Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Asian Journal of Plant Science and Research, 2012, 2 (2):180-186

ISSN : 2249-7412
CODEN (USA): AJPSKY

Multifarious antagonistic potentials of rhizosphere associated bacterial isolates against soil borne diseases of Tomato

B. Sasirekha, Ashwini and Srividya Shivakumar*

Department of Microbiology, Centre for PG Studies, Jain University, Bangalore

---

ABSTRACT

Biological control of soil borne plant pathogens is a potential alternative strategy to agrochemicals that are harmful to human health and the environment. Thus the present study focuses on the screening of indigenous soil isolates with multiple traits related to biocontrol of Xanthomonas, Fusarium oxysporum and Rhizoctonia solani. Rhizosphere soils were collected from different areas of Bangalore in India. Out of 12 actinomycetes and 51 bacterial isolates that were isolated, 1 actinomycete and 3 bacterial isolates showed maximum antagonistic activity against Xanthomonas spp (13- 20%) and Fusarium oxysporum (25-76%), Rhizoctonia solani (55- 83%) by dual culture technique. These isolates were identified as B. subtilis, P. aeruginosa, Streptomyces spp and P. fluorescens. The four isolates were further screened for PGPR traits. P.aeruginosa p6 showed positive for all PGPR traits and antagonism due to siderophore and HCN production. B.subtilis B2 showed mycolytic enzymes mediated antagonism. Streptomyces sp. 9p and P. fluorescens R showed concurrent production of both mycolytic enzymes and PGPR traits. All the four isolates showed increase in seed germination in the range of 28 to 71% when compared to control (14%). Present study reports the PGPR potential and biocontrol ability of the strains which can be used as biofertilizers as well as biocontrol agents.

Keywords: Antagonism, PGPR, chitinase, *Pseudomonas* spp, *B. subtilis*.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.), one of the most important vegetable in many countries has a worldwide economic and nutritive importance [1]. *Rhizoctonia solani* and *Fusarium oxysporum* are major soilborne fungal pathogens of both greenhouse and field grown tomatoes in the warm vegetable growing areas of the world [2]. Fusarium wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani* causing damping-off, cankers, root rots, fruit decay, foliage disease causes serious economic loss. *Fusarium oxysporum* penetrates the roots mainly through wounds and proceeds into and throughout the vascular system, leading to functional collapse, systemic wilting and often the death of the infected plant [3]. *Rhizoctonia solani* reduces plant growth by rotting the roots, and thus reducing the ability of the plants to take up water and nutrients; it may even lead to plant death or at any rate to significant yield losses in field conditions [4].

Bacterial spot of tomato caused by *Xanthomonas* spp is one of the serious diseases of tomato responsible for severe economic losses. This disease is economically devastating to growers, especially in warm and damp climates which are common in tropical and subtropical regions. Tomato plants are susceptible to this disease in all developmental stages [5].

Fungicides as a soil and seed treatment are used for the control of different plant pathogens [6,7] and the number of effective fungicides with negligible effect on the environment is rare. Fungicides are expensive, can cause environmental pollution and may cause the selection of pathogen resistance [8]. Biological control, therefore, holds promise as a strategy for disease management and it is environment friendly too.

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly. PGPRs promote plant growth directly by synthesizing plant growth promoting substances or by facilitating the uptake of certain plant nutrients from the environment. Indirectly by antagonizing pathogenic fungi by production of siderophores, chitinase, β-1,3-glucanase, antibiotics, fluorescent pigments, and cyanide [9]. In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil [10]. So keeping in view the above constraints, the present study was designed to screen for rhizospheric bacterial isolates having multiple mechanisms to antagonize tomato pathogens.

MATERIALS AND METHODS

Collection of samples

Thirty five different *Solanaceae* rhizospheric soil samples were collected from in and around Bangalore, Karnataka. The sample was collected in 1cm depth and it was packed in a sterile polythene bag and labeled properly for further processing [11]. 1g of soil sample was serially diluted in sterile distilled water, 0.1 ml of soil suspension from 10^{-3} to 10^{-7} were spread plated onto nutrient agar plate for bacteria and ISP-4 medium for actinomycetes. The plates were incubated for 24hrs at 37°C. Colonies showing different morphological types were picked and subcultured onto respective media for purification and they were stored at 4°C.

Phytopathogens

Bacterial pathogen, *Xanthomonas* spp was obtained as a kind gift from IIHR, Hessarghatta, Bangalore. Two fungal pathogens *Rhizoctonia solani* (MTCC 4633) and *Fusarium oxysporum* (MTCC 1755) were obtained from IMTECH, Chandigarh.

Antagonistic effect of the isolates

Antifungal activity

The bacterial isolates were screened for *in vitro* growth inhibition of phytopathogenic fungi *Fusarium oxysporum and Rhizoctonia solani.* One microlitre of 24 hrs old bacterial culture was inoculated in potato dextrose agar (PDA) plates and a 3cm diameter disc of mycelia was introduced in the plate centre and incubated for 10 days at 25°C. The culture plates were observed constantly, the radial growth of the pathogen recorded on the fifth day of inoculation and percentage inhibition was calculated using the formula,

Percentage inhibition=[(Control-Test)/Control] X 100

Antibacterial activity

The bacterial isolates were screened for *in vitro* growth inhibition of phytopathogenic bacteria *Xanthomonas.* One microlitre of 24 hrs old bacterial cultures was inoculated in Medium-9 plates containing the lawn culture of *Xanthomonas* spp incubated at 30°C and observed daily for formation of transparent halos around each colony for up to 4 days.

Identification of the isolates

Isolates showing maximum percentage inhibition were selected and identified by cultural, morphological and biochemical characteristics as described in Bergey's manual of Determinative Bacteriology [12]. Further the isolates were subjected to partial 16srDNA gene sequencing for phylogenetic analysis.

In vitro screening of Multiple Plant Growth Promoting Activities of isolates

Phosphate solubilization

Phosphate solubilizing efficiency of the isolates were measured following the method of described by Katznelson and Bose (1959) [13]. Plates containing nutrient agar medium supplemented with Ca_5 $(PO_4)_3$0H were inoculated with 10µl of 24 h old pure bacterial culture and incubated at 30°C for 7 days for the formation of transparent halos around each colony.

Zinc solubilization
Plates containing basal medium (glucose-1.0 g; ammonium sulphate - 0.1 g; potassium chloride - 0.2 g; dipotassium hydrogen phosphate-0.1 g; magnesium sulphate - 0.2 g; agar- 2g; distilled water -100 ml, pH 7.0) supplemented with 0.1% insoluble zinc compounds ZnO, $ZnCO_3$ and ZnS (Sphalerite ore material) were inoculated with 10µl of 24 h old pure bacterial culture and incubated at 30°C for 7 days for the formation of transparent halos around each colony.

IAA production
IAA production was detected by the modified method as described by Brick *et al*. (1991) [14]. The cultures were grown in nutrient broth and ISP broth supplemented with Tryptophan (5mg/ml) and incubated for 4 days. On 5th day Salkowski reagent (50ml 35% of perchloric acid, 1ml 0.5M $FeCl_3$ solution was added and development of pink colour indicates the IAA production.

Ammonia production
Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48- 72 h at 37°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production [15].

Siderophore production
Siderophore production efficiency of isolates was determined by the method described by Schwyn and Neilands (1987) [16]. For this, 10 µl pure bacterial cultures grown in nutrient broth were inoculated to Chrome Azurol S (CAS) agar plate and incubated at 30°C for 4 days for orange color formation around each colony.

HCN production
HCN production was tested according the method described by Kremer and Souissi (2001) [17]. Discoloration of the filter paper from orange to brown after incubation was considered as microbial production of cyanide [18].

Detection of Hydrolytic Enzymes
Chitinase activity
Isolates were screened for chitinase production on chitin agar plates according to Chernin *et al*. (1995) [19]. Chitinase activity was measured with colloidal chitin as a substrate. The culture broth was centrifuged and enzyme solution 1 ml was added to 1.0 ml of substrate solution, which was made by suspending 1% of colloidal chitin in Phosphate buffer (pH 7.0). The mixture was incubated at 37°C for 45 minutes and the amount of reducing sugar produced in the supernatant was determined by DNS method [20]. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar per min [21].

Glucanase activity
Glucanases (β 1,4 and β 1,3) were detected on microcrystalline cellulose and Azo-glucan containing plates, respectively as described by Teather *et al.*(1982) [26, 22] and Chen *et al.*(1997) [23]. The specific activity of β-1, 3-glucanase was determined by measuring the amount of reducing sugars liberated using dinitrosalicylic acid solution [20].

Other lytic enzymes
Cellulase, protease and lipase activities were tested on LB plates, with the respective substrate added [10, 24, 25]. Aliquots of bacterial culture (10µl), grown overnight in LB broth, were spot-inoculated onto the above mentioned plates. Plates were incubated for 2-8 days at 30°C and formation of a transparent halo zone around the colony was considered as a positive result for enzyme activity.

Germination activity in Tomato seeds
The selected bacterial isolates were screened to record germination percentage in Tomato (Arka vikas) under nursery. Tomato seeds were procured from IIHR, Hessarghatta, Bangalore. Tomato seeds were Surface sterilized with Sodium hypochlorite (3%) and soaked in the test cultures separately for 15 min and sown in the sterilized cocopeat in protray under nursery conditions and recorded germination percentage on 8th day from the date of sowing.

RESULTS

From 35 rhizosphereic soil samples 12 actinomycetes, 33 bacterial isolates were isolated. Of these, 4 isolates- B2, P6, 9P, R exhibited maximum inhibition percentage against fungal and bacterial phytopathogens (Figure 1). On the basis of biochemical and molecular characterization, the 4 isolates were identified as *B. subtilis*, *P. aeruginosa*, *Streptomyces* spp, *P. fluorescens* respectively. The strains *B. subtilis*, *P. aeruginosa* were allotted with accession number as JN861778 (*Pseudomonas aeruginosa* strain p6), JN032305 (*Bacillus subtilis* strain B2) by National Centre for Biotechnology Information (NCBI) GenBank.

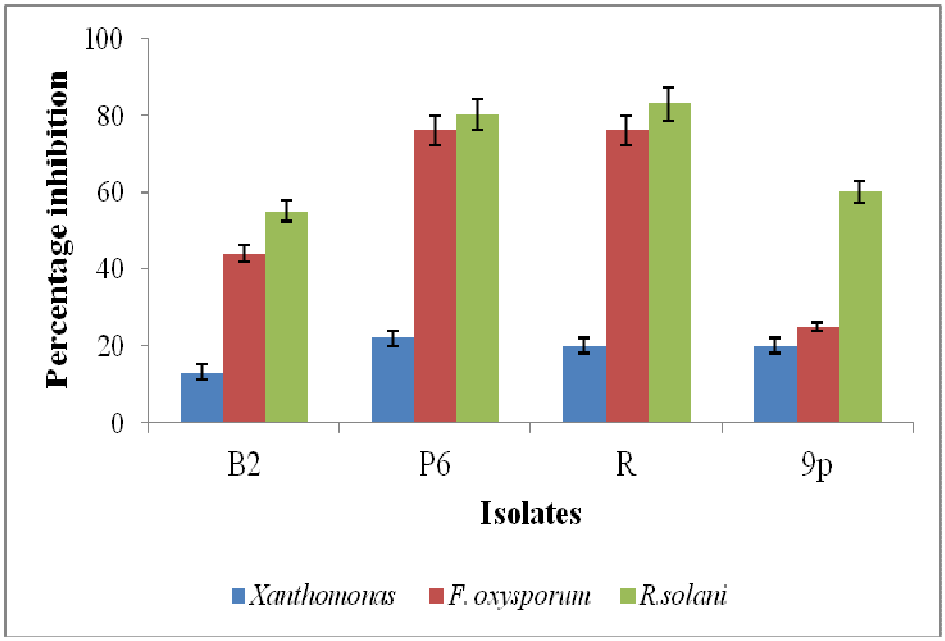

Figure 1. Dual plate assay showing the inhibition percentage of the selected isolates against three pathogens

PGPR traits

The isolates showed varied levels of PGPR traits such as phosphate solubilization, zinc solubilization, IAA, ammonia, siderophores and HCN production (Table 1).

Phosphate solubilization

Two of the four strains (P6 and R) exerted ability for phosphate solubilization on Pikovskaya medium with different efficacy. Strain P6 showed maximum degree of phosphate solubilization of 52 %. The phosphate-solubilizing activity characterizes the microorganisms with ability to produce and release metabolites such as organic acids that chelate the cations bound to phosphate, converting them into soluble forms [26]. Solubilization of different form of phosphates and improvement of its availability presents very important treat of plant-associated bacteria since possible increasing of mass and productivity of agriculture plants. None of the isolates were able to solubilize zinc phosphate.

IAA production

Auxin is the most investigated hormone among plant growth regulators. The most common, best characterized and physiologically most active auxin in plant is indole-3-acetic acid (IAA). IAA is known to stimulate both a rapid response (e.g. increased cell elongation) and a long-term response (e.g. cell division and differentiation) in plants [11]. In our study, three bacterial isolates (9P, R, P6) were able to produce indole-3-acetic acid (IAA) growing in medium without addition of tryptophan. Maximum IAA production was recorded in P6 strain (16µg/ml) as compared to other isolates.

Siderophore production

Microbial Siderophore stimulate the plant growth directly by increasing the availability of iron in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron-uptake system [27]. Siderophore production was detected only for isolates 9P, P6 and R. Sharma and Johri [28] showed increasing of shoot and root length as well as dry weights of maize seedlings inoculated with siderophore-producing strains. Siderophore mediated competition for iron was shown to be major mechanism of antagonistic activity of fluorescent Pseudomonads in the control of some parasitic fungi, limiting iron availability to them [29].

HCN production

Ability for hydrogen cyanide synthesis was observed for isolates R and P6. Hydrogen cyanide mediated antagonism was observed for isolate R and P6 which is in agreement to earlier reports [30]. The increased production of HCN by the efficient strain of *P. fluorescens* contributed to effective inhibition of mycelial growth of *Rhizoctonia solani in vitro* [31] and appears to be a major factor in control of soil-borne disease by *Pseudomonas fluorescens* CHA0 [30]. Three isolates (9P, R and P6) produced both ammonia and biosurfactant. Ammonia production by the plant growth promoting bacteria helps influence plant growth indirectly.

Hydrolytic enzymes

Strains R, 9P and B2 showed the presence of chitinase. When tested for the presence of other hydrolytic enzymes like glucanase (β-1, 4; β-1, 3), protease and lipase, 9p and B2 showed positive for all while strain R tested positive for only glucanase (β-1,3) in addition to chitinase. The strain 9p produced relatively high levels of chitinase (15.4 U/ml) and β -1, 3-glucanase (8.7 U/ml) on 4th day of incubation period. Strain B2 produced high level of chitinase (11U/ml) and glucanase (β-1, 4; β-1, 3) (30U/ml; 4.9U/ml) on the 3rd day of incubation. The enzyme production subsequently decreased slightly during the stationary phase. One of the possible antifungal mechanisms of the *Streptomyces* strain 9p and *B. subtilus* in this study may be associated with the production of extracellular chitinase and β -1, 3-glucanase enzymes. R produced significant amount of both chitinase and β-1, 3 glucanases in pure substrates. Maximum production of β-1, 3 glucanases (3.2 U/mL) was on day 1, while maximum chitinase production was on 5th day (3.73 U/mL). Moataza (2006) have also reported varied levels and types of mycolytic enzymes by different *Pseudomonas* strains with *P. capsici* and *R. solani*. [32]. Proteolytic enzyme production was detected as formation of a clear zone around the colony on skim milk agar medium for three strains- P6, 9P and B2. Strain 9P and B2 also showed lipase activity. The present study revealed the production of mycolytic enzymes viz. Chitinase, β-1, 3-glucanase, β-1,4- glucanase, protease and lipase. Mycolytic enzymes produced by antagonistic microorganisms are very important in biocontrol technology. There are many reports on production of lytic enzymes by microorganisms [33,34]. Antagonistic mechanism is mediated by variety of mechanisms. In this present study, strain 9p and B2 antagonistic mechanism is by production of mycolytic enzyme whereas in strain p6 and R the antagonistic mechanism is due to siderophore and HCN production. Strain p6 and R can also promote plant growth. Similar observation of antagonistism of chilli phytopathogen by soil fungi from chilli rhizosphere was also reported [35].

Table 1. Lytic enzymes production and Plant growth promotion traits of four isolates isolated from rhizospheric soil samples

Bacterial isolates	Hydrolytic enzyme production					PGPR traits				
	Chitinase	Glucanase	Cellulase	Protease	Lipase	Phosphate solubilization	IAA	Ammonia	HCN	Siderophore
B2	+	+	+	+	+	-	-	-	-	-
R	+	-	+	-	-	+	+	+	+	+
P6	-	-	-	+	-	+	+	+	+	+
9p	+	+	+	+	+	-	+	-	-	+

Seed germination

Seed inoculation significantly enhanced the tomato seed germination. Inoculation resulted in early seedling growth and development. However the rate of enhancement varied with bacterial strains. Seed germination percentage varied from 28 to 71% when compared to the control (Figure 2). Similar promotion in growth parameters and yields

of various crop plants in response to inoculation with PGPR were reported by Shaharoona *et al*. (2006) and Gravel *et al*. (2007) [36, 37, 38, 39].

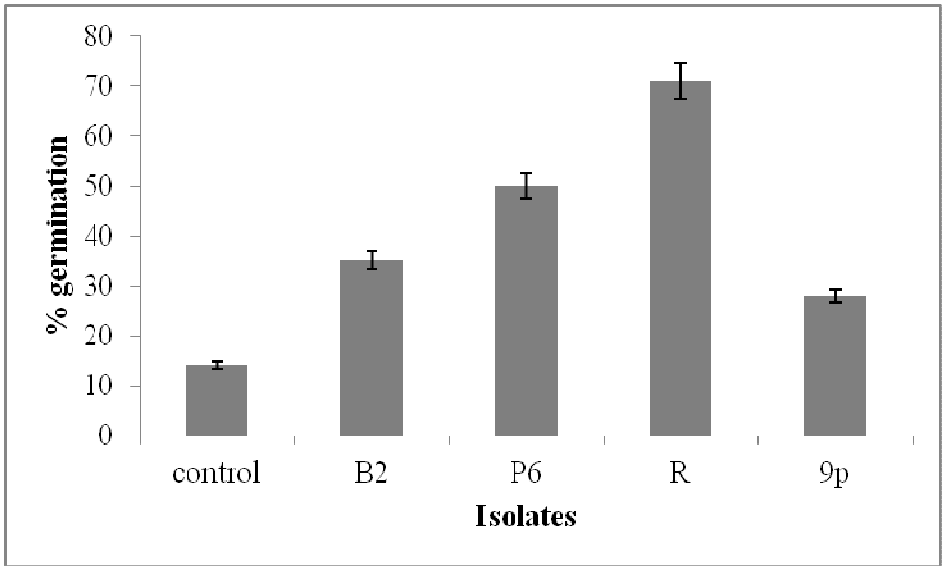

Figure 2. Effects of seed germination of tomato seeds with bacterial inoculants.

CONCLUSION

This study illustrates the significance of screening rhizobacteria under *in vitro* conditions for multiple PGPR traits. Isolate B2, 9P, p6 and R with antagonistic against *F. oxysporum*, *R. Solani*, *Xanthomonas* spp was isolated and the study further exhibited the diverse antagonistic mechanism exhibited by the strains, with functional properties distinctive for plant growth promoting rhizobacteria. These strains may represent precious biological alternative as single biocontrol agents (BCAs) or as a microbial consortia for harmful pesticides and chemical fertilizers application in agriculture fields due to crucial role of these rhizobacteria in plant health maintenance and soil fertility.

Acknowledgment
The authors wish to thank the management of Jain University for all the infrastructural and financial support rendered for this work.

REFERENCES

[1].A.W. Khoso, Growing vegetable in Sindh, 2nd ed., Allied Printing Corporation, Hyderabad, **1994**; pp.136.
[2]. B. Sneh, B.M. Zeidman, M. Ichielevich-Auster, I. Barash, Y. Koltin, *Can. J. Bot.*, **1986**, 64, 2372-2378.
[3]. J.H. Bowers, J.C .Locke, *Plant Dis.*, **2000,** 84:300-305.
[4]. B. Sneh, S. Jabaji-Hare, S. Neate, Djist G. In *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology and disease control, Kluwer Academic Publishers, Dordrecht, Netherlands,**1994;** pp. 247-559.
[5]. K. Pohronezny, R. B.Volin, *Hort. Science*, **1983**, 18: 69-70.
[6]. J. Liggit, P. Jenkinson, D.W.Parry, *Crop. Prot.*, **1997**,16: 679-685.
[7].T Diehl,; H. Fehrmann, *J. Plant Dis. Prot.*, **1999,** 96:393-407.
[8]. R.D. Lumsden, J.C.Locke, *Phytopathology*,**1989**,79:361-366.
[9]. K.K. Pal, K.V.B.R. Tilak, A.K. Saxena, R. Dey, C.S. Singh, *Microbiol. Res*, **2001**,156, 209- 223.
[10]. A.J. Cattelan, P.G. Hartel, J.J. Fuhrmann, *Soil Sci. Soc. Am. J.*, **1999**,63, 1670-1680.
[11]. F. Ahmad, I. Ahmad, M.S. Khan, *Turk. J. Biol.*, **2005**, 29: 29-34.
[12]. N. R. Krieg, J. G. Holt (ed.), Bergey's manual of systematic bacteriology. **1984,** Williams & Wilkins Co., Baltimore, Md.
[13]. H. Katznelson, B. Bose, *Can. J. Microbiol.*, **1959,** 5: 79-85.

[14]. J.M. Brick, R.M. Bostock, S.E. Silverstone, *Appl. Environ. Microbiol.*, **1991**, 57: 535-538.
[15]. J.C. Cappuccino, Sherman N. In: Microbiology: A Laboratory Manual, New York, **1992;** pp. 125-179.
[16].B. Schwyn, J. B.Neilands, *Anal. Biochem.*, **1987**,160: 47-56.
[17]. R.J. Kremer; T.Souissi, *Curr. Microbiol*., **2001**,43: 182-186.
[18]. J.T. De Souza, J.M.Raaijmakers, *FEMS Microbiol. Ecol*., **2003**,43: 21- 34.
[19]. L Chernin, Z. Ismailov, S. Haran, I.Chet, *Appl. Environ. Microbiol.*, **1995**, 61 (5): 1720–1726.
[20]. G.L. Miller, *Anal. Chem.*, **1959**, 31: 426-428.
[21]. N. Annamalai, S. Giji, Arumugam, T.Balasubramanian, *Afr. J. Microbiol. Res.*, **2010**, 4(24): 2822-2827.
[22]. R. M.. Teather, P.J.Wood, *Appl. Environ. Microbiol*., **1982**, 43(4): 777-780.
[23]. C.C. Chen, R. Adolphson, J.F.D. Dean, K.E. Ericksson, M.W.W. Adams, J. Westpheling, *Enz. Microb. Technol.*, **1997**, 20: 39–45.
[24]. R.S. Kumar, N. Ayyadurai, P. Pandiaraja, A.V. Reddy, Y. Venkatesvarlu, O. Prakash, N.Sakthivel, *J. Appl. Microbiol.*, **2005**,98: 145-154.
[25].N Rashid, Y. Shimada, S. Ezaki, H.A. Tomi, T.Y. Imanaka, *Appl. Environ. Microbiol*., **2001**, 67: 4064- 4069.
[26]. L.V. Kravchenko, T.S. Azarova, N.M. Makarova, I.A. Tikhonovich, *Microbiology*, **2004**,73(2): 156-158.
[27]. M. Marek- Kozaczuk, M. Deryto, A. Skorupska, *Plant and soil*, **1996**, 179: 269-74.
[28].A. Sharma, B.N. Johri, *Microbiol. Res.*, **2003**, 158: 243-248.
[29]. M. Miethke, M. A. Marahiel, *Microbiol. Mol. Biol. Rev.*, **2007**, 71: 413 – 451.
[30]. F. Jamali, A. Sharifi-Tehrani, M.I. Lutz, M. Maurhofer, *Microb. Ecol.*, **2009**, 57: 267-275.
[31]. J. Kuklinsky-Sobral, W.L. Araujo, R. Mendes, I.O. Geraldi, A.A. Pizzirani-Kleiner, J.L. Azevedo, *Environ. Microbiol.*, **2004**, 6: 1244-1251.
[32]. M.S..Moataza, *Res. J. Agri. Biol. Sci*., **2006**, 2(6): 274-281.
[33]. C.J. Huang, C.Y. Chen, *Annals of Microbiology*, **2004**, 54(3): 289-297.
[34]. V. Gohel, A. Singh, M. Vimal, P. Ashwini, H.S. Chhatpar, *Afr. J. Biotechnol*., **2006**, 5 (2): 54-72.
[35]. S.Gomathi, V. Ambikapathy, *Adv Appl Sci Res.*, **2011**, 2 (4): 291-297
[36]. B. Shaharoona. M. Arshad, Z.A. Zahir, A. Khalid, *Soil Biol. Biochem*, **2006**, 38: 2971-2975.
[37].V. Gravel, H. Antoun, R. J. Tweddell, *Soil Biol. Biochem.*, **2007**, 39: 1968-1977.
[38]. S. Ramyasmruthi, O.Pallavi, S. Pallavi, K. Tilak, S. Srividya, *Asian Journal of Plant Science and Research*, **2012**, 2 (1):16-24.
[39]. S. Srividya, Adarshana Thapa, V. B. Deepika, G. Kajingailu, D. Nilanjan, *Eur. J. Experimental Biol*., **2012**, 2 (1):163-173