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ISOLATION, PURIFICATION, CHARACTERIZATION AND IDENTIFICATION OF VIRULENCE FACTORS OF *PSEUDOMONAS SYRINGAE* PV. *SESAMI* AND *XANTHOMONAS CAMPESTRIS* PV. *SESAMI*

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Abstract

In the present study production, characterization and identification of virulence factors produced by virulent isolates of *Pseudomonas syringae* pv. *sesami* (*Psse*-08/NARC 1) and *Xanthomonas campestris* pv. *sesami* (*Xcs*-08/NARC) were carried out. Identification was made on the basis of earlier published reference data. Previously, toxins were detected by the phytotoxic as well as growth inhibition assays. HPLC analysis of cell free culture filtrates allowed characterizing toxins activity of a single active peak obtained from virulent isolate of *Psse*-08/NARC 1 that produced necrotic symptoms. In the present results, the retention time of peak obtained from *Psse*-08/NARC 1 was same as previously reported from mangotoxin, which causes apical necrosis of mango tree. However, there was no reference data for the active peak obtained from *Xcs* isolates, but the single active peak showed antibacterial activity. Later characterization of active fraction from *Xcs* was also performed in phytotoxic assay.

Introduction

Pseudoumonas syringae (*Ps*) is one of the model pathogens for the study of host specificity and virulence like *Xanthomonas campstri* (*Xc*). Both have wide host range and *Ps* infect nearly all of the terrestrial plant species (Hirano & Upper, 1990). On sesame, both *Ps* and *Xc* elicite disease collectively known as bacterial leaf blight, which is one of the major factors that limit sesame production in Pakistan.

Many fungal and bacterial plant pathogens are known to produce non host selective toxic metabolites in infected leaves as well as in culture media (Mitchell, 1984; Cazorla *et al*., 1997 and Bender *et al*., 1999). Bacterial toxins considered as secondary metabolites with diverse chemical structure and function at extremely low concentration (Mitchell, 1991). Non host selective phytotoxins act as virulence factor for producing pathogen (Mitchell, 1984) and increased symptoms such as chlorosis and necrosis in infected host (Gross, 1991; Durbin, 1991 and Bender, 1999).

Many *Pseudomonas* pathovars produced secondary metabolites such as phytotoxins. *P. syringae* pv. *tabaci* produce chlorosis inducing tabtoxin (Stewart, 1971). Many strains of pathovar coronafaciens also known to produce tabtoxin (Mitchell, 1991). Tabtoxin is a ß- lactam, an unusual dipeptide that inhibits glutamine synthesis (Turner, 1981; Thomas *et al*., 1983), which is the mode of action of the tabtoxin. Upon inhibition of glutamine synthesis, ammonia accumulates in infected tissues and lead to chlorotic symptoms (Turner & Debagge, 1982).

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Phaseolotoxin produced by *P. syringae* pv. *phaseolicola* (Patil *et al*., 1972; Staskawicz & Panopoulos, 1979) is a tripeptide and also elicited chlorosis symptoms on plants by the reversible inhibition of ornithine carbamoyl transferase (OCTase), an enzyme essential in orginine biosynthesis (Patil *et al*., 1972; Templeton *et al*., 1985; Tamura *et al*., 2002). Phaseolotoxin is also produced by *P. syringae* pv. *actinidae*, which causes bacterial canker of Kiwifruit (Swaeda *et al*., 1997). Coronatine (COR) is a polyketide molecule that acts as methyl jasmonate. It is also reported that the hypocotyles of mung leaves stimulate the activity of the enzyme 1-Amino cyclopropane carboxylic acid (ACC) upon treatment with COR. So the stimulation of the ACC synthase leading to ethylene formation and confirm the mode of action of the toxin COR (Kenyon & Turner, 1992).

Many other different toxins with structure similar to lipodepsipeptide produced by strains of *P. syringae* pv. *syringae* has been reported (Gross *et al*., 1977; Ballio *et al*., 1988, 1991; Gross, 1991) such as syringomycins and syringopeptins. Syringomycins produced by strains of *P. syringae* pv. *syringae* (Iacobilles *et al*., 1992a; Bender *et al*., 1999) elicited necrotic symptoms (Sinden *et al*., 1971; Iacoblilles *et al*., 1992) as a result of pore formation and inhibition of plant cell membrane functions (Iacobellis *et al*., 1992a; Hutchinson & Gross, 1997). Another class of lipodepsipeptidic toxins, such as syringopeptins elicited same symptoms as syringomycins by similar mode of action as syringomycins (Iacobellis *et al*., 1992a; Di Giorgio *et al*., 1996; Hutchinson & Gross, 1997).

The virulence factor produced by *Psse* and *Xcs* isolates was detected in previous studies in different bioassays (Firdous, 2009). These included chlorosis and necrosis producing symptoms, hypertrophy in potato tubers, phytotoxic and antibacterial bioassays as well as effects of culture filtrate on root and shoot sesame length in previous studies. The objective of the present study was the production, extraction, purification, characterization and identification of the phytotoxic and antibacterial substances from *Psse* and *Xcs* isolates that are the resultant cause of symptoms development in sesame.

Materials and Methods

Bacterial isolates and growth conditions: *Psse*-08/NARC 1 and *Xcs*-08/NARC used in this work were obtained from National Agricultural Research Centre (NARC) Pakistan and were maintained at 4ºC.

Toxins production on different media: Virulent isolates of *Psse*-08/NARC 1 that produce necrotic symptoms in sesame and *Xcs*-08 NARC were grown on different media such as Improved Minimal Medium (IMM) and Potato Dextrose Broth with Casamino acid (PDBCA).

Conditions for modified IMM: Isolates were grown in 1L Roux flasks containing 100 ml of modified IMM liquid (pH 5.5) as stationary cultures at 25ºC for 7 days. There were 3 flasks for each isolate inoculated with 1 ml (10^8 CFU /ml) of 24 old cultures on Nutrient Glucose Agar (NGA). The composition of IMM medium is described in Table 1.

Conditions for PDBCA: For toxins production on PDBCA, bacteria were grown on PDA plates for 24 h at 27ºC. After overnight growth, bacteria were replaced on PDB in falcon tubes of 15 ml with 6 ml each for 30 h at 25ºC. These bacteria were further used for inoculating the batch.

Table 1. Composition* of Improved Minimum Medium (IMM).

Ingredient	Quantity
L-histidine-HCL:	4.00g
Mannitol:	10.00g
MgSO4:	0.20g
$CaCl_2$:	0.10g
$FeSO^4$:	0.02g
KH_2PO_4:	0.80g

* The composition given in the table is for one litre

Preparation of batch on fresh potato broth (FPB) for PDBCA: Fresh potato weighing four hundred grams were boiled in one litre of distilled water after cutting into small pieces. The mixture was cooked and filtered through a muslin cloth. Distilled water was added in the filtrate until a final volume of two liters was obtained. Dextrose (40 gms) and casamino acid (8 gms) were added into it. The eight flasks containing 250 ml of broth were sterilized and closed with an aluminium foil. After sterilization, each flask was inoculated with 2 ml of bacteria, already prepared as described above and were incubated at 25-27ºC for seven days.

Peptides extraction, purification and chromatographic analysis: After incubation, cultures were acidified at pH 2 by 1 M HCl, cold acetone (1:1) was added in equal amount and the precipitate was stored overnight at 4ºC. The mixture was centrifuged at 5000 x g for 10 min. The supernatant was collected and concentration was done upto 1/10th volume at 40 to 45ºC with a rotary evaporator. Then 60% (v/v) concentration was prepared with the addition of acetone and kept the flasks at 4ºC, centrifuged again and then supernatant was concentrated again as before and then diluted to I L with SDW (Ballio *et al*., 1988). For partial purification of toxins, a method of Arrebola *et al*., (2003) was followed with slight modification. Fifty ml from acetone preparation concentrated samples were dissolved in 10 ml of 20% acetonitile and 20 µl of samples were injected and fractionated by reversed phase HPLC with an aquapore RP C-18 coulmn using a model 200 LC pump and a model SP-10 AV vp UV-VIS detector (Schimadzu, Japan). The mobile phase was 20% isocratic gradient of acetonitrile in water, with a flow rate of 1 ml/min at a wavelength of 254 nm. All peaks were collected with the help of fraction collector. The peaks having antibacterial and phytotoxic activities were collected and fractionation was done by TLC on silica plates. The spots were recovered from the silica plates, extracted in 20% acetonitrile and then concentrated as before. This concentrated extract was again fractionated by HPLC, and 500 µl fractions from *Xcs* samples were tested for the phytotoxic and same quantity from *Psse* samples were tested in antibacterial activity against unknown bacterial pathogen as previously described.

Results and Discussion

The isolates of *Psse* and *Xcs*, which were highly virulent and showed inhibition zones against unknown bacterium (Firdous, 2009a), were selected in the studies of production and isolation of the bioactive substances. Two media were used for the production of bioactive compound such as PDB-CA and IMM. IMM was used to isolate the lipodepsinonapeptides such as syringomycins and syringopeptins, but none of the isolates showed antibacterial activity against *Bacillus megaterium* (sensitive against lipodepsinonapeptides).

Similarly, none of the gradient fractionation by reverse phase HPLC of the above bioactive fractions gave rise to same elution pattern as that of typical syringomycin preparation (Gross & deVay, 1977) from strains of *P. syringae* pv. *syringae* (Ballio *et al*., 1988 and Iacobellis *et al*., 1992). So it is suggested that *Psse* and *Xcs* did not produce any lipodepsinonapeptides like Syringomycins, syringopeptins and Syringostatins.

Partial purification: Taking into account the bioassays for detection, further work was done on purification of bioactive substances from *Psse* and *Xcs* by chromatographic techniques. Crude extracts from acetone preparation of *Psse* and *Xcs* isolates were recovered, concentration and fractionation was done on silica TLC plates. After developing TLC dark spots were observed under U.V. light at 254 nm (data not shown). The spots were further checked against tested bacteria. Only unknown bacterium showed inhibition zones.

Further steps for purification of the substances were also executed. Cell free preparation from *Psse* and *Xcs* isolates were fractionated by HPLC and TLC. The spots containing bioactive substance were recovered from the TLC plates and then again tested for antibacterial activity. Further purification of the spots was made by HPLC analysis. The toxic activity obtained from the HPLC column after 10 min corresponding with the single active peak. Reverse phase HPLC of a *Psse* (chl^-) producing isolate extract partially purified produced an elution pattern as observed with mangotoxin from *P. syringae* pv. s*yringae* strain UMAF0158 (Fig. 1) (Arrebola *et al*., 2003). *Xcs* virulent isolates also showed peaks having phytotoxic activity obtained at approximately 12 minute retention time (Fig. 2). Results obtained in this study confirmed previous investigations (Arrebola *et al*., 2003) on the ability of *P. syringae* pv. *syringae* to produce mangotoxin *in vitro* which caused necrosis symptoms on mango. *Xcs* also produced substance with phytotoxic activity on applying fraction in sesame leaves.

Conclusions

The production of toxin from *Psse* similar to mangotoxin is a new finding as no previous studies on chromatographic analysis for any bacterial virulence factors in Pakistan as well as for the *P. sesami* is reported. In the present study necrosis producing toxins from *Psse* as detected further by using fraction in antibacterial bioassay, showed similar chromatographic analysis as those reported for mangotoxin produced by *P. syringae*. However, further work is required to confirm the structure and mode of action of these toxins. Although the coronatine or related toxin has been detected first time in the present studies in potato tuber and seedling bioassay for the sesame pathovar, but their chromatographic analysis still require further exploration. The active fractions of *Xcs* in phytotoxic assay also confirmed the involvement of any secondary metabolite in disease symptoms. *Psse* isolates showed chlorosis as well as necrosis symptoms on leaves that indicate the involvement of toxins. Lesions expand and bacteria also monitored to multiply over the sampling periods against both pathogens in all the tested genotypes, yet it is sure whether these bacteria act as 'pathogenicity' or 'virulence' factor. A suitable bioassay and detection is needed at this stage.

Once the role of toxins in host pathogen interaction is determined, the understanding of phytotoxins action (coronatine, phaseolotoxin or LPDs) in the pathogenesis of *Psse* infection can contribute in the development of strategies for effective control measure based on toxin resistance to plants. As previous studies reported with the tabtoxin resistance gene (ttr) and phaseolotoxin reistance gene (argk) of *Psphy* and their respective diseases.

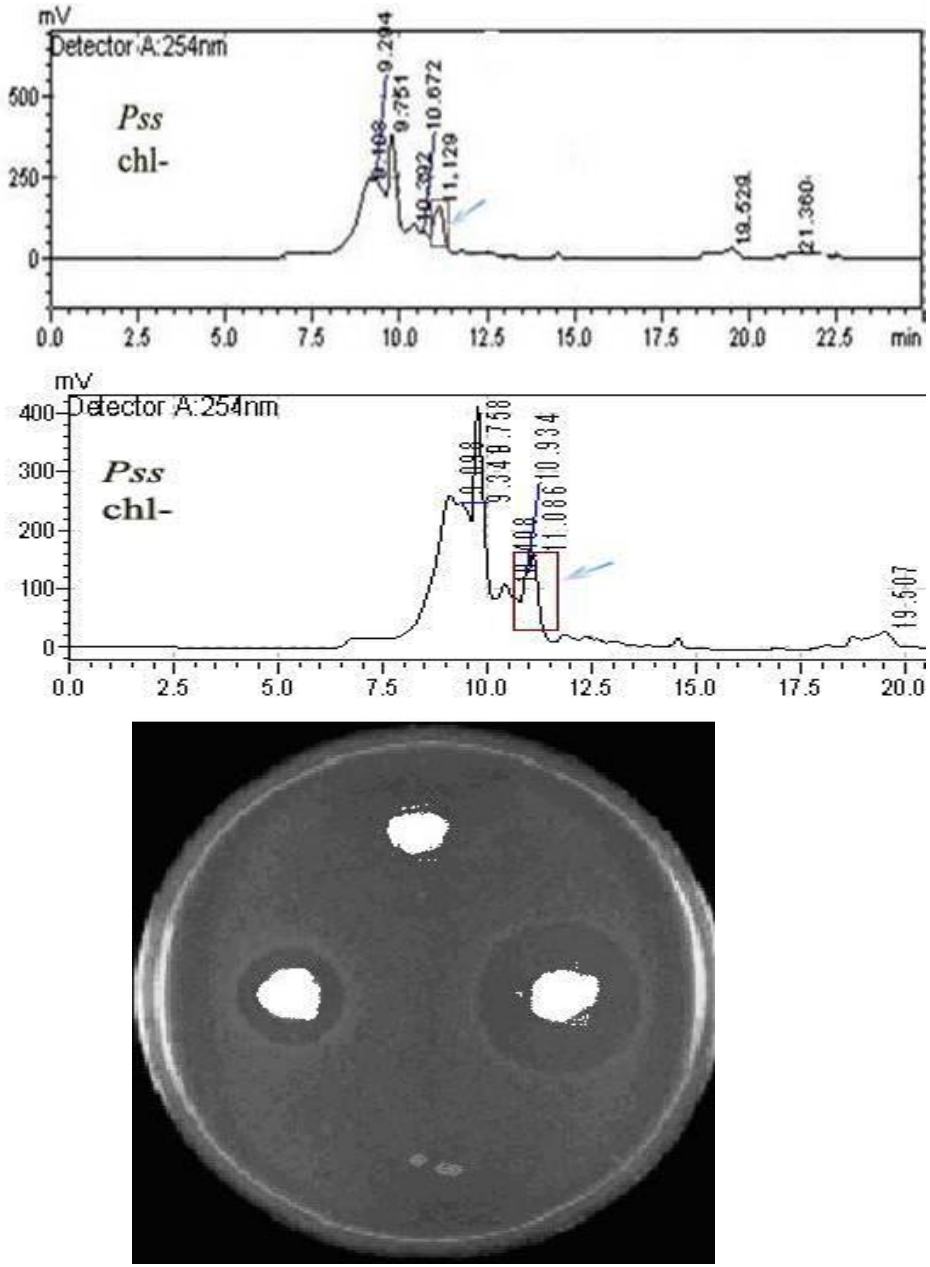

Fig. 1. HPLC separations of *Psse*–08/NARC 1 isolate. Toxin extractions from acetone of cell free culture filtrate of 7 day old culture. All the fractions obtained from different peaks were tested against unknown bacterium. The peak enclosed by a box showed toxic activity against unknown bacterium.

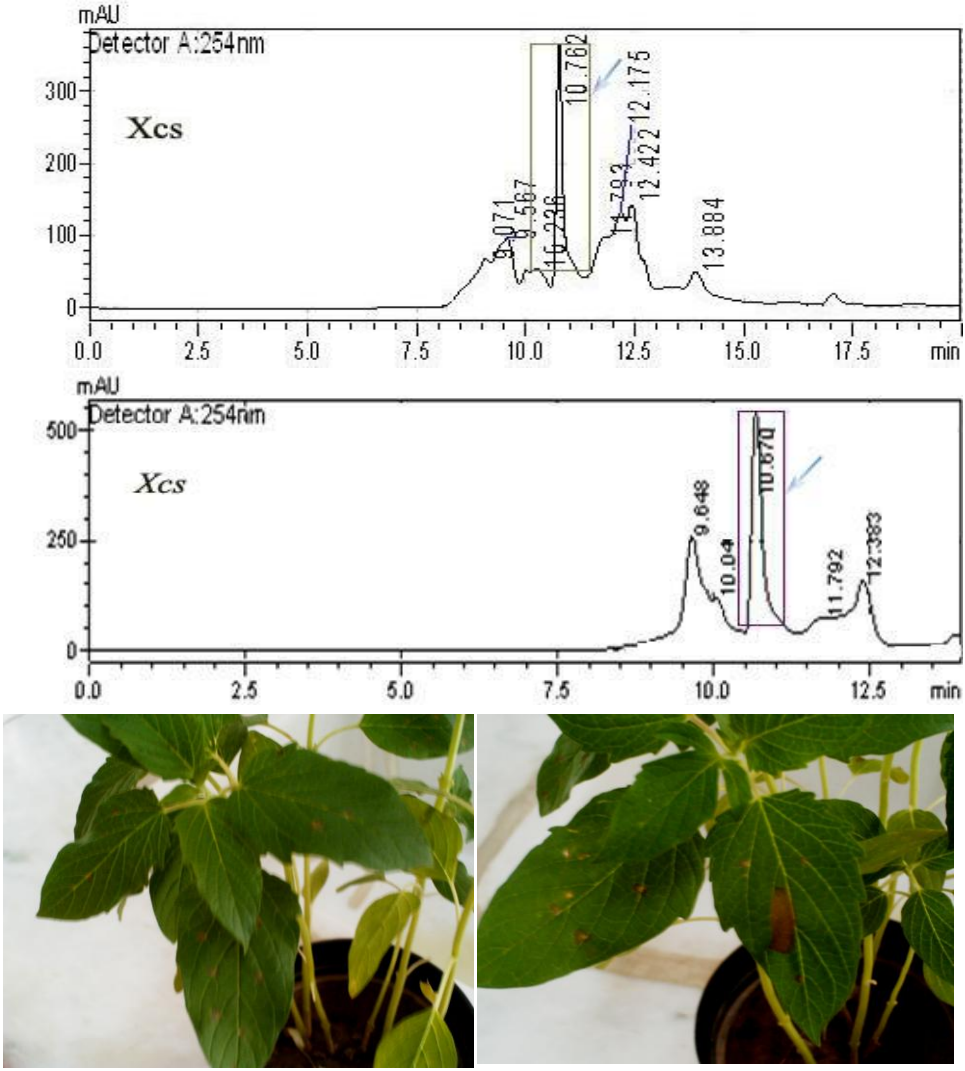

Fig. 2. Fractions of peak enclosed in box from *Xcs*-08/NARC showed phytotoxic activity on sesame plant.

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References

Arrebola, E., F.M. Cazorla, V.E. Duran, E. Rivera F. olea, J.C. Codina, A. Perez-Garcia and A. De vicente. 2003. Mangotoxin: a novel antimetabolie toxin produced by *Pseudomonas syringae* inhibiting ornithine/arginine biosynthesis. *Physiol. Mol. Plant Pathol*., 63: 117-127.

Ballio, A., D. Barra, F. Bossa, J.E. DeVay, I. Grugurina, N.S. Iacobellis, G. Marino, P. Pucci, M. Simmaco and G. Surico. 1988. Multiple forms of syringomycin. *Physiol. Mol. Plant Pathol*., 33: 493-6.

Ballio, A., D. Barra, F. Bossa, A. Collina, I. Grgurina, G. Marino, G. Moneti, M. Paci, P. Pucci, A. Segre and M. Simmaco. 1991. Syringopeptins, new phytotoxic lipodepsipeptides of *Pseudomonas syringae* pv. *syringae*. *FEBS Letters*, 291: 109-12.

Bender, C.L. 1999. Chlorosis inducing phytotoxins produced by *Pseudomonas syringae*. *Euro. J. Plant. Pathol*., 105: 1-12.

Bender, C.L., F. Alarcon-Chaidez and D.C. Gross. 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation and biosynthesis by peptide and polyketide synthesis. *Microbial. Mol. Biol. Rev*., 63: 266-292.

Bender, C.L., S.A. Young and P.E. Mitchell. 1992. Ecology and genetic studies of coronatine synthesis in *Pseudomonas syringae*. In: *Pseudomonas*: *Molecular Biology and Biotechnology*. (Eds.): E. Galli, S. Silver and B. Witholt. Am. Soc. for Microbiol, Washington, D.C. p. 56-63.

Cazorla, F.M., L. Olalla, J.A. Tores, J.C. Codina, A. Perez-Garcıa and A. de Vicente. 1997. *Pseudomonas syringae* pv. *syringae* as microorganism involved in apical necrosis of mango: characterization of some virulence factors. In: *Pseudomonas syringae pathovars and related species*. (Eds.): K. Rudolph, T.J. Burr, J.W. Mansfield, D. Stead, A. Vivian and J. von Kietzell. Dordrecht: Kluwer Academic Publishers, p. 82-87.

Di Giorgio, D., L. Cammoni, K.A. Mott and A. Ballio. 1996. Syringopeptins, *Pseudomonas syringae* pv *syringae* phytotoxins, resemble syringomycin in closing stomata. *Plant Pathol*., 45: 564-571.

Durbin, R.D. 1991. Bacterial phytotoxins: mechanism of action. *Experientia*, 47: 776-83.

Firdous, S.S. 2009. Virulence anlysis of *Xanthomonas campestris* pv. s*esami* and *Pseudomonas syringae* pv. s*esami* the causal organisms of Sesame (*Sesamum indicum* L.) bacterial blight. (Unpublished) Ph. D. thesis, Department of Botany, PMASAAU Rawalpindi.89-108 pp.

Firdous, S.S. 2009a. Virulence anlysis of *Xanthomonas campestris* pv. *sesami* and *Pseudomonas syringae* pv. s*esami* the causal organisms of Sesame (*Sesamum indicum* L.) bacterial blight. (Unpublished) Ph. D. thesis, Department of Botany, PMASAAU Rawalpindi.97-99 pp.Gross, D.C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. *Annu. Rev. Phytopathol*., 29: 247-278.

Gross, D.C. and J.E. DeVay. 1977. Production and purification of syringomycin, a phytotoxin produced by *Pseudomonas syringae*. *Physiol. Plant Pathol*., 11: 13-28.

Gross, D.C., M.L. Hutchinson, B.K. Scholz and J.H. Zhang. 1997. Genetic analysis of the role of toxin production by *Pseudomonas syringae* pv. *syringae* in plant pathogenesis. In: *Pseudomonas syringae pathovars and related pathogens*. (Eds.): K. Rudolph, T.J. Burr, J.W. Mansfield, D. Stead, A. Vivan, J. von Kietzell. Kluwer Academic Publishers, Dordrecht, Netherlands, 163-169.

Hirano, S.S. and C.D. Upper. 1990. Population biology and epidemiology of *Pseudomonas syringae*. *Annu. Rev. Phytopathol*., 28: 155-157.

Hutchinson, M.L. and D.C. Gross. 1997. Lipopeptide phytotoxin produced by *Pseudomonas syringae* pv. *syringae*: comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. *Mol. Plant–Microbe Interact*., 10: 347-54.

Iacobellis, N.S., P. Lavermicocca, I. Grgurina, M. Simmaco and A. Ballio. 1992. Phytotoxic properties of *Pseudomonas syringae* pv. *syringae* toxins. *Physiol. Mol. Plant Pathol*., 40: 107-16.

Iacobellis, N. S., P. Lavermicocca, G. Surico and R. D. Durbin. 1992a. The occurrence and characterization of a syringomycin-macromolecular complex in cultures of *Pseudomonas syringae* pv. *syringae*. *Physiol. Mol. Plant Pathol*., 40: 91-105.

Kenyon, J.S. and J.G. Turner. 1992. The stimulation of ethylene synthesis in *Nicotiana tabacum* leaves by the phytotoxin coronatine. *Plant Physiol*., 100: 219-224.

Mitchell, R.E. 1984. The relevance of non-host toxins in the expression of virulence by pathogens. *Annu. Rev. Phytopathol*., 22: 215-45.

Mitchell, R.E. 1991. Implications of toxins in the ecology and evolution of plant pathogenic microorganisms: Bacteria. *Experientia*, 47: 791-803.

Patil, S.S., L.Q. Tam and W.S. Sakai. 1972. Mode of action of the toxin from *Pseudomonas phaseolicola*. I. Toxin specificity, chlorosis and ornithine accumulation. *Plant Physiol*., 49: 803-7.

Sawaeda, H., T. Takeuchi and I. Matsuda. 1997. Comparative analysis of *Pseudomonas syringae* pv. *actinidae* and pv. *Phaseolicola* based on phaseolotoxin resistant ornithine carbamoyltransferase gene (erg K) and 16S-23S rRNA intergenic spacer sequences. *Appl. and Environ. Microbiol*., 63: 282-288.

Sinden, S.L., J.E. DeVay and P.A. Backman. 1971. Properties of syringomycin, a wide spectrum antibiotic and phytotoxin produced by *Pseudomonas syringae* and its role in the bacterial canker disease of peach trees. *Physiol. Plant Pathol*., 1: 199-213.

Stasawicz, B.J. and N.J. Panopoulos. 1979. A rapid and sensitive microbiological assay for phaseolotoxin. Phytopathology, 69: 663-666.

Stewart, W.W. 1971. Isolation and proof of structure of wildfire toxin. *Nature*, Lond. 219: 275-85.

Tamura, K., M. Imamura, K. Yoneyama, Y. Kohno, Y. Takikawa, I. Yamaguchi and H. Takahashi. 2002. Role of phaseolotoxin production by *Pseudomonas syringae* pv. *actinidae* in the formation of halo lesions of kiwifruit canker disease. *Physiol. Mol. Plant Pathol*., 60: 207-14. doi: 10.1006/pmpp. 2002-0405.

Templeton, M.D., R.E. Mitchel, P.A. Sullivan and M.G. Shepherd. 1985. The inactivation of ornithine transcarbamoylase by N'L (N'-sulphodiaminophosphinyl)-Lomithine. *Biochem. J*., 228: 347-352.

Thomas, M.D., P.J. Langston-Unkefer, T.F. Uchytil and R.D. Durbin. 1983. Inhibition of glutamine synthetase from pea by tabtoxinine-b-lactam. *Plant Physiol*., 71: 912-5.

Turner, J.G. 1981. Tabtoxin, produced by *Pseudomonas tabaci*, decreases *Nicotiana tabacum* glutamine synthetase *In vivo* and causes accumulation of ammonia. *Physiol. Plant Pathol*., 19: 57-67.

Turner, J.G. and J.M. Debbage. 1982. Tabtoxin-induced symptoms are associated with accumulation of ammonia formed during photorespiration. *Physiol Plant Pathol*., 20: 223-233.

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