Rivoal C, Cesbron S et al. Characterization of *Pseudomonas syringae* pv. actinidiae (Psa) isolated from France and assignment of Psa biovar 4 to a de novo pathovar: *Pseudomonas syringae* pv. actinidifoliorum pv. nov. *Plant Pathol*. 2015; 64: 582–596.
6. Daub, Margaret E, and Hagedorn DJ. Epiphytic populations of *Pseudomonas syringae* on susceptible and resistant bean lines. *Phytopathol.* 1981; 71: 547-550.
7. Fahy P, and Hayward A. Pseudomonas: the fluorescence pseudomonas. 1983. In Plant Bacterial Disease: A diagnostic Guide, (eds). Fahy P, and Persley A. Academic Press Inc, Australia, 141-178
8. Gardan L, Shafic H, Belouin S, Broch F et al. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of tremae sp. Nov. and P.cannabina sp. nov. (ex Sutic and Dowson 1959). *Int J of Syst Bact.* 1999; 49: 469-478.
9. Gasson J. Indicator technique for antimetabolite toxin production by plant pathogenic species of Pseudomonas. *Appl Enviro Microbiol.* 1980; 39: 25-29.

10. Gross D, Cody L, Procbsting J and Spott A. Ecotypes and pathogenicity of ice-nucleation active *Pseudomonas syringae* isolated from deciduous fruit tree orchards. *Phytpathol*. 1984; 74: 241-248.
11. He SY. Elicitation of plant hypersensitive response by bacteria. *Plant Physiol*. 1996; 112: 865–869.
12. Hildebrand D. Pectate and pectin gels for differentiation of Pseudomonas sp. and other bacterial plant pathogens. *Phytopathol.* 1970; 61: 1430-1436.
13. Isabel M, Roos M and Hattingh M. Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains, isolated from deciduous fruit trees. *Phytopathol*. 1987; 77: 900-908.
14. Isabel M, Roos M and Hattingh M. Systemic invasion of cherry leaves and petioles by *Pseudomonas syringae* pv. *Morsprunorum*. *Phytopathol.* 1987; 77: 1246-1252.
15. Kang, In Jeong. Effective selection of soybean cultivars to wildfire disease pathogen *Pseudomonas amygdali* pv. *tabaci*. *J of crop sci and biotechnol*. 2015; 18(4): 279-284.
16. Khalaf H. Olive Knot Disease in Jordan. *Jordan J of Agri Sci*. 2006; 2(4): 387-400.
17. Kings EO, Ward MK and Raney DE. Two simple media for the demonstration of pyocyanin fluorescin. *J Lab Clin Med*. 1954; 44: 301-307
18. Klement Z. Rapid detection of the pathogenic pseudomonades. *Nature*. 1964; 199: 299-300.
19. Lelliott A, Billing G and Hayward EC. A degenerative scheme for the fluorescent plant pathogenic pseudomonades. *J of Appl Bact.* 1966; 29: 470-489.
20. Lindow E, Hirano S and Upper D. Relationship between ice nucleation frequency of bacteria and frost injury. *Plant Physiol*. 1982; 70: 1090-1093.
21. Ma Z, Geudens N, Kieu NP, Sinnaeve D et al. Biosynthesis, chemical structure, and structure-activity relationship of orfamide lipopeptides produced by Pseudomonas protegens and related species. *Front Microbiol*. 2016; 7: 382.
22. Mahaureh B. Isolation and characterization of Pseudomonads isolation from soil and root of the citrus trees. Master thesis, *Yarmook Univ*. 1987; 126
23. Margaret E and Hagedom D. Epiphytic populations of *Pseudomonas syringae* on susceptible and resistance bean lines. *Phytopathol.* 1981; 70: 5-8.
24. Mushtaq S, Shafiq M, Ashraf T, Haider MS et al. Characterization of plant growth promoting activities of bacterial endophytes and their antibacterial potential isolated from citrus. *The J of Animal & Plant Sci.* 2019; 29(4): 978-991.
25. Morris CE, Kinkel LL, Xiao K, Prior P et al. Surprising niche for the plant pathogen *Pseudomonas syringae*. *Infect Genet Evol*. 2007; 7(1): 84-92.
26. Nandi M, Selin C, Brawerman G, Dilantha F et al. Hydrogen cyanide, which contributes to *Pseudomonas chlororaphis* strain PA23 biocontrol, is upregulated in the presence of glycine. *Biol Control*. 2017; 108: 47–54.
27. Nobutaka S, Masaharu K, Kasumi T, Yusuke U et al. Diversity of Antibiotic Biosynthesis Gene-possessing Rhizospheric Fluorescent Pseudomonads in Japan and Their Biocontrol Efficacy. *Micro Env*. 2020; 35(2): doi:10.1264/jsme2.ME19155
28. Schippers B, Schippers A and Bakker P. Interactions of deleterious and Beneficial rhizosphere microorganisms and the effect of cropping practices. *Ann Rev Phytopathol*. 1987; 25: 339-358.
29. Skerman VBD. A guide to the Identification of the Genera of Bacteria. Baltimore, Maryland, Williams and Wilkins. 1961. 2nd ed. https://doi.org/10.1002/jobm.19610010307.
30. Skerman D, Mcgowan V and Sneath A. Approved list of bacterial name. *Int J of Syst Bact.* 1980; 30: 225-420
31. Takeuchi K, Noda N, Katayose Y, Mukai Y et al. Rhizoxin analogs contribute to the biocontrol activity of newly isolated Pseudomonas strain. *Mol Plant-Microbe Interact.* 2015; 28: 333–342.
32. Turner JG, and Taha RR. Contribution of tabtoxin to the pathogenicity of *Pseudomonas syringae* pv. *tabac*i. *Physiol Plant Pathol.* 1984; 25(1): 55-69.
33. Wolberts DP, Denny TP and Schell MA. Cloning of egl gene of *Pseudomonas selanacearum* and analysis of its role in phytopathogenicity. *J Bact*. 19881; 70: 1445-1451.
34. Xin, Xiu-Fang, Brian K, and Sheng YH. *Pseudomonas syringae*: what it takes to be a pathogen. *Nat Rev Microbiol.* 2018; 16(5): 316.
35. Xu A and Gross D. Evaluation of the role of syringomycin in plant pathogenisis by using Tn 5 mutants of *Pseudomonas syringae* pv *syringae*. *Appl Enviro Microbiol.* 1988; 84: 1345-1353.
36. Young JM, Saddler GS, Takikawa Y, Boer SH et al. Names of plant pathogenic bacteria 1864-1995. *Rev Plant Pathol.* 1996; 75(9): 721–763
37. Young J, Takikawa L, Gardan L and Stead D. Changing concepts the Taxonomy of plant pathogenic bacteria. *Annu Rev Phytopathol.* 1992; 30: 67-105.