International Journal of Scientific & Engineering Research, Volume 6, Issue 2, February-2015
ISSN 2229-5518

Molecular identification and evaluation of inhibitory activity of antagonistic Bacteria isolated from Pomegranate field of Rajuluru village against Phyto-pathogens

Vulasi Indrani [1], Madhuri T[1], Lakshmi kalyani B[1], Sreedevi A[1], Nagalakshmi Devamma M[2] and Suvarnalatha Devi P[1]
Department of Applied Microbiology[1]
[1]Sri Padmavathi Mahila Visvavidyalayam (Women's University), Tirupati- 517502

[2] Department of Botany, S.V.University[2], Tirupati- 517502

Email of Corresponding Authors:drsuvarnapallipati@gmail.com

Abstract— Phyto- pathogens are the causal agents of plant diseases, which is one of the most destructive crop loss through worldwide. The yield of crop plants is severely diminished by the regular outbreaks of plant diseases, a large part of which is caused by pathogenic fungi and bacteria. Biological control is thus being considered as an alternative way of reducing the use of chemicals in agriculture. The present study was aimed to evaluate the antagonistic activity of bacteria isolated from pomegranate soils from Rajuluru village of Chittoor district. Thirty two bacterial isolates were isolated from soil sample. To evaluate the antagonistic activity of all the isolates, they were screened against Phyto pathogens i.e *Colletotrichumgloeo sporioides* from Pomegranate, *Helminthosporiumturcicu* from sorghum, *Phytoptheradrechsleri* from Red gram, *Colletotrichum lindemuthianum* from Dolichos, *Xanthomonasphaseoli var. sojense* from Horse gram, *Xanthomonas axonopodis* from lemon. Among all the isolates PI 9, PI 12, PI 27, PI 28 shows antagonistic activity. Isolate PI12 suppressed the growth of all tested pathogens potentially. Based on16S rRNA gene sequencing and phylogenetic analysis the above potent antagonistic isolate PI 12 were identified as *pseudomonas aeruginosa.*

Index Terms— Phyto pathogens, Antagonistic activity, Biocontrol agent,16S rRNA, Phylogenetic analysis.

1 INTRODUCTION

Soil is a rich medium on which a variety of microorganisms can grow and multiply [1].Microorganisms display interesting competitive mechanisms, of which antagonism has been commonly referenced [2],[3]. Antagonistic bacteria are common soil inhabitants with potential to be developed into biofungicides for the management of seedling damping-off, root rot, and other soil-borne diseases of various crops [4]**.** Pathogens are estimated to account for a loss in crop yield that could feed tons of millions of people, the socio-economic impact of microbial plant pathogens cannot be overestimate. The mechanisms of biological control of plant pathogens by antagonistic bacteria and fungi have been the subjects of many studies in the past two decades [5].Antagonists are biocontrol agents such as bacteria, fungi, actinomycetes, viruses, and nematodes that reduce the number of disease producing activities of the pathogens [6]. Germicides are usually used as a solution to the problems of pathogen attack, however their use results in serious environmental problems. Recently, there has been an increasing interest in using beneficial microorganisms as a solution to the overuse of potentially harmful pesticides [7],[8],[9].Potential use of naturally occurring bacteria, actinomycetes and fungi replacement or supplements for chemical pesticides have been addressed in many studies [10],[11],[12],[13].Even though chemical inputs such as pesticides showed promising results in controlling the disease, phytotoxicity and chemical residues may pose a serious threat to the environment and human health [14].Control of phytopathogens by biological means was environmentally advantageous in comparison to chemical control methods which had many risks on human health and environment [15].The present work was undertaken with an effort to isolate Antagonistic bacteria from pomegranate soil samples of Rajuluru village, chittoor (district) and evaluate their antagonistic potentials against phyto pathogenic bacteria.The potential isolate was identified by 16S rRNA partial gene sequence.

2 MATERIALS AND METHODS

2.1 collection of soil sample

Samples were taken randomly to a depth of 5 cm from the top soil from Pomegranate field using sterile spatula and soil were sealed in sterile polythene bags.

2.2 Serial dilution of soil sample

The samples were serially diluted from 10^{-1} to 10^{-9}. 0.1 ml of sample was spreaded on the nutrient agar plates using sterile glass rod and incubated for 24 hours at room temperature until colonies were developed.

2.3 Collection of Phytopathogens

The Different Phyto pathogens selected for study such as *Colletotrichumgloeo sporioides*, *Helminthosporiumturcicu*, *Phytopthera drechsleri*, *Colletotrichum lindemuthianum*, *Xanthomonasphaseoli var. sojense*, *Xanthomonas axonopodis*.

2.4 Screening of Antagonistic Bacteria against Phytopathogens by Dual culture technique

In vitro evaluation of antagonistic activity against the phytopathogens was carried by Dual culture method [16]. Culture of the pathogen was placed at the centre of a Petri dish containing nutrient agar medium. The isolates was inoculated at opposite corner. Plates were incubated for24 hours at 37°C and growth of the pathogen was measured and compared to control growth [17].

2.5 Molecular Identification of antagonistic bacteria

The potent Antagonistic isolate was confirmed using gene sequencing of 16S ribosomal RNA. DNA extraction, polymerase chain reaction (PCR) and sequencing were done at Credora life sciences, Bangalore,India. The 16S rRNA region was sequenced using primers pA andpH and gene sequence was analysed using CLUSTAL analysis

.

3 RESULT AND DISCUSSIONS

3.1 Isolation and screening of antagonistic bacteria

A total of 32 bacterial isolates were obtained from Pomegranate fields. All the isolates were screened for antagonistic activity against phyto pathogens.Only four isolates named as PI 9, PI 12, PI 27 and PI 28 showed antagonistic activity, among four isolates PI 12 showed potent antagonistic activity against as shown below Fig 1(*Colletotrichum gloeosporioides*), Fig 2 (*Helminthosporium turcicum*), Fig 3 (*Phytopthera drechsleri),* Fig 4 (*Colletotrichum lindemuthianum*), Fig 5 (*Xanthomonasphaseoli var. sojense*) and Fig 6 (*Xanthomonas axonopodis*).

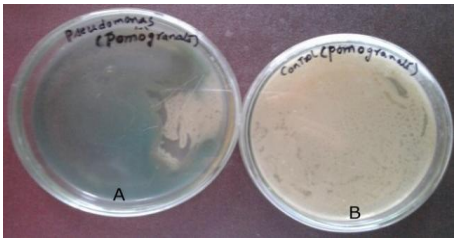

Fig 1: Antagonistic activity against *Colletotrichum gloeosporioides*
A)Inhibition of growth B) Control

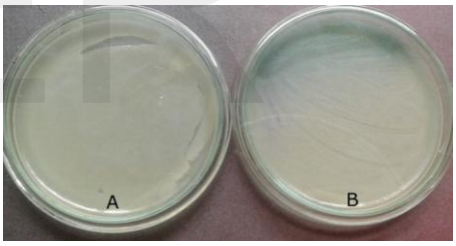

Fig2:Antagonistic activity against *Helminthosporum turcicum*
A)Control B) Inhibition of growth

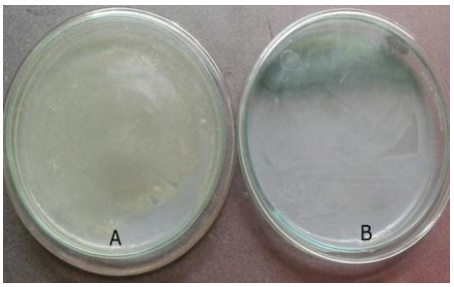

Fig3:Antagonistic activity against *Phytoptheradrechsleri*
A) Control B) Inhibition of growth

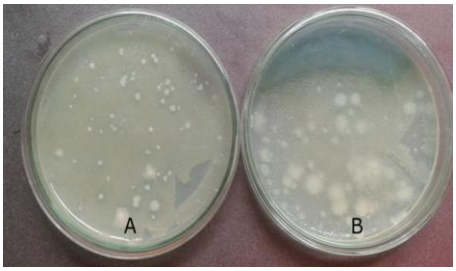

Fig4:Antagonistic activity against *Colletotrichum lindemuthianum*
A) Control B) Inhibition of growth

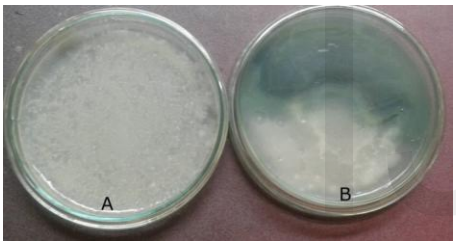

Fig5:Antagonistic activity against *Xanthomonas phaseoli var. sojense*.
A) Control B) Inhibition of growth

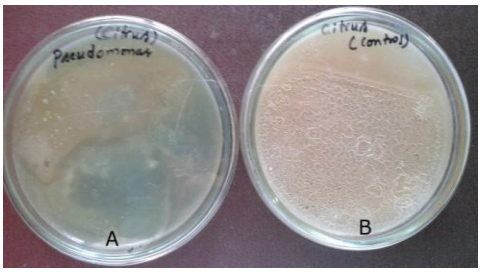

Fig 6. Antagonistic activity against *Xanthomonas axanopodis*
A) Inhibition of growth B)Control

3.2 Molecular identification of Antagonistic bacteria

3.2.1PCR amplification of 16SrRNA gene

One band of Chromosomal DNA was found on the agarose gel after illumination with the UV light. Lane one indicates the DNA marker and lane 2 indicates the bacterial chromosomal DNA. The isolated chromosomal DNA was 1500 bp which compared with DNA marker (Fig 7).

PrimerDesination	Position (*E.coli* 16S rRNA)	Sequences
pA	19-38	**5′AGA GTT TGA TCC TGG CTC AG3′**
pH	1541-1581	**5′AAG GAG GTG ATC CAG CCG CA3′**

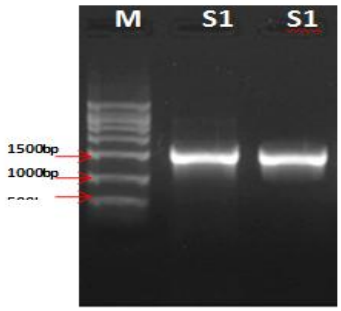

Fig7: PCR amplification of IS12 isolate using 16SrRNA gene.

3.2.2 Chromatogram file

5′TAAAAAATCCGCAGCCTACCATGCAGTCGAGCGGATG
AAGGGAGCTTGCTCCTGGATT-
CAGCGGCGGACGGGTGAGTAATGCCTAG-
GAATCTGCCTGGTAGTGGGGGA-
TAACGTCCGGAAACGGGCGCTAATACCGCA-
TACGTCCTGAGGGA-
GAAAGTGGGGGATCTTCGGACCTCACGCTATCAGAT-
GAGCCTAGGTCGGATTAGC-
TAGTTGGTGGGGTAAAGGCCTACCAAGGCGAC-
GATCCGTAACTGGTCTGAGAGGATGATCAGTCA-
CACTGGAACTGAGACACGGTCCA-
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA-
CAATGGGCGAAAGCCTGATCCAGC-
CATGCCGCGTGTGTGAA-
GAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAG-
GAAGGGCAGTAAGTTTATTCCTGCTGTTGACGTAA-
CAACGGAATAAGCATCGGTAACTTCGTGCCAG-
CAGCTCGTTAATACGAAGGTGCAAGCGTTAATG-
GAAATTTCTGGCGTAAGGCGCGCGTAGGGGGTTCAG-
CAATTGGATGGGAAATCCCCGGGGCTCAACCTGGGAAC
TGCATCCAAAACTACTGAGCTAGAGTACGGTA-
GAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTA
GATATAGGAAGGAACAACAGTGGCGAAGGCGAC-
CACCTGGACTGATACTGACACT-
GAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA-
TACCCTGGTAGTCCACGCCGTAAACGATGTCGAC-
TAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGC-
TAACGCGATAAGTCGACCGCCTGGGGAG-
TACGGCCGCAAGGTTAAAACTCAAATGAATT-
GACGGGGGCCCGCACAAGCGGTGGAG-
CATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCA-

GAGATGGATTGGTGCCTTCGGGAACTCAGACA-
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGA-
GATGTTGGGTTAAGTCCCGTAAC-
GAGCGCAACCCTTGTCCTTAGTTACCAG-
CACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGA-
CAAACCGGAGGAAGGTGGGGATGACGTCAAGTCAT-
CATGGCCCTTACGGCCAGGGCTACACACGTGCTA-
CAATGGTCGGTA-
CAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCA-
TAAAACC-
GATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTG
AAGTCGGAATCGCTAGTAATCGTGAATCA-
GAATGTCACGGTGAATACGTTCCCGGGCCTTGTACA-
CACCGCCCGTCACACCATGGGAGTGGGTTGCTCCA-
GAAGTAGCTAGTCTAACCGCAAGGGGGACGGTTAC-
CACGGAGTGATTCATGACTGGGGGAAGTCTTAA-
CAAGTGGACAATGGCCTC3

3.3 Phylogenetic relationship

After the 16S rRNA gene sequencing,it was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to Gene Bank database. The highest degree of similarity found was 97%, which was the value obtained with the 16S rRNA gene of *Pseudomonas aeruginosa*. On the basis of CLUSTAL results, Phylogenetic analysis (Fig 8) the isolate PI 12 was identified as *Pseudomonas aeruginosa.*

Fig 8. Phylogenetic analysis of PI 12 isolate

Plant growth promoting bacteria such as *Azospirillium, Bacillus, Pseudomonas, Rhizobium, Arthrobacte*r also inhibit fungal plant pathogens including the production of antibiotics, iron-chelating siderophores which reduce the population of major root pathogen [18].*Pseudomonas*sp., reduce root rot infection through several mechanisms, such as the induction of systemic resistance against phytopathogens in the host plant [19].*Pseudomonas* spp. commonly inhabits in soil and has been applied for biocontrol, promoting plant growth and bioremediation. Environmental *P. aeruginosa* isolates have been considered as potential biological control agents or inducers of systemic acquired resistance [20],[21],[22]. *Pseudomonas aeruginosa* isolates investigated stand out as potent antagonists of *Vibrio sp.,* [23].

Nucleotide sequence accession number

The strain deposited in Genbank with accession number KP636653.

4. CONCLUSION

The indiscriminate use of synthetic pesticides has brought undesired problems to human health, agriculture, and the environment. Plant diseases are the result of interactions among the components of disease triangle i.e. host, pathogen and environment. The present study conclude that *Pseudomonas aeruginosa* has potent antagonistic activity against all the pytopathogens selected for study. Biological control agents are the organisms that interact with the components of disease triangle to manage the disease. Bio control agents involve a bewildering array of mechanisms in achieving disease control. However the conclusive evidences for the involvement of a particular factor in biological control is determined by the strict correlation between the appearance of factor and the biological control. Future outlooks of biocontrol of plant diseases play an important role, it is possible to use the biological control as an effective strategy to manage plant diseases.

ACKNOWLEDGEMENT

The authors wish to thankful to authors/ editors/ publishers of all those articles, journals and books from where the literature for this article has been reviewed and discussed and also todepartment of Applied Microbiology,DST-FIST & CURIE, Sri Padmavathi Mahila Visvavidyalayam (Women's University), Tirupati, Andhra Pradesh, India for providing Research facilties.

REFERENCES

[1] M. Oelbermann, and R. Paul Voroney, "Antibacterial activity of five fungal strains isolated from a leguminous soil field against nitrogen-fixing bacteria ".*Ecological Engineering*., 29 (4) : pp. 342–349,2007.

[2] T.L. Czaran, R.F. Hoekstra, and L. Pagie,"Chemical warfare between microbes promotes biodiversity". *Proc. Natl. Acad. Sci*.,99: pp. 786–790, 2002.

[3] B.C. Kirkup, and M.A. Riley,"Antibiotic-mediated antagonism leads to a bacterial game of rock-paper – scissors". *in vivoNature*.,x 428 : pp.412–414,2002.

[4] Khabbaz,Abbasi PA,"Isolation, characterization, and formulation of antagonistic bacteria for the management of seedlings damping-off and root rot disease of cucumber. Can J Microbiol., 60(1): PP.25-33, 2014.

[5] W.J.Janisiewicz, T.J. Tworkoski, and C. Sharer, "Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients. Phytopathology"., 90(11): pp. 1196-2000, 2000.

[6] J.M. Whipps, and J.M. Lumsden, "Commercial use of fungi as plant disease biological control agents: status and prospects". In: Fungi as biocontrol agents: progress, problems and

potential Eds. Butt, TM, Jackson C, Magan N, Wallingford, UK: CABI Publishing., pp. 9–22, 2001.
[7] D.R. Fravel,"Role of antibiosis in the biocontrol of plant diseases". Annu Rev Phytopathol.26: pp.75–91, 1988.
[8] N. Sakthivel, and S.S. Gnanamanicham,"Evaluation of *Pseudomonas fluorescenes* for suppression of sheath rot disease and for enhancement of grain yields in rice *(Oryza sativa L.)".Appl Environ Microbiol.,* 53: pp. 2056–9, 1987.
[9] G.W. Xu, and D.C. Gross, "Selection of fluorescent *pseudomonas* antagonistic to *Erwiniacartovora* and suppressive of potato seed piece decay". Phytopathology.,76:pp.414–22, 1986..
[10] M. Schoeman, J. Webber, and D. Dickinson,"The development of ideas in biological controlapplied to forest products*". Intl. Biodeter. Biodegr.,*43:pp. 109-123, 1999.
[11]Berg,G.,P.Marten,andG. Ballin,"*Stenotrophomonas maltophilia*in the rhizosphere of oilseed rape: Occurrence, characterization and interaction with phytopathogenic fungi". *Microbiol. Res.,* 151: pp.19-27, 1996.
[12] S. Moricca, A. Ragazzi, K.R. Mitchelson, and G. Assante, "Antagonism of the two-needle pine stem rust fungi *Cronartium flaccidum* and *Peridermiumpini* by *Cladosporium tenuissimum* invitro and in planta". *Phytopathol.,*91: pp. 457-468, 2001.
[13] J.A. Lucas-GarciaA. B. Probanza, and J.J. Ramos, "Colón-Flores and F.J. Gutierrez-Mañero, Effects of Plant Growth Promoting Rhizobateria (PGPRs) on the biological nitrogen fixation, nodulation and growth of *Lupinusalbus*I. cv. Multolupa". *Eng. Life Sci.,*4 ; pp.71-77, 2004.
[14] Y. Wang,Q. Zeng, and Z .Zhang, "Antagonistic bioactivity of an *Endophytic* bacterium H-6".*African J. Biotechnol*., 9(37):pp. 6140-6145, 2010.
[15] K. Parani, and B.K. Saha, "Studies on interaction of *Serratiam arcescens* strain(SR1) with fungal pathogens" .*J. Agri. Environ. Sci*., 5(2): pp. 215- 218, 2009.
[16] N.J. Fokkema," fungal antagonism in the *phyllosphere". Ann.appl.Biol*.,89 :pp. 115-117, 1978 .
[17] F.M. Cazorla1,D. Romero, A. Pe´rez-Garcı´a, B.J.J. Lugtenberg A, de Vicente and G. Bloemberg, "Isolation and characterization of antagonistic Bacillus subtilis strains from the avocado rhizoplane displayingbiocontrol activity" *J. Appl Microbiol.,* 103: pp.1950–1959, 2007.
[18] Weller D.M, R.J. Cook, "Suppression of take all of wheat by seed treatment with fluorescent *Pseudomonads" . Phytopathology.*73, 463-469,1983.
[19] Ramamoorthy, V, Viswanathan, R, Raguchander, T, Prakasam, V, Samiyappan, R,"Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases". *Crop Prot*, 20:1-11, 2001.
[20] Anjaiah V, Cornelis P, Koedam N,"Effect of genotype and root colonization in biological control of *fusariumwilt*s in pigeonpea and chickpea by *Pseudomonas aeruginosa*.PNA1". Can *J Microbiol.,* 49:85-91, 2000.
[21] Bano N, MusarratJ "Characterization of a new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent". *Curr Microbiol.,* 46:324-328,2003.
[22] De Meyer G, Capieau K, Audenaert K, Buchala A, Métraux JP, Höfte M "Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean". *Mol Plant-Microbe Interact,* 12:450- 458, 1999.
[23] Prabhakaran Priyaja, Puthumana Jayesh, Neil Scolastin Correya, BalachandranSreelakshmi, Naduthalmuriparambil S Sudheer, Rosamma Philip, Isaac Sarogeni Bright Singh. Antagonistic effect of *Pseudomonas aeruginosa* isolates from various ecological niches on *Vibrio* species pathogenic to crustaceans, *J. Coast. Life Medi.*, 2(1): 76-84, 2014.