JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2017. Vol. **11**(3), p. 1293-1298

Activation of Syringomycin and Syringopeptin, Two Major Toxins of *Pseudomonas syringae* pv. *syringae* by three Cherry Cultivars

Maasoomeh Erfaninik, Rasool Rezaei* and Habiballah Charehgani

Department of Plant Protection, College of Agriculture, Yasouj University, Yasouj, Iran.

http://dx.doi.org/10.22207/JPAM.11.3.09

(Received: 15 June 2017; accepted: 08 August 2017)

***Pseudomonas syringae* pv. *syringae* (*Pss*) is a gram negative plant pathogenic bacteria that cause severe disease on more than 150 various plant species. *Pss* is the most frequently bacterial pathogens on crops that make high economical damages in Iran. Studies showed that this bacterium cause significant yield losses in main crops in Iran. During infection of host plants by this organism, damage to plant cells will occur by production of virulence factors. This pathovar produce two cyclic lipodepsipeptide phytotoxin families including syringomycins (SR) and syringopeptins (SP). The strains of *Pss* were isolated from apricot, cherry, peach and wheat in Southwest Iran. To find out whether production of syringomycin and syringupptin in different isolates is affected by plant material, inhibitory effect of these toxins on *Geotrichum candidum* and *Bacillus megaterium* were studied. When no plant extracts were added to the SRM medium, the most intense inhibitory effect on the *G. candidum* was observed in isolate C1. The highest production of syringomycin was achieved by this strain in the presence of Takdane cultivar leaf extract. The production of syringomycin and syringopeptin by all isolates was significantly higher in the presence of plant extracts on SRM and PDA agar medium.**

Keywords: Iran; Plant extracts; *Pss*; Toxin.

Due to its geographic location and climate, Iran is one of the major producers of stone fruits in the world. One of the ways to increase garden products is to fight against pests and diseases of these plants. One of the most dangerous diseases of stone fruits is bacterial canker caused by *Pseudomonas syringae* pv. *syringae* (*Pss*). This disease reduces the quality and quantity of the product and reduces the life of the garden, especially in cherries, and can also damage young and old gardens sometimes up to 85 percent (Bultreys & Kaluzna, 2010). Currently, bacterial canker of stone fruits in most parts of the world damage apricots, plums, peaches and cherries. In Iran, bacterial canker disease was reported for the first time from apricot trees in Isfahan and *P. syringae* was reported as causal agent (Bahar *et al.,* 1982). It seems that the concept of pathovar for this bacteria is not applicable, because it infect more than 150 different plant species (Kennelly *et al.,* 2007). Epidemiologic studies indicate that the disease has two epiphytic and endophytic phases. Therefore, the bacteria can be safely multiply in the leaves of the host plants throughout the growth season. During contamination, *Pss* uses various secretion systems to transfer proteins into plant cells (Feil *et al.,* 2005). This bacterium produces many pathogenicity factors that can infect wide range of plants. Many strains of *Pseudomonas syringae* pv. syringae are known to produce cyclic lipodepsipeptides (CLPs) as secondary metabolites such as syringomycin and syringopeptin. The CLPs are considered to be plant virulence factors

* To whom all correspondence should be addressed.
E-mail: rrezaei@yu.ac.ir

and antifungal agents (Quigley & Gross, 1994). They affect plant membrane activities and induce necroses at relatively high concentrations (Guenzi *et al.,* 1998), but the relationship of these effects to plant diseases has not been clearly established. Both toxins have strong antibacterial and antifungal activity. It has been shown that these secondary metabolites help colonizing of bacteria in the host and promote bacterial growth in intercellular space (Lu *et al.,* 2002). Apparently, syringomycin production was unique to *Pss* strains and was not reported by other *pseudomonas* spp. The aim of this study was to investigate the effect of leaf extracts of three different cherry cultivars (cultivated in Iran) on the production of syringomycin and syringupoptin by *Pseudomonas syringae* pv. *syringae*.

MATERIAL AND METHODS

Bacterial strains

The bacterial isolates used in the study are listed in Table 1. The strains of *Pss* were isolated from apricot, cherry, peach and wheat in Fars and Kohgiluyeh and Boyer-Ahmad provinces. The isolates were examined for gram reaction, catalase, oxidase, ultraviolet fluorescence, argenin dihydrolase, levan production, gelatin liquefaction, aesculin hydrolysis, hypersensitive reaction on tobacco leaves and pectolytic capability (Schaad *et al*., 2001). All isolates were stored in water suspensions (10^6 cells/ml) at 4°C and subcultured on king medium B (Little *et al*., 1998).

Virulence testing on sweet cherry fruitlets

Freshly collected immature sweet cherry fruits cv. 'Takdane' were disinfected by dipping in 50% ethanol for 3 min and then rinsed three times in sterile distilled water. Afterwards, fruitlets were wrapped with paper towel to remove the excess of water. Each fruitlet was inoculated by pricking in two places to the depth of 2 mm with sterile needle previously immersed in suspension of each strain. After inoculation each fruitlet was immediately placed on moist filter paper in sterile Petri dish and incubated at 24°C for four days. The reference strains and sterile distilled water were included as positive and negative control, respectively. Ten fruitlets were used for testing of each strain.

Bioassays for syringomycin production

Different strains of *Pss* were used to determine the amount of syringomycin and syringopeptin production in the absence of leaf extract and in the presence of leaf extract of Cherry cultivars Takdane, Surati and Ghaheri. These strains were evaluated for syringomycin production on SRM (Syringomycin minimal) media by using standard bioassays as previously described by Scholz-Schroeder and associates (2001). 15 ml of this culture medium was transferred in each Petri dish. 10 µl of 10^7 CFU/ml suspension from each bacterium was placed in the center of each SRM medium and kept at 23°C for five days. Petri dishes were sprayed with the suspension of the fungus *Geotrichum candidum* and after 24 to 48 hours the diameter of the inhibition zones was measured (Schaad *et al.,* 2001). For the detection of syringopeptins *Bacillus megaterium* should be used as toxin indicator strain, since *G. candidum* is insensitive to syringopeptins. In order to investigate the effect of leaf extract on the production of toxin, 10 g of leaf tissue in 50 ml sterilized distilled water was completely dissolved. The extract was then sterilized through 0.45 µL pore size filter. For each 15 ml of SRM medium, 5 ml of herbal extract was used.

Bioassays for syringopeptin production

The PDA (Potato Dextrose Agar) medium was used to study the production of syringopeptin. *P. fluorescense* was also used as a negative control. For each 15 ml culture medium, 5 ml of leaf extract of different cultivars of cherry was added. In each Petri dishes, about 15-20 ml of medium was used. 10 µl of bacterial suspension was placed in the center of each Petri dishes, and stored for six days at 23°C. After six day, the suspension of *Basillus megaterium*, sprayed onto Petri dishes. After 24-48 hours, the inhibition zone diameter was measured.

Statistical analysis

The experiment was carried out in a completely randomized design and data were subjected to ANOVA and means were separated according to the Duncan's multiple range test. Data were analyzed by SAS software.

RESULTS AND DISCUSSION

The strains of *Pss* were isolated from apricot, cherry, peach and wheat in Fars and Kohgiluyeh and Boyer-Ahmad provinces. All *P. syringae* pv. *syringae* strains used in this study

were negative for oxidase, potato rot, and arginine dihydrolase, but, positive for levan production and the hypersensitive response on tobacco. All strains caused symptoms showing disorders around wounds on fruitlets. After 24 h from fruit inoculations with strains of *Pss* deep black brown necroses were observed, while strain of *P. fluorescens* did not induce any symptoms. To find out whether production of syringomycin and syringupptin in different isolates is affected by plant material, inhibitory effect of these toxins on *G. candidum* and *B. megaterium* were studied. When no plant extracts were added to the SRM medium, the most inhibitory effect on the *G. candidum* was observed in isolate C1 (Fig. 1). The highest production of syringomycin was

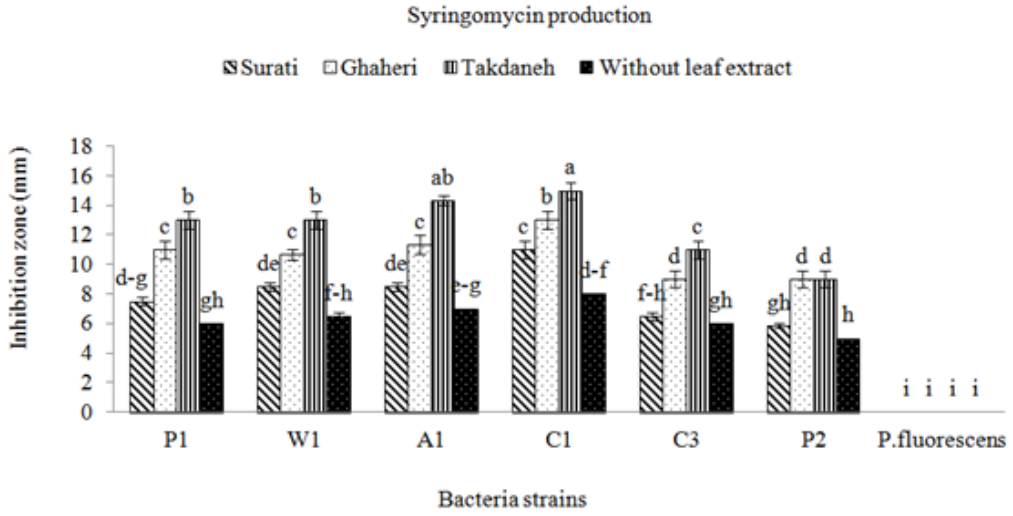

Fig. 1. Effects of plant extracts on syringomycin production by different strains of *Pseudomonas syringae* pv. *syringae.* The amounts of syringomycin produced were measured as the diameters of inhibition zones of the fungus *G. candidum* on SRM agar medium

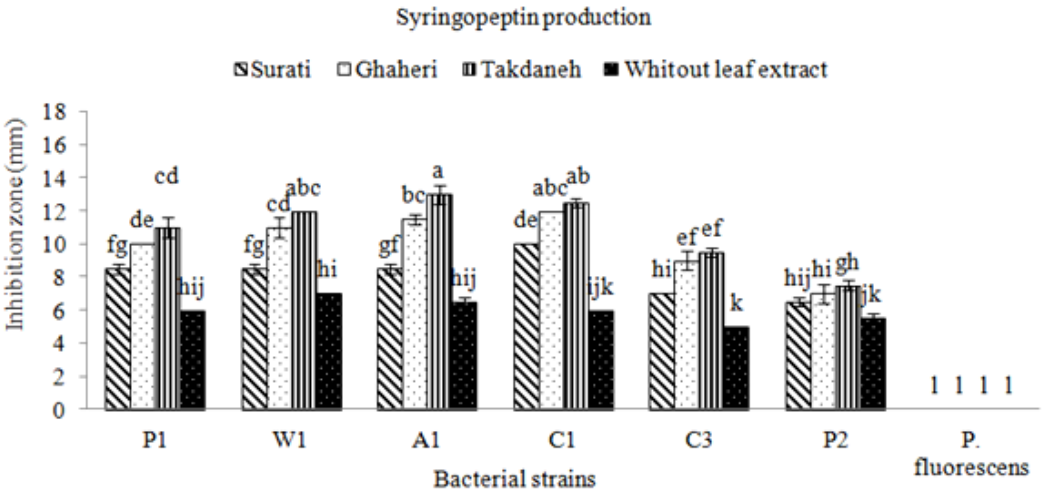

Fig. 2. Effects of plant extracts on syringopeptin production by different strains of *Pseudomonas syringae* pv. *syringae.* The amounts of syringopeptin produced were measured as the diameters of inhibition zones of the bacterium *B. megaterium* on PDA agar medium

achieved by this strain in the presence of Takdane cultivar leaf extract. Most of the isolates produced more syringomycin in the presence of Takdane leaf extract. Syringomycin inhibition was not significantly different between three strains A1, W1 and P1 on the media containing leaf extract of Ghaheri cultivar (Fig. 1). The inhibitory difference of all isolates in the presence of leaf extract of all three cherry cultivars except P2 was significant in comparison to absence of leaf extract. leaf extract of Surati variety had a lower effect on the increasing of syringomycin and syringopeptin production (Fig. 1 and 2). After Takdane cultivar, Ghaheri and Surati cultivars increased the production of toxin. As a result, Takdane, Qahiri and Surati cultivars were more susceptible to bacterial canker of stone fruits respectively. Significant increase in the production of syringomycin and syringopeptin in the presence of leaf extract in comparison to the conditions without the presence of leaf extract indicates the high effect of plant signal molecules on the expression of pathogenicity genes of *Pss*. The production of syringomycin and syringopptin is significantly related to the amount of plant signal molecules, especially phenolic and sugar compounds (Wang *et al.,* 2006). Among the isolates tested, inhibition zones were observed for all strains of *P. syringae* pv. *syringae* (Fig. 1 and 2). The largest inhibition zones were obtained with strains A1 and C1 in presence of plant extracts. The zones of inhibition were generally larger on medium supplemented with extracts of cherry cultivar Takdaneh, but the sizes of the inhibition zones varied by strain. The strains A1 and C1 produced substantially more syringomycin and syringopeptin on SRM and PDA agar media in presence of all plant extracts. In SRM medium without any extracts, the strain A1 formed 7-mm zones of inhibition of *G. candidum*, compared to 14-mm zones on medium supplemented with Takdaneh extracts and 11mm zones on medium with Ghaheri extracts (Fig. 1). Similar results were observed on PDA agar developed specifically for the production of syringopeptin under defined culture conditions (Fig. 2). Syringomycin and syringopeptin production by the strains was relatively sensitive to plant extracts. The production of syringomycin and syringopeptin by all isolates was significantly higher in the presence of plant extracts on SRM and PDA agar medium. On SRM agar medium, strains A1 and C1 produced zones of antifungal activity whose diameters were almost two times those produced in the absence of the plant extracts. Also on PDA agar medium, strains W1, A1 and C1 produced zones of antibacterial activity whose diameters were almost two times those produced in the absence of the plant extracts. There is growing evidence that virulence genes in bacteria respond to environmental stimuli (Mo *et al.,* 1995), and *Pss* is no exception. Because virulence determinants are not constitutively expressed in most bacteria, activation of virulence genes upon perception of a specific chemical or physical stimulus imparts order and balance to pathogenesis that will optimize the bacterium's chances for long term survival. The induction of toxigenesis in *P. syringae* pv. *syringae* by specific plant signal molecules reflects an ability of the bacterium to adapt to a dynamic plant environment. It was reported that syringomycin production is activated by specific plant signal molecules in diverse strains of *P. syringae* pv. *syringae* (Quigley & Gross, 1994). It recently was established that phytotoxin production by *Pss* is modulated by the

Table 1. Characteristics of bacterial strains used in this study

Strain	Isolate	Host of isolate	Location collected
Pseudomonas syringae pv. *syringae*	A1	Apricot	Yaouj (Iran)
Pseudomonas syringae pv. *syringae*	P1	Peach	Shiraz (Iran)
Pseudomonas syringae pv. *syringae*	P2	Peach	Yasouj (Iran)
Pseudomonas syringae pv. *syringae*	W1	Wheat	Yasouj (Iran)
Pseudomonas syringae pv. *syringae*	C1	Sweet cherry	Yasouj (Iran)
Pseudomonas syringae pv. *syringae*	C3	Sweet cherry	Yasouj (Iran)
Pseudomonas fluorescens	CHA0	Soil	Switzerland

perception of specific plant metabolites (Mo & Gross, 1991). Certain phenolic glucosides, such as arbutin, serve as signals that induce production of syringomycin, a cyclic lipodepsinonapeptide toxin that causes necrotic symptoms in host plants. In addition, the syrB gene, which is conserved in toxigenic strains of *Pss* and is predicted to encode a synthetase (Quigley & Gross, 1994), is activated by phenolic signal compounds. Cherry tissues appear to contain plant signal molecules that are perceived by *Pss* based on evidence that the syrB-ZucZ fusion is transcriptionally activated in an environment containing plant constituents (Mo & Gross, 1991). Inoculations of immature cherry fruits demonstrated that there is a rapid and strong expression of syrB in situ. When SRM medium was not amended with the cherry leaf extracts, strains failed to increase syringomycin production. This demonstrated that the cherry leaf extract contained a constituent or signal that was sensed by the bacterium, eventually leading to activation of genes responsible for toxin induction. Studies by Krzesinska *et al*., (1993) indicated that susceptibility of cherry genotypes to bacterial canker is correlated with signal activity. Extracts from the stems of 12 cherry genotypes were tested for syrB-inducing activity, and the genotypes most susceptible to bacterial canker contained higher signal activities than resistant genotypes. Consequently, both the quantity and quality of plant metabolites with signal activity may have an acute effect on disease development. Because high amounts of flavonoid glycoside signals occur in cherry leaves, one can speculate that their sudden release would be quickly sensed by the bacterium and lead to activation of genes, such as *syrB*, necessary for phytotoxin production. In addition, it appears that a broad spectrum of *Pss* strains would be capable of recognizing the flavonoid glycoside signals from cherry. This is based on evidence that most strains of *Pss* attack a wide range of plant hosts and that they recognize phenolic signal molecules that are found in the leaves, bark, and flowers of many plant species (Mo & Gross, 1991; Quigley & Gross, 1994). Host resistance as observed, for example, in cherry cultivars (Krzesinska *et al.,* 1993) may reflect qualitative and quantitative differences in signal molecules or the balance of plant substances that antagonize induction by plant signals.

In conclusion, it was found that the cultivars of Takdaneh, Ghaheri and Surati respectively induce more toxin production by *Pss* strains. These cherry cultivars in southwest Iran have the highest cultivation area. In susceptible cultivars, the pathogenicity factors of *Pss* is produced at a higher level compared to resistant cultivars.

ACKNOWLEDGEMENTS

The authors would like to thank the Yasouj University, Iran, for the providing support for this study.

REFERENCES

1. Bahar, M., Mojtahedi, H., Akhiani, A. Bacterial Canker of Apricot in Isfahan. Iran. *J. Plant Pathol.,* 1982; **18**: 58-68.
2. Bultreys, A., 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; **92**: 21-33.
3. Feil, H., Feil, W.S., Chain, P., Larimer, F., DiBartolo, G., Copeland, A., Lykidis, A., Trong, S., Nolan, M., Goltsman, E. Comparison of the complete genome sequences of *Pseudomonas syringae pv. syringae* B728a and pv. tomato DC3000. Proceedings of the National Academy of Sciences, 2005; USA **102**: 11064-11069.
4. Guenzi, E., Galli, G., Grgurina, I., Gross, D.C., Grandi, G. Characterization of the syringomycin synthetase gene cluster: a link between prokaryotic and eukaryotic peptide synthetases. *J. Biol. Chem.,* 1998; **273**: 32857-32863.
5. Hösel, W. Glycosylation and glycosidases, 1981; p. 725-753. In: E.E. Conn (ed.). The biochemistry of plants. vol. 7. Secondary plant products. Academic, New York.
6. Kennelly, M.M., Cazorla, F.M., De Vicente, A., Ramos, C., Sundin, G.W. *Pseudomonas syringae* diseases of fruit trees: progress toward understanding and control. *Plant Dis.,* 2007; **91**: 4-17.
7. Krzesinska, E.Z., Azarenko, A.N., Gross, D.C. Inducing the syrB gene in Pseudomonas syringae pv. syringae in twig extracts from cherry genotypes. *Hortscience.,* 1993; **28**: 335-337.
8. Little, E.L., Bostock, R.M., Kirkpatrick, B.C. Genetic characterisation of *Pseudomonas syringae* pv. *syringae* strains from stone fruits

in California. *App. Environ. Microbiol.,* 1998; **64**: 3818-3823.

9. Lu, S.E., Scholz-Schroeder, B.K., Gross, D.C. Characterization of the *salA*, *syrF*, and *syrG* regulatory genes located at the right border of the syringomycin gene cluster of *Pseudomonas syringae* pv. *syringae*. *Mol. Plant-Microbe Interact.,* 2002; **15**:43-53.
10. Mo, Y.Y., Gross, D.C. Plant signal molecules activate the *syrB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.,* 1991; **173**: 5784-5792.
11. Mo, Y.Y., Geibel, M., Bonsall, R.F., Gross, D.C. Analysis of sweet cherry (*Prunus avium* L) leaves for plant signal molecules that activate the *syrB* gene required for synthesis of the phytotoxin, syringomycin, by *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.,* 1995; **107**: 603- 612.
12. Quigley, N.B., Gross, D.C. Syringomycin production among strains of *Pseudomonas syringae* pv. *syringae*: conservation of the *syrB* and *syrD* genes and activation of phytotoxin production by plant signal molecules. *Mol. Plant-Microbe Interact.,* 1994; **7**: 78-90.
13. Schaad, N.W., Jones, J.B., Chun, W. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3nd Edition, 2001; American Phytopathological Society Press, St. Paul., Minnesota, USA, 373 PP.
14. Scholz-Schroeder, B.K., Hutchison, M.L., Grgurina, I., Gross, D.C. The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. *syringae* strain B301D on the basis of *sypA* and *syrB1* biosynthesis mutant analysis. *Mol. Plant-Microbe Interact.*, 2001; **14**: 336-348.
15. Wang, N., Lu, S.E., Yang, Q., Sze, S.H., Gross, D.C. Identification of the *syr-syp* Box in the promoter regions of genes dedicated to syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae* B301D. *J. Bacteriol.,* 2006; **188**: 160-168.