Pl. Dis. Res. 38 (2) : 222-230
DOI No. 10.5958/2249-8788.2023.00029.X

ISSN 0970-4914 (Print)
ISSN 2249-8788 (Online)

In-vitro screening of native strains of fluorescent pseudomonads against *Xanthomonas axonopodis* pv. *punicae*, causing bacterial blight of pomegranate

MADHU S. GIRI[1*], V. I. BENAGI[2], V. B. NARGUND[2] AND P. JONES NIRMALNATH[3]

[1] Central Coffee Research Institute, Coffee Research Station Post, Chikkamagaluru District-577 117
[2]Department of Plant Pathology, [3]Department of Agricultural Microbiology,
University of Agricultural Sciences, Dharwad- 580 005.
** E-mail: madhuccri@gmail.com*

ABSTRACT

Fluorescent pseudomonads isolated from rhizosphere soils of northern Karnataka and border areas of Maharashtra were screened against *X. axonopodis* pv. *punicae,* the causal agent of bacterial blight of pomegranate by dual culture technique *in-vitro*. Out of 99 colonies of fluorescent pseudomonads screened for the antagonistic activity, 83 colonies were found inhibitory against pathogen. The zone of inhibition ranged from 1.47 cm to 4.35 cm. The top nine strains of fluorescent pseudomonads were selected which showed maximum inhibitory zone and where pathogen failed to show re-growth in the inhibitory zone after 144 hours after inoculation. Among the nine selected strains FP-87 showed a maximum zone of inhibition of 4.35 cm in diameter followed by FP-64, FP-19 and FP-03 with a zone of inhibition 4.07 cm, 3.58 cm and 3.48 cm in diameter respectively. However, inhibition zone produced by FP-87 strain was significantly superior over remaining strains except FP-64 where they were at par with each other. Remaining strains were at par with each other.

Key words: Bacterial blight, fluorescent pseudomonads, pomegranate, *Xanthomonas axonopodis* pv. *punicae*

Pomegranate (*Punica granatum* L.) is an ancient fruit belongs to the family Punicaceae. It is regarded as the "Fruit of Paradise". In India it is grown as a commercial crop in Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Madhya Pradesh and Uttar Pradesh. Cultivation of pomegranate in recent years has met with different traumas such as pest and diseases. Among the diseases infecting pomegranate, the bacterial disease popularly known as 'bacterial blight' caused by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh,1959; Vauterin *et al*., 1995) is a major threat for successful cultivation of pomegranate in India. From 2002 to till date the disease has reached the alarming stage and hampering the Indian economy vis-à-vis export of quality fruits. Pomegranate "the boon commercial fruit crop to the farmer turned as a big bane" after the severe outbreak of bacterial blight.

To date, copper based products are applied to manage the plant diseases caused by *Xanthomonas* spp. However, these have negative effects on both human and animal health.

In the recent years the demand for chemical-free products by consumers has increased and this has resulted in the restricted use of chemicals (Ramesh *et al*., 2009; Buttimer *et al.*, 2017). In addition, the absence of chemical residues is also necessary requirements to export food. For these reasons, alternative technologies to copper bactericides are being investigated. At present, an increased interest had developed in natural biological agents to replace the conventional agricultural practices for achieving more sustainable pomegranate production.

The use of rhizosphere resident microbial antagonist specifically the fluorescent pseudomonads is noted as a promising management strategy. Among the rhizosphere organisms fluorescent pseudomonad strains are often selected for biological control strategies because of their ability to utilize varied substrates under different conditions and short generation time. Moreover, they produce active extracellular compounds such as siderophores responsible for the biological suppression of several

Received: 25-10-2023
Accepted: 28-12-2023

soil-borne plant pathogens. In the recent days biological control is considered as a best alternative way of reducing the use of chemicals in agriculture (Yang *et al*., 2007; Vega *et al*., 2008; Misk and Franc, 2001). Several strains of *Pseudomonas fluorescens*, *P. putida*, *P. cepacia*, *P. aeruginosa, P. aureofaciens* and *P. corrugata* have been widely used for the biological control of plant diseases (Leeman *et al.,* 1995; Vidhyasekaran and Muthamilan, 1999; Meena *et al*., 2001; Vidhyasekaran *et al*., 2001).

In this context, the present study was undertaken to isolate and evaluate native strains of fluorescent pseudomonads isolated from soil samples collected from pomegranate rhizospere against *X. axonopodis* pv. *punicae* at Plant Pathology Division, University of Agricultural Sciences, Dharwad.

MATERIALS AND METHODS

Isolation of *X. axonopodis* pv. *punicae*

Infected plant parts such as leaf showing typical symptoms of bacterial blight were subjected to isolation of *X. axonopodis* pv. *punicae* (*X.a.p*). The diseased samples were washed thoroughly with tap water and dried under shade. The infected portion along with healthy part was cut into small pieces and was surface sterilized with 1.0% sodium hypochlorite solution for 60 seconds and washed three times serially in sterile distilled water to remove the traces of sodium hypochlorite. The diseased bits were then suspended in a Petri plate containing 1 ml of sterilized distilled water and crushed with sterilized scalpel and allowed for 2-3 minutes. When the sufficient oozing of bacterial masses were released from the crushed tissues, a loop-full of sap was taken and streaked on to Petri plate containing Nutrient Glucose Agar medium (NGA). The inoculated plates were incubated at 28±1°C for 72-96 h. The young and fast growing colonies were saprophytic ones. Thus observations were made for the development of well separated, typical, light yellow coloured bacterial colonies resembling that of typical *Xanthomonas* sp. at 96 h.

Purification and preservation of *X. axonopodis* pv. *punicae* culture

The bacterial colonies showing typical characters of *X.a.p* were picked up with the help of sterilized inoculation loop and streaked onto the surface of sterilized NGA Petri plates. The inoculated plates were incubated at 28±1°C for 72-96 h. Observations were made for the development of well separated, typical, bright yellow, mucoid colonies. Such pure colonies were further streaked onto the agar slants containing the NGA medium and incubated at 28±1°C for 72 h. Then the cultures were stored in the refrigerator at 4 °C. For long term preservation, a loopful of bacterial culture was suspended in 600 µl of nutrient glucose broth in 2 ml cryo vials and added with 600 µl of glycerol (50 %) and stored at –80 °C for further use.

Proving pathogenicity

To prove the pathogenicity, *X. axonopodis* pv. *punicae* was multiplied in nutrient glucose broth (200 ml) in Erlenmeyer flasks by inoculating a loopful of bacterial culture. The inoculated flask was incubated for three days at 28±1°C. Two months old susceptible variety (Bhagwa) plants were sprayed with water and then covered with a polyethene sheet for 24 h before inoculation. The pre-incubated pomegranate plants were sprayed with $4x10^7$ cfu/ml of bacterial suspension (200 ml: 800 ml; broth culture: sterile distilled water) by using hand sprayer. Further, sprayed plants were covered with polyethene bags and kept in glass house for the next 48 h. Plants sprayed with sterile distilled water with nutrient broth served as control. Inoculated plants were sprayed twice a day with distilled water to maintain humidity. Observations were recorded every day for the appearance and development of symptoms. When artificially inoculated leaves expressed symptoms, re-isolation of the pathogen was done and compared with the original culture. The experiment was repeated twice for confirmation.

Isolation of native fluorescent pseudomonads

Soil samples from rhizosphere of 10 healthy pomegranate plants were collected at random from each field and finally were mixed and made into each sample consisted of 100 g soil. Fluorescent pseudomonads were isolated from rhizospheres of pomegranate crop according to the method of Weller and Cook (1983).

The colonies grown on plates containing King's B medium fluorescing (Fig. 1) under UV light (365 nm wavelength) were picked up, purified and preserved. These strains were maintained at 4°C with sterile distilled water.

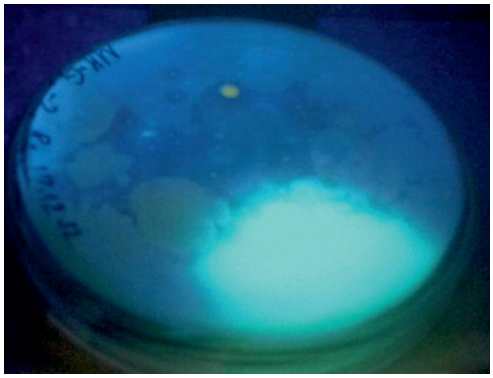

Fig. 1. Identification of fluorescent pseudomonads under UV light (365 nm wave length)

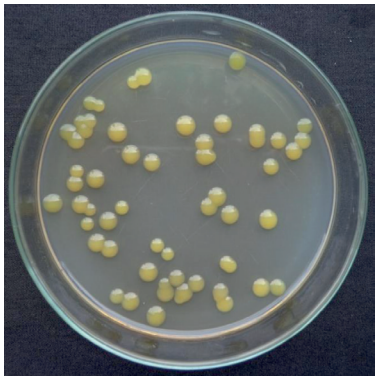

Fig. 2. Pure culture of *X. axonopdis* pv. *punicae* on Nutrient Glucose Agar medium

In vitro screening of native fluorescent pseudomonads against *X. axonopodis* pv. *punicae*

Ninety nine colonies of fluorescent pseudomonads were screened for their ability to inhibit the growth of *X.a.p* by following dual culture assay. Five loopfull of 74 h old *X.a.p* culture multiplied on nutrient agar plate were mixed with 1.50 ml of sterile distilled water in micro centrifuge tube. Three hundred microliter (3×10^8 cfu/ml) of this suspension was spread on NGA plates by using L-spreader. Two loopfull of 74 h old fluorescent pseudomonad cultures were mixed in 0.50 ml of sterile distilled water in a 1.50 ml micro centrifuge tube. Further, 10 µl of the aliquote of these suspensions were placed on the surface of NGA plates which was prior seeded with *X.a.p*. The inoculated plates were incubated at 28±1 °C for 144 h. Inhibition zone was recorded as diameter in cm.

RESULTS AND DISCUSSION

Isolation and proving pathogenicity of *X. axonopodis* pv. *punicae*

The *X.a.p* colonies developed on Petri plates containing NGA medium after 72 h of incubation at 28±1 °C were light yellow, raised, convex and glistening (Fig. 2). On inoculation of active, pure culture of freshly isolated *X.a.p* on susceptible pomegranate plant produced characteristic symptoms on leaves at six days after incubation period. Initially the symptoms on leaves appeared as small, water soaked lesions, later turned to brown to black colour. Later on such spots developed into angular to irregular shaped spots along the veins and veinlets of the leaf lamina leading to marginal necrosis. On re-isolation of pathogen from artificially inoculated plants yielded the bacterial colonies similar to the original one.

Isolation of fluorescent pseudomonad strains

A total of ninety nine fluorescent pseudomonas strains were isolated from soil samples obtained from 24 different locations representing northern Karnataka and border areas of Maharashtra. Isolated fluorescent pseudomonas were designated as FP-1 to FP-99 and were preserved at Plant Pathology Department, UAS-Dharwad.

In vitro screening of native fluorescent pseudomonads against *X. axonopodis* pv. *punicae*

The chemical disease management strategies are harmful to the environment. So, biological control assumes special significance in being an environment-conscious, cost-effective alternative strategy for bacterial blight management. This can also be used in integration with other strategies to afford greater levels of protection. The use of rhizosphere resident microbial antagonists specifically the fluorescent pseudomonads is noted as a promising disease management strategy. Among many biocontrol agents, fluorescent pseudmonads have received special attention because of their potential to produce a wide variety of anti-microbial metabolites. Pseudomonads are most diverse and ecologically significant group of bacteria on the planet (Vanitha *et al*., 2009; Erdogan and Benlioglu, 2010)

In the present study, out of 99 colonies of fluorescent pseudomonads collected from different rhizosphere soils of pomegranate growing areas of northern Karnataka and border areas of Maharashtra (Table 1), 84 colonies showed antagonistic activity against *X. axonopodis* pv. *punicae* under *in vitro*

Table 1. Isolation of fluorescent pseudomonad (FP) strains from pomegranate rhizosphere soil

State	District	Location	Number of FP colonies collected	Total No. of FP colonies collected	Colony code
KARNATAKA	Bagalakote	Ankalagi	6	29	FP-09, FP-10, FP-11, FP-12, FP-13, FP-14, FP-15, FP-16, FP-17, FP-18, FP-19 FP-20, FP-21, FP-22, FP-23, FP-24, FP-25, FP-26, FP-27, FP-28, FP-36, FP-37, FP-38, FP-39, FP-40, FP-41, FP-43, FP-44, FP-45
		Cholachagudda	7		
		Govindakoppa	1		
		Kaladagi	3		
		Kundaragi	11		
		Sokanadagi	1		
	Bellary	Ballahunasi	4	4	FP-5, FP-0 6, FP-07, FP-08
	Gadag	Hulihyder	1	3	FP-59, FP-60, FP-79
		Kotabal	2		
	Koppal	Hanumasagar	1	9	FP-02, FP-3, FP-4, FP-31, FP-32, FP-33, FP-34, FP-35, FP-01
		Menedal	2		
		Navali	2		
		Vadaki	3		
		Yelabunachi	1		
	Raichur	Kadagammanadoddi	1	18	FP-29, FP-30, FP-42, FP-46, FP-47, FP-48, FP-49, FP-50, FP-51, FP-52, FP-53, FP-54, FP-55, FP-56, FP-57, FP-58, FP-77, FP-78
		Hutkur	1		
		Neeramanvi	16		
	Vijayapur	Babanagar	7	16	FP-67, FP-68, FP-69, FP-70, FP-71, FP-72, FP-80, FP-81, FP-82, FP-83, FP-84, FP-92, FP-FP-93, FP-94, FP-95, FP-96
		Kanamadi	9		
MAHARASHTRA	Sangli	Shegoan	3	5	FP-75, FP-76, FP-97, FP-98, FP-99
		Singanahalli	2		
	Solapur	Kadlas	3	15	FP-61, FP-62, FP-63, FP-64, FP-65, FP-66, FP-73, FP-74, FP-85, FP-86, FP-87, FP-88, FP-89, FP-90, FP-91
		Madvash	2		
		Pandarpur	10		

(Table 2). The zone of inhibition ranged from 1.47 cm to 4.35 cm. The top nine strains which showed maximum inhibitory zone and where pathogen failed to show re-growth in the inhibitory zone after 144 h after inoculation were selected for further studies. Among the nine selected strains, FP-87 showed a maximum zone of inhibition of 4.35 cm in diameter followed by FP-64, FP-19 and FP-03 with a zone of inhibition of 4.07 cm, 3.58 cm and 3.48 cm in diameter respectively (Table 3, Fig. 3 and 4). However, inhibition zone produced by FP-87 strain was significantly superior over remaining strains except FP-64 where they were at par with each other. Various research workers also demonstrated the role of fluorescent pseudomonads in the inhibition of different species of *Xanthomonas*. Similar observation was made by Jagadeesh (2000) wherein out of 431 rhizobacterial isolates screened against *Ralstonia solanacearum*, 38 strains were found to be potent antagonists with the zone of inhibition of the pathogen varying from 8-20 mm in diameter. Further, studies indicated that antibacterial mechanism was due to production of antibiotic siderophore or HCN. Study conducted by Suneesh (2004) revealed that out of 48 different strains of fluorescent pseudomonads collected from Western Ghats of Uttar Kannada districts only two isolates were found to be potential antagonists against *R. solanaceaium*. Megha (2006) reported that out of 52 strains of fluorescent pseudomonads collected from Western Ghats of Uttar Kannada district only 14 were inhibitory to *Ralstonia solanacearum* and 10 were inhibitory to *Xanthomonas*

Table 2. *In-vitro* screening of fluorescent pseudomonads against *Xanthomonas axonopodis* pv. *punicae*

Fluorescent pseudomonads	Mean inhibitory zone (cm) after 72 hours of incubation	Regrowth of pathogen after 144 hours of incubation
FP-1	0	0
FP-2	3.27	+
FP-3	3.48	-
FP-4	3.28	+
FP-5	3.43	++
FP-6	3.40	++
FP-7	3.47	-
FP-8	3.17	+
FP-9	2.97	+
FP-10	3.25	-
FP-11	3.05	++
FP-12	3.13	++
FP-13	3.18	+
FP-14	3.18	-
FP-15	3.23	++
FP-16	3.18	++
FP-17	1.77	++
FP-18	2.87	++
FP-19	3.58	-
FP-20	1.72	++
FP-21	3.27	++
FP-22	3.03	++
FP-23	2.85	++
FP-24	2.17	++
FP-25	2.10	++
FP-26	0.00	0
FP-27	0.00	0
FP-28	0.00	0
FP-28	1.47	++
FP-29	0.00	0
FP-30	0.00	0
FP-31	0.00	0
FP-32	1.85	++
FP-33	1.83	++
FP-34	1.92	++
FP-35	0.00	0
FP-36	0.00	0
FP-37	1.55	++
FP-38	1.98	++
FP-39	1.67	++
FP-40	1.80	++
FP-41	1.65	++
FP-42	0.00	0
FP-43	0.00	0
FP-44	0.00	0
FP-45	2.30	++
FP-46	2.40	++
FP-47	3.20	++
FP-48	2.65	++
FP-49	2.42	++

Fluorescent pseudomonads	Mean inhibitory zone (cm) after 72 hours of incubation	Regrowth of pathogen after 144 hours of incubation
FP-50	2.30	++
FP-51	2.20	++
FP-52	2.28	++
FP-53	1.87	++
FP-54	1.53	++
FP-55	2.65	++
FP-56	2.27	++
FP-57	2.08	++
FP-58	2.42	++
FP-59	2.20	++
FP-60	4.22	+
FP-61	4.20	+
FP-62	4.23	+
FP-63	4.07	+
FP-64	4.07	-
FP-65	3.92	++
FP-67	2.37	++
FP-68	1.98	++
FP-69	2.35	++
FP-70	2.07	++
FP-71	2.42	++
FP-72	2.50	++
FP-73	2.13	+
FP-74	2.75	++
FP-75	2.63	++
FP-76	2.80	++
FP-77	1.77	++
FP-78	2.08	++
FP-79	3.40	-
FP-80	2.23	++
FP-81	2.23	++
FP-82	2.27	++
FP-83	2.23	++
FP-84	1.95	++
FP-85	3.92	+
FP-86	4.07	+
FP-87	4.35	-
FP-88	4.00	-
FP-89	3.98	-
FP-90	4.13	-
FP-91	0.00	0
FP-92	0.00	0
FP-93	0.00	0
FP-94	3.22	-
FP-95	3.07	-
FP-96	0.00	-
FP-97	2.07	++
FP-98	2.02	++
FP-99	1.88	++

Note: '-' No re-growth of pathogen; '+' Slight re-growth of pathogen; '++' Full re-growth of pathogen; '0' No inhibition of pathogen.

Table 3. Efficient strains of fluorescent pseudomonad in terms of zone of inhibitions

Fluorescent pseudomonad strains	Name of the village	Zone of inhibition (cm)	Remarks (Numerical ranking)
FP-03	Hanumasagar	3.48	4
FP-07	Ballahunasi	3.47	5
FP-10	Govindakoppa	3.25	7
FP-14	Cholachagudda	3.18	9
FP-19	Kundaragi	3.58	3
FP-64	Kadlas	4.07	2
FP-79	Hulihyder	3.40	6
FP-87	Pandarpur	4.35	1
FP-94	Jumnal	3.22	8
S.Em. ±		0.14	
CD @1 %		0.59	

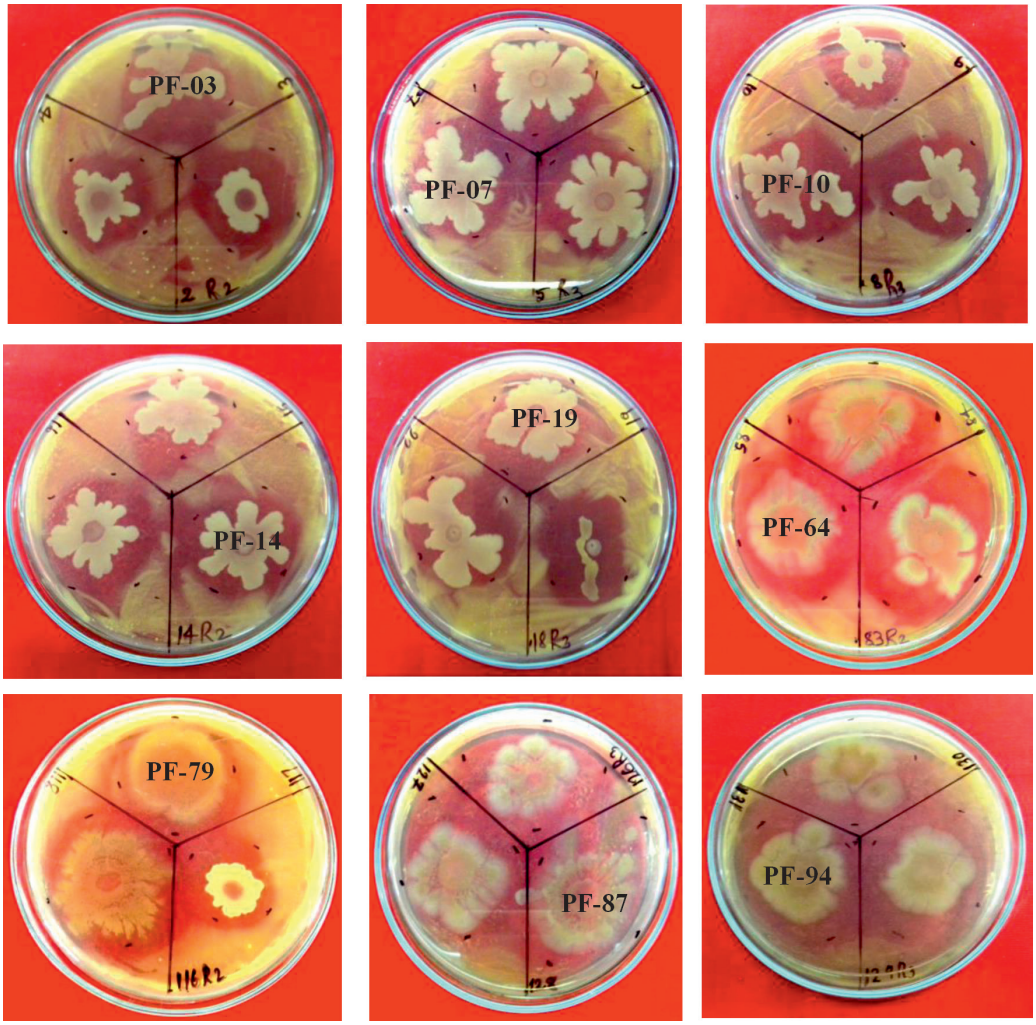

Fig. 4. *In-vitro* screening of fluorescent pseudomonads against *X. axonopodis* pv. *punicae*

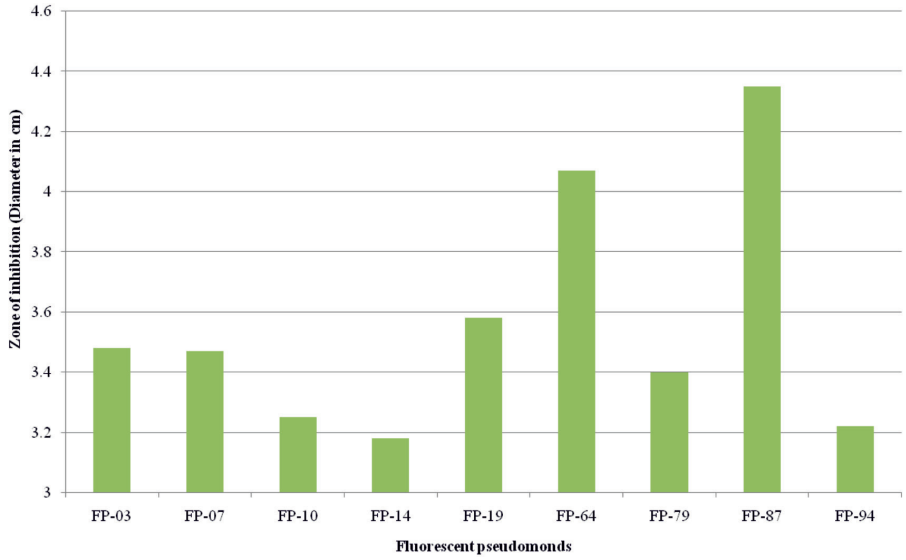

Fig. 3. Efficient strains of fluorescent pseudomonads in terms of zone of inhibition

campestris. Al-Saleh (2014) found that out of the 22 fluorescent pseudomonads screened against *X. citri* subsp *citri* under *in-vitro,* only five were capable of inhibiting the growth of the pathogen while others did not showed any antibiosis activity. The diameter of inhibition zones ranged from 45 (KSA14) to 62 mm (KSA1). The isolates KSA1 from Abha and KSA17 from Jazan showed the highest antibiosis activities.

The bio-control potential of these native fluorescent pseudomonads which were found effective under *in-vitro* need to be tested under field condition on large scale to identify the promising isolate for further adaption in the integrated management of bacterial blight.

REFERENCES

Al-Saleh, M.A. (2014). Evaluation of Saudi fluorescent pseudomonads isolates as a biocontrol agent against citrus canker disease caused by *Xanthomonas citri* subsp. *citri* A. *Egypt. Acad. J. Biol. Sci.* **6**: 1 – 7.

Buttimer, C., McAuliffe, O., Ross, R.P., Hill, C., O'Mahony, J. and Coffey, A. (2017). Bacteriophages and bacterial plant diseases. *Front. Microbiol.* **8**: 1-15. http://dx.doi.org/10.3389/fmicb.2017.00034.

Erdogan, O. and Benlioglu, K. (2010). Biological control of Verticillium wilt on cotton by the use of fluorescent *Pseudomonas* spp. under field conditions. *Bio. Control* **53**: 39-45.

Hingorani, M.K. and Singh, N.J. (1959). *Xanthomonas punicae* sp. Nov. on pomegranate (*Punica granatum* L.). *Indian J. Agric. Sci.* **29**: 45-48.

Jagadeesh, K.S. (2000). Selection of rhizobacteria antagonistic to *Ralstonia sonaceraum* E. F. Smith causing bacterial wilt in tomato and their biocontrol mechanisms. *Ph.D. Thesis*, Univ. Agric. Sci., Dharwad, Karnataka (India).

Leeman, M., Van Pelt, J.A., Den Ouden, F.M., Heinsbroek, M., Bakker, P.A.H.M. and Schippers, B. (1995). Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to Fusarium wilt, using a novel bioassay. *Eur. J. Plant Pathol.* **101**: 655 – 664.

Meena, B., Marimuthu, T., Vidhyasekaran, P. and Velazhahan, R. (2001). Biological control of root rot of groudnut with antagonistic *Pseudomonas fluorescens*. *J. Plant Dis. Protect.* **108**: 369 – 381.

Megha, Y.J. (2006). Functional and molecular diversity of fluorescent pseudomonads from Western Ghats regions of Uttar Karnataka district. *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Dharwad, Karnataka (India).

Misk, A. and Franc, C. (2001). Biocontrol of chickpea root rot using endophytic actinobacteria. *Bio. Control* **56**: 811-822.

Ramesh, R., Joshi, A.A. and Ghanekar, M.P. (2009). *Pseudomonas*: Major endophytic bacteria to suppress bacterial wilt pathogen *Ralstonia solanacearum* in the eggplant (*Solanum melongena* L.). *World J. Microbiol. Biotechnol.* **25**: 47-55.

Suneesh, K. (2004). Biodiversity of fluorescent pseudomonads in soils of moist-deciduous forests of Western Ghats of Uttar Kannada district. *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Dharwad, Karnataka (India).

Vanitha, S.C., Niranjan, S.R., Mortensen, C.N. and Umesha, S. (2009). Bacterial wilt of tomato in Karnataka and its management by *Pseudomonas fluorescens*. *Bio. Control* **54**: 685-695.

Vauterin, L., Haste, B., Kersters, K. and Swings, J. (1995). Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology* **45**: 475-489.

Vega, F.E., Posada, F., Aime, M.C., Pava, R.M., Infante, F. and Rehner, S.A. (2008). Entomopathogenic fungal endophytes. *Bio Control* **46**: 72-82.

Vidhyasekaran, P. and Muthamilan, M. (1999). Evaluation of powder formulation of *Pseudomonas fluorescens* Pf1 for control of rice sheath blight. *Biocontrol Sci. Techn.* **9**: 67 – 74.

Vidhyasekaran, P., Kamala, N., Ramanathan, A., Rajappan, K., Paranidharan, V. and Velazhahan, R. (2001). Induction of systemic resistance by *Pseudomonas fluorescens* Pf1 against *Xanthomonas oryzae* pv. *oryzae* in rice leaves. *Phytoparasitica* **29**: 155 – 166.

Weller, D.M. and Cook, R.J. (1983). Suppression of take all of wheat by seed treatments with fluorescent Pseudomonds. *Phytopathology* **73**: 403.

Yang Xie J, Jiang D, Fu Y, Li G. and Lin F. (2007). Antifungal substances produced by *Penicillium oxalicum* strain py-I potential antibiotics against plant pathogenic fungi. *World Journal of Microbial Biotechnology* **24**: 909-915.