Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6967

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (5):** 272-280 (2018)

International Journal of Pure & Applied Bioscience

Research Article

OPEN ACCESS

In vitro Evaluation of Antagonists Against *Xanthomonas axonopodis* pv. *punicae*

A. Chowdappa[1*], A. Kamalakannan[1], S. Kousalya[2], C. Gopalakrishnan[1], K. Venkatesan[3] and G. Shali Raju[4]

[1]Department of Plant Pathology, Center for Plant Protection Studies,

[2]Vanavarayar Institute of Agriculture, Pollachi

[3] Department of Horticulture, Tamil Nadu Agricultural University, Coimbatore,

[4]Krishi Vigyan Kendra, Dr. Y.S.R. Horticultural University, Venkataramannagudem

*Corresponding Author E-mail: chowdaagri008@gmail.com

Received: 3.06.2018 | Revised: 29.07.2018 | Accepted: 7.07.2018

ABSTRACT

Bacterial blight of pomegranate caused by Xanthomonas axonopodis pv. *punicae is an important disease in India. The Biocontrol agents such as six isolates of Bacillus subtilis, seven Pseudomonas fluorescens and six Streptomyces were isolated from the pomegranate rhizosphere soil and identified based on the cultural, biochemical and molecular characters. These biocontrol agents were screened under In vitro condition. Among the six Bacillus subtilis isolates, thehighest inhibition zone was recorded in the BS5 isolate this is followed by BS6 isolate. Among the seven isolates of Pseudomonas fluorescens, the maximum inhibition zone recorded in the Pseudomonas fluorescens PF5 this is followed by PF3 and PF1 isolates. Among the six Streptomyces species screened, the maximum inhibition was recorded in the isolate GG3 followed by GG4 isolate.*

Key words: *Xanthomonas axonopodis* pv. *punicae, Bacillus subtilis, Pseudomonas fluorescens, Streptomyces*

INTRODUCTION

Pomegranate is one of the most adaptable fruit crop and its cultivation is increasing very rapidly. It thrives well in dry tropics and sub tropics and comes up very well in soils of low fertility status as well as on saline soils. The most popular varieties suitable for processing and table use are Ganesh, Mridula, Arakta, Bhagwa, Kesar, G-137 and Khandari. Pomegranate is a good source of carbohydrates and minerals such as calcium, iron, sulphur, vitamin-C and citric acid is the most predominant organic acid[5]. In recent years, pomegranate cultivation has met with different constraints such as pest and diseases.Itis severely infected with pathogen like *Xanthomonas axonopodis* pv. p*unicae* causes bacterial blight and affects leaves, twigs and fruits. Disease is characterized by the appearance of small, irregular water soaked, dark coloured spots on leaves resulting in premature defoliation.

Cite this article: Chowdappa, A., Kamalakannan, A., Kousalya, S., Gopalakrishnan, C., Venkatesan, K. and Shali Raju, G., *In vitro* Evaluation of Antagonists Against *Xanthomonas axonopodis* pv. *punicae*, *Int. J. Pure App. Biosci.* **6(5):** 272-280 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6967

Pathogen also infects stem and branches causing girdling and cracking symptoms. Spots on fruit are dark brown, irregular slightly raised with oily appearance, which split opens with 'L'/'Y' shaped cracks at final stages. Under severe condition it destroys the entire orchard and causes heavy economic losses. Pomegranate bacterial blight is a daunting problem for pomegranate farmers in India. Though several management practices have been developed to control the pathogen, the present investigation was concentrated with an objective to manage the bacterial blight of pomegranate through biocontrol agents such as *Bacillus, Pseudomonas* and actinomycetes.

MATERIAL AND METHODS

Isolation of biocontrol agents from rhizosphere soil

One gram of soil from rhizosphere region of the plants were serially diluted up to10^{-7}. One ml of the soil suspension from the dilutions of 10^{-5}-10^{-7}were pipette out intothe sterile Petri plates. Sterilized nutrient agar and King's B media were dispensed into the plates for isolation of *Bacillus* and *Pseudomonas*, respectively. The plates were rotated in a clock wise and anti clock wise direction for uniform mixing of soil suspension with the media. The plates were incubated at 28± 2° C for 2 days. Dull white coloured colonies with serrated margins (*Bacillus*) were further sub cultured on NA and the fluorescent colonies were transferred to KB media for further use.

Biochemical characterization of Biocontrol agents

KOH test

A drop of bacterial suspension was thoroughly mixed with a drop of 3% KOH on a glass slide. Gram negative bacteria became gummy upon mixing due to separation of chromosomes as thin strands[8].

Gelatin hydrolysis

Cultures were inoculated in gelatin medium and incubated at room temperature for 2 days. After that kept the culture at 20°C for 30 min. Positive gelatin hydrolysis asshown by medium liquefaction[8].

Utilization of citrate

The slants with Simmons citrate agar were streaked with cultures and incubated atroom temperature. When the inoculated green colour of the medium turned blue after24-48 hours, it indicated the utilization of citrate by the bacteria[8].

Starch hydrolysis

Nutrient agar containing 0.2 percent soluble starch was used for this test. The test cultures were spotted on the Petri plates. Starch hydrolysis was tested after 48 hours of incubation by flooding the agar surface with Lugol's iodine solution. A colourless zone around the bacterial growth in contrast to the blue background of the medium indicated the positive reaction[8].

Catalase test

Smear of 24 hours old cultures were prepared on clean slide and covered with a few drops of three per cent hydrogen peroxide. Effervescence indicated the presence of catalase in the culture[8].

Molecular identification of biocontrol agents (*Bacillus* and *Pseudomonas*)

Identification of *Bacillus* through genus specific primer

Genus specific primers B-K1/F (5'-TCACCAAGGCRACGATGCG – 3');B-K1/R (5'-CGTATTCACCGCGGCATG-3') were used to amplify the gene product of1000 bp as reported by the 10 µl reaction mixture contained 2 µl of DNA (50 ngconcentration), 20 pmol of 1 µl of each forward and reverse primers and 5 µl of red dyePCR master mixer (Genei Bangalore, India). PCR amplification was performed in athermocycler (Eppendorf Master cycler) using the conditions: Initial denaturation at95°C for 5 min, 30 cycles consisting of 94°C for 1min (denaturation), 56°C for 45 sec(annealing), 72 °C for 1 min (primer extension) and final extension 72°Cfor 10 min. FinalPCR product was electrophoresed on 1.2 per cent agarose gel stained with ethidium bromideand was analysed using gel documentation system[9].

Identification of *Pseudomonas* through genus specific primer

Genus specific primers PS F (5'-GGTCTGAGAGGATGATCAGT – 3'); PS R(5'-TTAGCTCCACCTCGCGGC-3'), were used to amplify the gene at 990 bp. The10 µl mixture contained 2 µl of DNA (50 ng concentration), 20 pmol of 1 µl of each forward and reverse primers and 5 µl of red dye PCR master mixer (Genei Bangalore, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler) using the condtions: Initial denaturation at 95°C for 5 min, 35 cycles consisting of 94°C for 1min (denaturation), 57°C for 1 min (annealing), 72 °C for 1 min(primer extension)and final extension 72°C for 10 min. Final PCR product was electrophoresed on 1.2 per cent agarose gel stained with ethidium bromide and was analysed using gel documentation system.

In vitro* screening of biocontrol agents against *Xanthomonas axonopodis* pv. *punicae

Biocontrol agents (*Bacillus*, *Pseudomonas*) were multiplied in Nutrient and King's B broth respectively and incubated in incubator cum shaker at 28± 2° C for 2 days at 120 rpm. Individual *Streptomyces* isolates were multiplied in Ken Knight broth and incubated in incubator cum shaker at 28± 2° C for 7 days at 120 rpm[2].Meanwhile, bacterial pathogen was also inoculated in Nutrient broth and incubated in shaker for 24 hours and was mixed with nutrient agar at lukewarm temperature (40°C) at the rate of 1 ml for 100 ml of the media. Media seeded with bacterial culture was dispensed into the sterile Petri plates and was allowed to solidify. Sterilized filter paper disc of 5 mm diameter was dipped in the broth containing different strains of Biocontrol agents and was placed in the four corners of the plates. Three replications were maintained and incubated at room temperature (28± 2°C) for 2 days and inhibition zone around the filter paper disc was measured in cm.

RESULTS

Isolation and characterization of biocontrol agents

Thirteen isolates of antagonistic bacteria were isolated from the rhizosphere soilsof pomegranate. They were subjected to different biochemical tests. Based on the results of biochemical tests, Six of them identified as *Bacillus subtilis* and remaining sevenisolates identified as *Pseudomonas fluorescens* (Table 1, Table 2). These six isolates were confirmed as *Bacillus subtilis* through amplication by genus specific primers which produced an amplicon of 1000 bp. Similarly, *Pseudomonas fluorescens* was confirmed using genus specific primers which amplified a product of990 bp (Plate 1a and 1b).

In vitro* screening of biocontrol agents against *Xanthomonas axonopodis* pv. *punicae

Six isolates of *Bacillus subtilis* and seven isolates of *Pseudomonas fluorescens* were screened against growth of *X. axonopodis* pv. *punicae* by using filter paper discmethod on seeded medium. The results revealed that all the *Bacillus subtilis* and *Pseudomonas fluorescens* isolates inhibited the growth of the pathogen by producing clear inhibition zone around the filter paper. Among the six *Bacillus subtilis* isolates, the highest inhibition zone was recorded in the BS5 (2.08 cm). This is followed by BS6which recorded an inhibition zone of 2.01 cm. The other isolates were also inhibited the growth of *X. axonopodis* pv. *punicae* to an extend of (1.97cm to 1.47cm). The least inhibition zone was recorded in *Bacillus subtilis* BS2(1.33cm) (Table 3, Fig. 1 and Plate 2).

Among the seven isolates of *Pseudomonas fluorescens*, the maximum inhibition zone recorded in the *Pseudomonas fluorescens* PF5 (2.98 cm). This is followed by PF3(2.03 cm), PF1 (1.83cm) and PF2 (1.77cm). The minimum inhibition was observed in thePF7 (1.47cm) (Table 4, Fig. 2 and Plate 3).Most of the *Streptomyces* species screened in this study were able to inhibit the growth *X. axonopodis* pv. *punicae*. Among the six *streptomyces* species screened, the maximum inhibition was recorded in the isolate GG3 (3.03 cm) followed by GG4 (2.98 cm).The other isolates were also effective in reducing growth of *X. axonopodis* pv. *punicae* tolesser extent (Table 5 and Plate 4).

DISCUSSION

In vitro screening of biocontrol agents

In vitro screening of biocontrol agents will helpful for the identification of effective and elite isolates. Identification of effective isolates will facilitate the formulation of effective biocontrol agents and their shelf life. In the presence study seven isolates of *Pseudomonas fluoreoscens*, six isolates of *Bacillus subtilis* and six isolates of *Streptomyces* spp. were screened against growth of *X. axonopodis* pv. *punicae.* All the biocontrol agents exerted inhibitory effect on the growthof *X. axonopodis* pv. *punicae*. The inhibitory effect of *Pseudomonas fluorescens* might be due to competition, saprophytic ability, and production of cell wall degrading lytic enzymes, production of antibacterial, antibiotics and HCN production[3].The effect of *Pseudomonas* on the bacterial growth of *X.axonopodis* pv. *malvaciarum* was studied by Salaheddin *et al.*[7], and found that *Pseudomonas* and *Bacillus* isolates recorded on inhibition zone of ranging from 2.6 mm to 25 mm in *Pseudomonas* and 3.12 to 6.1 mm inhibition zone in *Bacillus subtilis. Bacillus subtilis* are known to produce a wide arsenal of antimicrobial substances including peptide and lipopeptide, antibiotics and bacteriocins[1]. Actinomycetes are prolific producers of useful bioactive metabolites with broad spectrum antimicrobial activitiy. The antibacterial activities of actinomycetes have been reportedon several phytopathogenic bacteria such as *Erwinia*, *Pseudomonas, Xanthomonas* and*Clavibacter*[4]. Poovarasan *et al.*[6], studied inhibitory effect of eight actinomycetes against the growth of *X. axonopodis* pv. *punicae* and found that *Streptomyces fradiae* recorded higher inhibition zone (2.41 cm).

Table 1. Biochemical characterization of *Bacillus subtilis* isolates

S. No.	*B. subtilis* isolates	Starch hydrolysis	Citrate utilization	Catalase	KOH test	Gelatin liquefaction
1	BS1	+	++	+	-	+
2	BS2	+	++	+	-	++
3	BS3	+	+	+	-	++
4	BS4	+	+	+	-	+
5	BS5	+	+++	+	-	+
6	BS6	+	+	+	-	+

+++ Strong production, ++ Medium Production, + Normal Production, - Negative

Table2. Biochemical characterization of *Pseudomonas fluorescens* isolates

S. No.	*P. fluorescens* Isolates	Starch hydrolysis	Citrate utilization	Catalase	KOH test	Gelatin liquefaction
1	Pf1	+	+	+	+	+
2	Pf2	+	+	+	+	++
3	Pf3	+	+	+	+	+
4	Pf4	+	+	+	+	+
5	Pf5	+	+	+	+	+
6	Pf6	+	+	+	+	+
7	Pf7	+	+	+	+	+

+++ Strong production, ++ Medium Production, + Normal Production, - Negative

Table 3. *In vitro* screening of *Bacillus subtilis* against *Xanthomonas axonopodis* pv. *punicae*

S. No.	*Bacillus subtilis* isolates	Inhibition Zone (cm)*
1	BS1	1.97 (1.57)^{ab}
2	BS2	1.33 (1.35)^{c}
3	BS3	1.47 (1.40)^{c}
4	BS4	1.57 (1.44)^{bc}
5	BS5	2.08 (1.61)^{a}
6	BS6	2.01 (1.58)^{ab}
7	Control	0.00 (0.71)^{d}
SEd		**0.07**
CD (0.05)		**0.15**
CV%		**6.39**

***** - Means of three replication

Values in the parentheses are square root transformed values

Table4. *In vitro* screening of *Pseudomonas fluorescens* against *Xanthomonas axonopodis* pv. *punicae*

S. No.	Isolates	Inhibition Zone (cm)*
1	PF1	1.83 (1.53)^{b}
2	PF2	1.77 (1.51)^{b}
3	PF3	2.03 (1.59)^{b}
4	PF4	1.53 (1.43)^{b}
5	PF5	2.98 (1.87)^{a}
6	PF6	1.50 (1.41)^{b}
7	PF7	1.47 (1.40)^{b}
8	Control	00 (0.71)^{c}
SEd		**0.12**
CD (0.05)		**0.25**
CV%		**10.35**

*Means of three replication

Values in the parentheses are square root transformed values

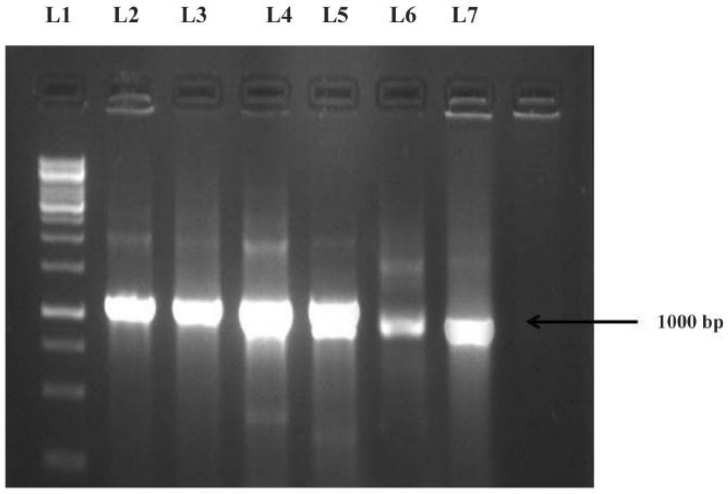
Table 5. ***In vitro*** **screening of** ***Streptomyces*** **isolates against** ***Xanthomonas axonopodis*** **pv.** ***punicae***

S. No.	*Streptomyces* species isolates	Zone of inhibition (cm)*
1	GG1	2.84 (1.83)[a]
2	GG2	0.00 (0.71)[b]
3	GG3	3.03 (1.88)[a]
4	GG4	2.98 (1.86)[a]
5	GG5	0.00 (0.71)[b]
6	GG6	0.00 (0.71)[b]
7	Control	0.00 (0.71)[b]
SEd		**0.03**
CD (0.05)		**0.05**
CV%		**3.28**

*- Means of three replication

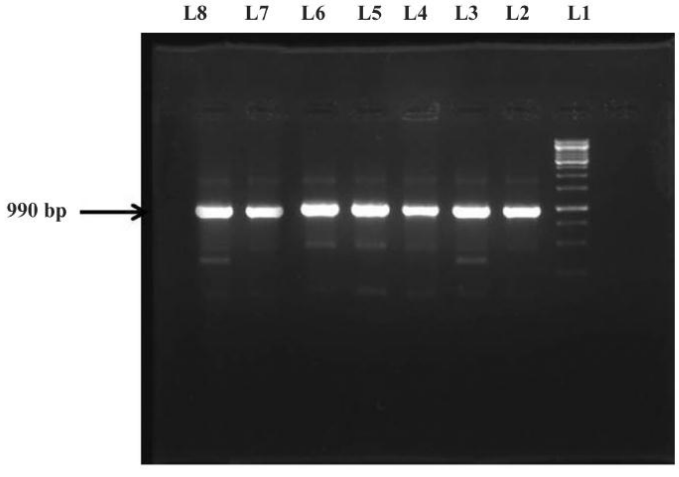
Values in the parentheses are square root transformed values

Plate 1a. Molecular confirmation of *Bacillus subtilis*



L1- 1kb Ladder, L2- BS1, L3- BS2, L4- BS3, L5-BS4, L6-BS5, L7- BS6

Plate 1b. Molecular confirmation of *Pseudomonas fluorescens*



L1- 1kb Ladder, L2- PF1, L3- PF2, L4- PF3, L5-PF4, L6-PF5, L7- PF6, L8- PF7

Plate 2. ***In vitro*** **screening of** ***Bacillus subtilis against Xanthomonas axonopodis*** **pv.** ***punicae***

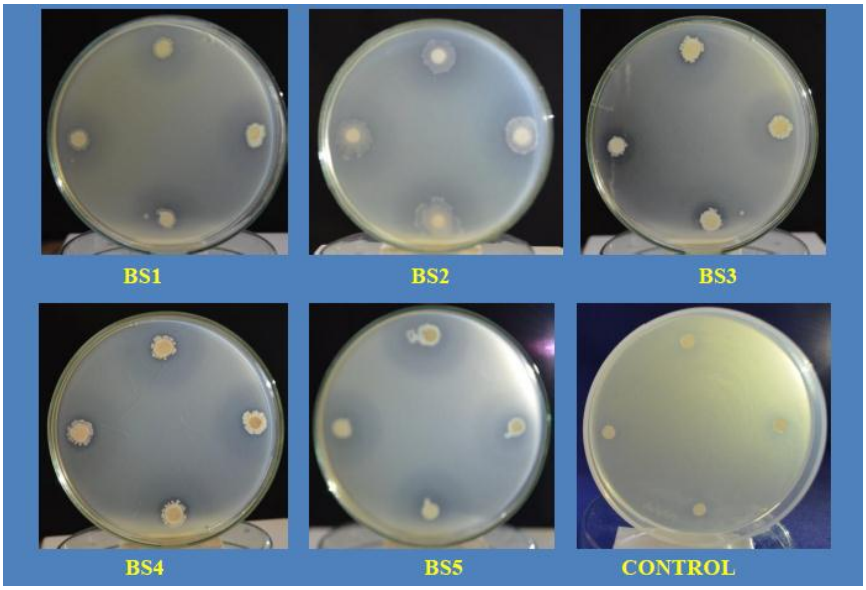


Plate 3. ***In vitro*** **screening of** ***Pseudomonas fluorescens against Xanthomonas axonopodis*** **pv.** ***Punicae***

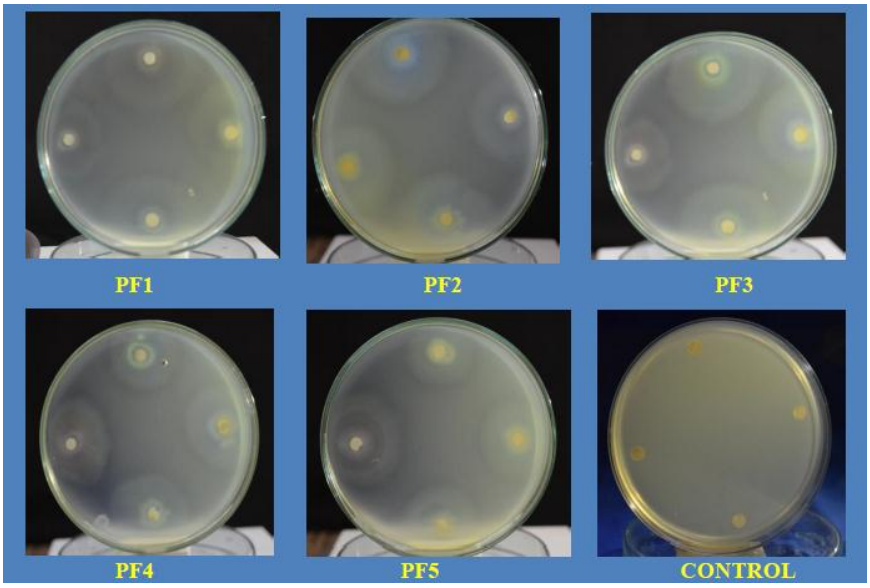


Plate 4. ***In vitro*** **screening of** ***Streptomyces against Xanthomonas axonopodis*** **pv.** ***punicae***

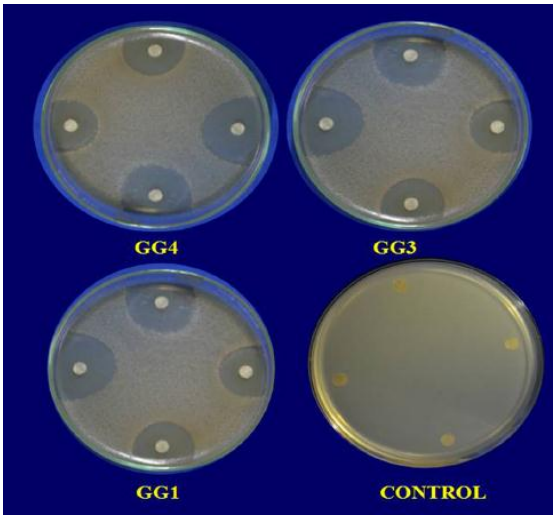


Fig. 3: *In vitro* screening of *Bacillussubtilis*against *Xanthomonas axonopodis* pv. *punicae*

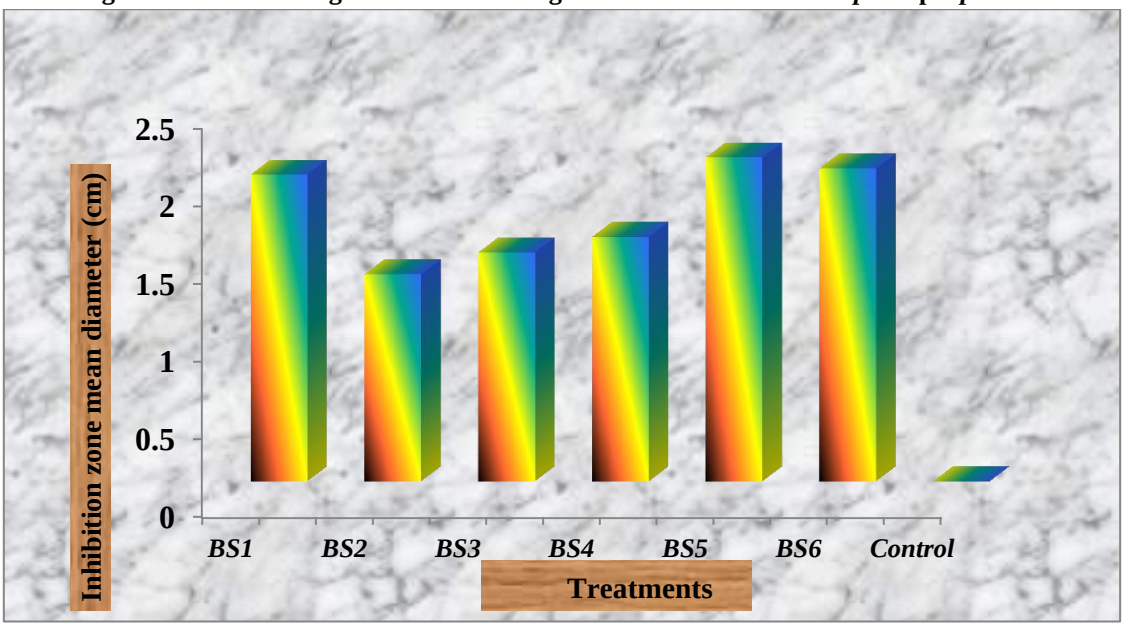
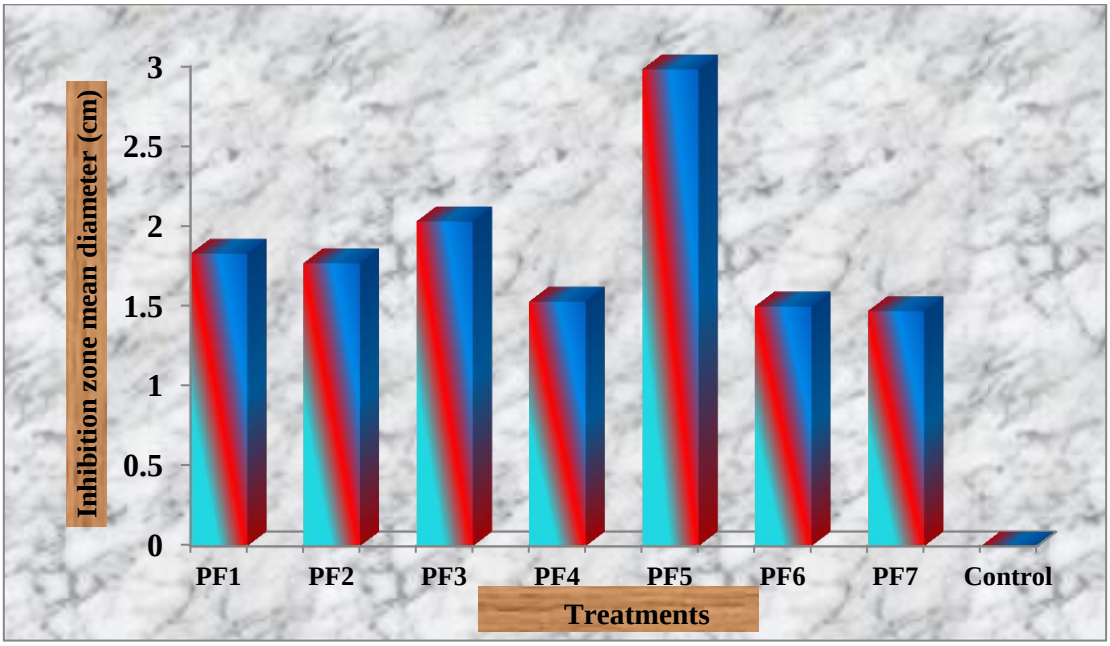


Fig. 4: *In vitro* screening of *Pseudomonas fluorescens* against *Xanthomonas axonopodis* pv. *punicae*



REFERENCES

1. Abrouel, H., Franz, C. M., Ben Omar, N. and Galvez, A., Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiology Reviews* **35(1):** 201-232 2011.
2. Bauer, A.W., Kriby, W.M., Sherries, J. C. and Turck, M., Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology* **36:** 493-496 (1966).
3. Bural, G. I., Dixon, D. G. and Glick, B. R., Plant growth promoting bacteria that decrease heavy metal toxicity in plants. *Canadian* Journal of *Microbiology* **46:** 237-245 (2000).
4. Kieser, T., BibbM, J., Buttner, M. J., Chater, K. F. and Hopwood, D, A., Practical *Streptomyces* Genetics. *John Innes Foundation, Norwich, England, ISBN* 0-7084-0623-8 (2000).
5. Malhotra, H. K., Khajuria, H. H. and Jawanda, J. S., Studies on physic chemical characteristics of pomegranate cultivars. *Punjab Horticulture Journal* **23:** 153-161 (1983).

6. Poovarasan, S., Sukhada, M., Paneerselvam, P., Sarith, a B., Ajay, K.M., Mycorrhizae colonizing actinomycetes promote plant growth and control bacterial blight disease of pomegranate. (*Punica granatum* L. cv Bhagwa), *Crop Protection* **53:** 175-181 (2013).
7. Salaheddin,K., Valluvaparidasan, V., Ladhalakshmi, D. and Velazhahan, R., Management of bacterial blight of Cotton using a mixture of *Psuedomonas fluorescens* and *Bacillus subtilis. Plant Protection Science* **46(2):** 41-50 (2010).
8. Schaad, N.W., Laboratory Guide for Identification of Plant Pathogenic Bacteria. *Second Ed. International Book Distributing* Co, Lucknow, p 165 (1992).
9. Taale, E., Savadogo, A., Zongo, C., Somda, M. K., Sereme, S. S., Karou, S. D., Soulama Iand Traore, A. S., *International Journal of Advanced Research in Biological Sciences. International Journal of Advanced Research in Biological Sciences* **2(4):** 279-290 (2015).