Journal of Applied Biological Sciences 4 (3): 25-30, 2010
ISSN: 1307-1130, E-ISSN: 2146-0108 www.nobel.gen.tr

Isolation and Characterization of *Pseudomonas* Isolates for Antagositic Activities

İnci GÜLER[1] Çiğdem KÜÇÜK[2*]

[1]Harran University, Graduate School of Natural and Applied Sciences, Şanlıurfa, TURKEY
[2]Harran University, Faculty of Science and Art and Science, Department of Biology, Şanlıurfa, TURKEY

[*]**Corresponding Author**
e-mail: cdmkucuk@yahoo.com

Received : July 08, 2010
Accepted : September 30, 2010

Abstract

Pseudomonas spp. isolates were isolated from rhizosphere of maize, chickpea and pepper grown in field locations in Şanlıurfa, Turkey. B1, B5, B8, B17, B22, B26, N13, N14, D1, D8, D11, BA2 and BA15 isolates grew in 4% of NaCl. 88.9 % of isolates grew at 41°C. Isolates were resistant chloramphenicol (27.8%), ampicillin (27.8%) and cycloheximide (38.9%). The isolates produced different enzymes and were antagonistics against some plant pathogenic fungi. The highest mean inhibition values, 99.77% RI, were obtained *D.sorokiniana* with B1, B5, M4 and BA2 isolates.

Key words: Isolation, rhizosphere soil, phytopathogonic fungi, antagonistic activity

INTRODUCTION

The antagonistic microorganisms of the rhizosphere are important determinants of plant healt and soil fertility because of their participation in several key processes such as those involved in the biocontrol of pathogens, nutrient cycling and seedling establishment [1]. As most of the soil borne plant pathogens are fungi, biocontrol by bacteria has been investigated [1,2]. *Pseudomonas, Bacillus, Streptomyces, Rhizobium, Azospirillium, Pantoea* and *Enterobacter* have been reported to be bacterial control agents [2].

Pseudomonas species have been used recently as biocontrol agents and their isolates have become commercially available of late [3,4]. This development is lagely the result of a change in public attitude towards the use of chemical pesticides and fumigates [5]. In this respect, *Pseudomonas* spp. have been studied as biological control agents against plant pathogenic fungi such as *Rhizoctonia solani, Fusarium culmorum, Fusarium moniliforme, Sclerotium rolfsii* [6-8]. *Pseudomonas* spp. with broad spectrum antagonistic activity are able to colonize rapidly roots of a range crops and spread in the rhizosphere [4]. Isolates of *Pseudomonas* species can produce lytic enzymes [4,9-11] and antibiotics [12-15] and they can be competitors of fungal pathogens [7,16-20] and promote plant growth [4,11,21].

In this study we aimed to examine the antimicrobial effects, physiological and biochemical features of *Pseudomonas* isolates isolated from rhizosphere soils in Şanlıurfa, Turkey.

MATERIALS and METHODS

Isolation of *Pseudomonas* spp. from rhizosphere soil

A total 18 isolate was isolated from maize, chickpea and pepper rhizosphere soil samples in Şanlıurfa, Turkey. *Pseudomonas* selective medium was used for their isolation [22].

Identification

The cultures were identified according to their biochemical characteristics. According to Bergey's Manual Systematic of Bacteriology [22] the isolates had been identified using the following criteria: Gram reaction, growth temperature range (4, 28, 37 and 41°C), salt resistance (0, 1, 2, 3 and 4 % (v/w) NaCl), catalase and oxidase activity, carbohydrate assimilations (maltose, mannitol, tryptophan, fructose, xylose, myo-inositol and glucose) were added to the medium to a final concentration of 0.1%, reduction nitrate, production of levan and citrate. The cultures were inoculated with 1 % (v/v) inocula. The results were scored after 7 days incubation at 28°C.

Antibiotics resistance of isolates

King's B agar with various concentrations of test antibiotics was used. Fresh solutions of filtered sterilized (0.45 µg ml-1): ampicillin, streptomycin, penicillin, chloramphenicol and cycloheximide 100, 250, 500, 1500 [23]. Each bacterial culture was replicated twice per antibiotic concentration, by dispensing 10 µl (of a 10^{-6} dilution) per petri dishes. Petri dishes were incubated at 28°C and scored after 3 days [17].

Determination of antagonistic activity

Antagonistic bacterial isolates were screened on potato dextrose agar (PDA, MERCK) for their ability to inhibit fungal growth in petri dish as described by Ahmad et al. [24]. Briefly, two bacterial isolates were streaked at equidistant points along the perimeters of the each petri. Dishes incubated at 28°C for 2 days [18]. Following bacterial growth, mycelial plug (5 mm) from the edge of the 7 days old fungal culture on PDA were inoculated in the centre of the bacterial isolates. Plates without bacteria were used as control. Plates were incubated at 28°C for 7 days. The radii of the fungal colony towards and away from the bacterial colony were measured. The percentage of growth inhibition was calculated in relation to the control treatment [25].

Production enzyme

Colonies were screened for chitinases by plating on chitin agar plates (Nutrient broth, 1.62 g; NaCl, 0.6 g; Na_2HPO_4, 0.2 g; NH_4Cl, 0.1 g; chitin, 5 g; agar, 15 g; distilled water, 1 l) [26]. Hydrolysis of Tween 80 was tested on Sierra's medium containing (per liter) 20 g $CaCl_2$ and 10 ml of Tween 80 [11]. Liquefaction of gelatin was tested by shabbing cultures into 2 ml nutrient gelatin medium (beef extract 3.0 g; peptone 5.0 g; gelatin 120 g) and incubating for one week at 28°C [27]. Clear zones in skimmed milk agar were measured and used as indicators of protease activity [28]. The method of Jayasanka and Graham [29] was used to determine the presence of cellulase. Urease activity was detected using Christiansen's method [30]. Arginine dihydrolase was performed according to the method to Thornley [31].

RESULTS and DISCUSSION

Many studies have reported on the isolation of pseudomonads from the rhizosphere [6,11,15,16,23]. *Pseudomonads* may have a favorable, neutral or deleterious influence on plant growth [16,21,27]. It has been suggested that plant growth promoting *Pseudomonas* species act mostly by antagonizing (production of antibiotics, cyanide or chelating agents) or displacing deleterious microorganisms. Eighteen isolates of *Pseudomonas* spp. were isolated from rhizosphere soils. Morphological and physiological characteristics of 18 isolates from rhizosphere soils were detectived. All of the isolates were motile and Gram negative. After Gram staining, *Pseudomonas* identification were confirmed by growing on King's B medium. Green fluorescence of *Pseudomonas* spp. was very clear on King's B medium.

Monosaccharides (glucose, maltose, fructose, xylose, mannitol, tryptophan and myo-inositol) were utilized by the isolates. Hovewer some isolates showed variable response to tryptophan, mannitol and myo-inositol (Table 1). Characteristic tests for identification of *Pseudomonas* showed that 18 isolates (B1, B5, B8, B16, B17, B22, B26, N13, N14, D1, D5, D8, D9, D11, M2, M4, BA2, BA15) were possitive for growth at 28 and 37°C, levan formation and identified as *Pseudomonas fluorescens*. When biochemically characterized all the isolates were motile rods, testing possitive for catalase, oxidase, reduction of nitrate. Based on these morphological and physiological characteristics, isolates closely resembled to *Pseudomonas* spp.

Table 1. Growth on some carbon sources of isolates.

Isolates	Carbon sources						
	Maltose	Mannitol	Trypto-phan	Fructose	Xylose	Myoino-sitol	Glucose
B1	+	+	+	+	+	+	+
B5	+	+	+	+	+	+	+
B8	+	-	-	-	+	-	+
B16	+	+	+	+	+	-	+
B17	+	+	+	+	+	+	+
B22	+	+	-	+	+	-	+
B26	+	-	-	-	-	-	+
N13	+	+	+	+	+	+	+
N14	+	+	-	+	+	+	+
M2	+	+	+	+	+	+	+
M4	+	-	+	+	+	+	+
D1	+	+	+	-	-	-	+
D5	+	+	+	+	+	-	+
D8	+	+	+	+	-	+	+
D9	+	+	+	+	+	+	+
D11	+	-	w	-	-	+	+
BA2	-	-	+	+	+	+	+
BA15	+	+	+	+	+	-	+

+ : growth - : no growth w: weak growth

Table 2. Growth at different temperature and NaCl concentrations

Isolate	Temperature (°C)				NaCl (% v/w)				
	4	28	37	41	0	1	2	3	4
B1	+	+	+	+	+	+	+	+	+
B5	-	+	+	+	+	+	+	+	+
B8	+	+	+	+	+	+	+	+	+
B16	-	+	+	+	+	+	+	+	-
B17	-	+	+	+	+	+	+	+	+
B22	+	+	+	+	+	+	+	+	+
B26	+	+	+	+	+	+	+	+	+
N13	-	+	+	+	+	+	+	+	+
N14	-	+	+	+	+	+	+	+	+
M2	-	+	+	-	+	+	+	+	-
M4	-	+	+	-	+	+	+	+	-
D1	+	+	+	+	+	+	+	+	+
D5	-	+	+	+	+	+	+	+	-
D8	-	+	+	+	+	+	+	+	w
D9	+	+	+	+	+	+	+	+	-
D11	-	+	+	+	+	+	+	+	+
BA2	+	+	+	+	+	+	+	+	+
BA15	-	+	+	+	+	+	+	+	+

+ : growth - : no growth w: weak growth

The growth temperature range for isolate B1, B8, B22, B26, D1, D9 and BA2 were 4 - 41°C (Table 2). B1, B5, B8, B17, B22, B26, N13, N14, D1, D8, D11, BA2 and BA15 isolates were able to growth in medium with 4% NaCl (Table 2). The 13 isolates from this study are able to tolerance high salt concentration, these isolates a potential competitive advantage to survival in soils.

The sensitivity of these 18 isolates was checked against five antibiotics, i.e. penicillin, streptomycin, chloramphenicol, ampicillin and cycloheximide (Table 3). In our study, the effects of antibiotic concentrations were variable, depending on the antibiotics (Table 3). 83.3 % isolates grew at 100 µg ml-1 ampicillin. 38.9 % isolates showed resistance against cycloheximide (1500 µg ml-1). The results of Pandey et al. [23] agree with the above findings. They reported that isolates of *Pseudomonas* could tolerate ampicillin, carbenicillin and penicillin concentration upto 1500 µg ml-1. In our study 1000 and 1500 µg ml-1 streptomycin concentrations were found to be inhibitory to all of the isolates. Sindhu et al. [17] also found that all the tested six isolates of *Pseudomonas* spp. were resistant to ampicillin and chloramphenicol and sensitive to streptomycin. 50 % of the isolates were resistant to streptomycin (250 and 500 µg ml-1) and 44.4 % of the isolates were resistant chloramphenicol (250 and 500 µg ml-1). 66.7 % of the isolates were resistant to 250 500 and 1000 µg ml-1 of ampicillin concentration (Table 3). Similar observations have been reported [8, 17,18].

Table 3. Percentage of isolates resistance to tested antibiotics.

Antibiotics	Concentrations (µg/ ml)				
	100	250	500	1000	1500
Penicillin	61.1	61.1	61.1	55.6	0
Streptomycin	66.7	50	50	0	0
Chloramphenicol	66.7	44.4	44.4	33.3	27.8
Ampicillin	83.3	66.7	66.7	66.7	27.8
Cycloheximide	72.2	61.1	61.1	50	38.9

Total number of isolates: 18

In this study, the effect of 18 isolates of *Pseudomonas* were studied on *Fusarium culmorum, Fusarium solani, Fusarium accuminatum, Fusarium moniliforme, F.chlamydosporum, Dreschlera sorakiniana, Alternaria alternata* and *Ascochyta rabiei* (Table 4). The highest mean inhibition values, 96.77% RI, were obtained *D.sorokiniana* with B1, B5, M4 and BA2 isolates (Table 4). B16, M2 and BA15 showed inhibitory effects against *F. chlamydosporum,* 83.3 % and 75 %, respectively (Table 4). We observed that the *Fusarium* species showed more resistance to the selected *Pseudomonas* spp. isolates than the other plant pathogenic fungi. A similar result was also obtained for Gu et al. [18].

In the study of Egamberdieva et al. [27] it was shown that the development of isolates in the medium showed variations depending on enzyme activity such as proteinase, chitinase, urease, arginine dihydrolase.

Table 4. Inhibition effects ([a]RI) of *Pseudomonas* spp. on some plant pathogenic fungi

	Plant pathogenic fungi							
Isolates	*F. culmorum*	*F. solani*	*F. accuminatum*	*F. moniliforme*	*F. chlamydosporum*	*D. sorakiniana*	*A. alternata*	*A. rabiei*
B1	75	34.61	62.85	52.94	58.33	96.77	50	54.9
B5	11.11	23.07	44.9	26.47	ND	96.77	45	58
B8	55.5	26.92	68.57	41.17	58.3	58.06	75	80.76
B16	11.11	ND	3.12	8.82	83.3	51.61	45	45.65
B17	ND	ND	2.77	17.64	16.6	64.51	62.5	4.25
B22	67.56	23.07	60	41.34	25	61.29	55	60
B26	ND	30.76	50	35.29	ND	93.54	50	ND
N13	48.64	50	25	11.76	66.6	74.19	65	6.25
N14	51.93	57.69	33.3	50	25	51.61	37.5	25
M2	23.07	ND	25.92	47.05	75	51.61	ND	13.04
M4	50	ND	66.6	11.76	66.66	96.77	25	42.5
D1	37.5	26.92	33.3	26.47	25	70.96	50	55
D5	23.91	38.46	47.68	26.47	50	45.16	37.5	11.11
D8	57.69	30.76	13.3	11.76	50	48.38	45	25
D9	33.3	26.92	31.25	26.47	ND	80.64	65	57.49
D11	56.52	23.07	52.38	41.17	50	51.61	57.5	57.69
BA2	55.55	23.07	60	41.17	ND	96.77	62.5	64
BA15	ND	ND	62.5	2.94	75	64.51	25	10.63

[a]RI= Range Inhibition ND =not detectable

Table 5. Enzyme activities of *Pseudomonas* spp. isolates

	Enzymes						
Isolate	**Urease**	**Liquefaction of Gelatin**	**Hydrolysis of Tween 80**	**Protease**	**Cellulase**	**Arginine dihyrolase**	**Chitinase**
B1	-	-	-	-	+	+	-
B5	+	-	-	-	+	+	-
B8	-	-	+	-	+	+	-
B16	-	-	+	-	+	+	+
B17	-	-	+	+	+	-	+
B22	+	-	-	-	-	+	-
B26	-	-	+	-	+	-	+
N13	-	-	-	-	-	+	-
N14	+	-	-	+	+	+	+
D1	+	-	+	-	-	+	-
D5	+	-	+	-	+	-	-
D8	+	-	-	+	+	-	-
D9	-	-	-	-	+	-	-
D11	-	-	-	+	+	+	+
M2	-	-	+	-	-	-	+
M4	-	-	+	-	-	+	-
BA2	+	-	+	+	+	+	+
BA15	-	-	+	-	+	-	+

+ : positive - : negative

It was shown that *Pseudomonas* produced chitinase, cellulase enzymes and degraded the glucans in the walls of pathogenic fungi [4,17,19]. De Boer et al. [26] demonstrated that *Pseudomonas* was antagonistic to Fusarium and produced lytic enzymes. For eight isolates, chitinolytic activity could be detected, whereas thirteen showed activity of cellulase activity (Table 5). None of the *Pseudomonas* isolates produced liquefaction of gelatine. Eleven isolates (B1, B5, B8, B16, B22, N13, N14, D1, D11, M4 and BA2) positive for arginine dihydrolase but five isolate were positive for protease (Table 5). In summary, the made of action and quality of the activity produced were isolate specific.

In conclusion, many studies in the recently have found the *Pseudomonas* species to be potential biological control agents of phytopathogenic fungi [3, 18, 19, 23, 25] and further studies will be carried out to evaluate the potential use of our isolates in biological control.

Acknowledgements

The autors are grateful to Harran University Research Foundation for financial support the Project number of 902.

REFERENCES

[1] Vessey JK. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255 (2): 571-586.

[2] Avis TJ, Gravel V, Antoun H, Tweddell R J. 2008. Multifacated beneficial effects of rhizosphere microorganisms on plant health and productivity. Soil Biol. Biochem. 40: 1733-1740.

[3] Hafeez FY, Yasmin S, Ariani, D, Rahman M, Zafar Y, Malik KA. 2006. Plant growth promoting bacteria as biofertilizer. Agron. Sustain. Dev. 26:143-150.

[4] Whipps JM. 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52: 487–511.

[5] Fravel DR. 2005. Commercialization and implementation of biocontrol. Ann. Rev. Phytopathol. 43: 337–359.

[6] Hass D and Defago G. 2005. Biological control of soilborne pathogens by fluorescent Pseudomonads. Nat. Rev. Microbiol. 3: 307–319.

[7] Fravel D R. 1988. Role of Antibiosis in the biocontrol of plant diseases. Annu. Rev. Phytopathol. 26: 75-91.

[8] Jayaswal RK, Fernandez M, Upadhyay RS, Visihtin L, Kurz M, Webb J, Rinehart K. 1993. Antagonism of *Pseudomonas cepacia* aganist phytopathogenic fungi. Curr. Microbiol. 26: 17-22.

[9] Chen C, Belanger RR, Benhamou N, Paullitz TC. 2000. Defence Enzymes Induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR). Physiol. Molecular Plant Pathol. 6: 13-23.

[10] Vivekananthan R, Ravi M, Ramananthan A, Samiyappan R. 2004. Lytic enzymes Induced by *Pseudomonas fluorescens* and other biocontrol organisms mediate defence aganist the anthracnose pathogen in mango. World J Microbiol. Biotechnol. 20:235-244.

[11] Egamberdieva D. 2005. Plant growth promoting rhizobacteria isolated from a calcisol in a semi arid region of Uzbekistan: biochemical bharacterization and effectiveness. J. Plant Nutr. Soil Sci. 168: 94-99.

[12] Raaıjmaker JM, Weller DM. 2001. Exploiting genotypic diversity of 2,4-diacetylphlorolucinol producing *Pseudomonas* spp. characterization of superior root colonizing *P. fluorescens* strain Q8r1-96. Appl. Environ. Microbiol. 67: 2545-2554.

[13] Weller DM, Cook R. J. 1983. Supression of take-all of wheat by seed treatments with Fluorescent Pseudomonads. Phytopathol. 26: 379-407.

[14] Ozturk A, Çaglar O, Sahin F. 2003. Yield response of wheat and barley to inoculation of plant growth promoting rhizobacteria at various levels of nitrogen fertilization. Plant Nutr. Soil Sci. 166:1-5.

[15] De Souza JT, Weller DM, Raaıjmaker JM. 2003. Frequency, diversity, and activity of 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. in Dutch take-all decline soils. Phytopathology 93: 54-63.

[16] Kloepper JW, Lifshitz R, Zablotowicz RM. 1999. Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. 7:39-43.

[17] Sindhu S.S, Gupta SK, Dadarwal KR. 1999. Antagonistic effect of *Pseudomonas* spp. on pathogenic fungi and enhancement of growth of green gram (*Vigna radiata*). 29: 62-68.

[18] Guo Y, Zheng H, Yang Y, Wang H. 2007. Characterization of *Pseudomonas corrugata* strain P34 ısolated from soil in Beijing as a potential biocontrol agent. 55: 247-253.

[19] Trivedi P, Pandey A, Palni LMS. 2006. *In vitro* evaluation of antagonistic properties of *Pseudomonas corrugata*. Microbiol. Research. 163: 329-336.

[20] Muleta D, Assefa F, Garnhall U. 2007. In vitro antagonism of rhizobacteria isolated from *Coffea arabica* L. aganist emerging fungal coffee pathogens. Eng. Life Sci. 7(6): 577-586.

[21] Siddiqui ZA, Baghel G, Akhtar MS. 2007. Biocontrol of *Meloidogyne javanica* by *Rhizobium* and plant growth-promoting rhizobacteria on lentil. World J Microbiol Biotechnol. 23: 435-441.

[22] Holt GJ, Krıeg NR, Sneath PH, Staley JT, Williams ST. 1994. In: Bergey's manual of determinative bacteriology. Ninth Ed. The Williams and Wilkins Pub., M. D., USA.

[23] Pandey A, Palni LMS, Hebbar KP. 2001. Suppression of damping-off in maize seedlings by *Pseudomonas corrugata*. Microbiol. Res. 156: 191–194.

[24] Ahmad F, Ahmad I, Khan MS. 2008. Screening of free living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Areas. 163:173-181.

[25] Idriss HA, Labuschagne N, Korsten L. 2007. Screening rhizobacteria for biological control of *Fusarium* root and crown rot of sorghum in Ethiopia. Biol Control. 40: 97-106.

[26] De Boer W, Gunnewiek J, Lafeber P, Janse JD, Woldendrop JW. 1998. Anti-fungal properties of chitinolytic dune soil bacteria. Soil Biol. Biochem. 30: 193-203.

[27] Egamberdieva D, Kamilova F, Validov S, Gafurova L, Kucharova Z, Lugtenberg B. 2008. High indicence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan. Environmental Microbiol. 10:1-9.

[28] Costa R, Gotz M, Mrotzek N, Lottmann J, Berg G, Smalla K. 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbiol guilds. FEMS Microbiology Ecology 56: 236-249.

[29] Jayasanka NP, Graham PH. 1970. An agar plate method for screening and enumerating pectinolytic microorganisms. Can J Microbiol. 16: 1023-1025.

[30] Cowan ST. 1974. Cowan and Steel's manual for the identification of medical bacteria. Cambridge University Pres, Cambridge.

[31] Thorney M. 1960. The differentiation of *Pseudomonas* from other gram negative bacteria on the basis of arginine metabolism. J Appl. Bacteriol. 23: 37-52.