J. Agric. Sci. Mansoura Univ., 33(5): 3365 - 3383, 2008

SCREENING OF FLUORESCENT PSEUDOMONADS BACTERIA ISOLATED FROM RHIZOSPHERES OF CULTIVATED AND WILD PLANTS *IN VITRO* FOR PLANT GROWTH PROMOTING TRAITS

Gamal-Eldin, H. ; M.Elbadry; S. Mahfouz and S.A.Abdelaziz
Department of Agricultural Microbiology, Faculty of Agriculture, Fayoum University, Fayoum, Egypt

ABSTRACT

Utilization of fluorescent pseudomonads bacteria (FPB) as plant growth promoting agents offers a promising alternative solution to the application of potential harmful agrochemicals. In this study, FPB inhabiting ectorhizosphere and endorhizosphere of different cultivated and wild plants species were enumerated. Rhizospheres of wild plants, in general, were found to harbor high FPB population density compared to rhizospheres of cultivated plants. From counting plates, ninety five FPB isolates representing different morphological types were obtained. The isolates were *in vitro* screened for activities related to plant nutrition and plant growth regulation, for antifungal traits and for antagonistic potential towards different phytopathogenic fungi. Results showed that varied proportions of the FPB isolates are putative nitrogen fixers. solubilize phosphate and zinc, produce indoleacetic acid, produce varies antifungal traits and inhibit phytopathogenic fungi. Based on the results of screening, isolates were assessed for their ability to function as plant growth promoting rhizobacteria (PGPR) and ranked. Among the top 20 FPB isolates, 8 were isolated from rhizosphere of Rotrait (*Zygophyllum coccineum*), 6 from Kokia (*Spergularia marina* L.), 3 from Akol (*Alhagi graecorum, boiss*), and 3 from Maize (*Zea mays* L.) plant.

Keywords: fluorescent pseudomonads bacteria, cultivated and wild plants, ectorhizosphere and endorhizosphere, PGPR traits

INTRODUCTION

The estimated number of prokaryotic cells in our planet's soil is $2.6x10^{29}$, providing an enormous capacity for diversity (Whitman et al., 1988) and a great potential for exploitation. The soil around the plant roots, the root surface and the root tissues are relatively rich in nutrients because as much as 40% of plant photosynthates are lost from the root regions support large microbial populations. One such population is free-living microorganisms which has been extensively investigated are "Rhizobacteria" which are the subset of rhizosphere bacteria known to aggressively colonize plant roots (Schroth and Hancock, 1982). Rhizobacteria have been shown to be efficient microbial competitors that can displace native root-colonizing microorganisms and persist throughout some or all of the crop season (Kloepper and Schroth, 1981). The general effects of rhizobacteria on host-plants range from deleterious to neutral or beneficial (Lavarovits and Nowak, 1997). Rhizobacteria that exert beneficial effects on plant development are termed "Plant Growth-Promoting Rhizobacteria" (PGPR) (Kloepper and Schroth, 1978) because their application is usually associated with increased rates of

plants growth. PGPR promote plant growth and yield either directly or indirectly (Glick, 1995). The direct growth promoting mechanisms are as follows: i) nitrogen fixation, ii) solubilization of phosphate, iii) sequestering of iron by production of siderophores, iv) production of phytohormones such as auxins, cytokines, gibberellins; v) lowering of ethylene concentration (Glick *et al.*, 1999).The indirect mechanisms of plant growth promotion by PGPR include i) antibiotic production, ii) depletion of iron from the rhizosphere, iii) synthesis of antifungal metabolites, iv) production of fungal cell wall lysing enzymes, v) competition for sites on roots vi) induce systemic resistance (Liu *et al.*, 1995). Most PGPR consist of Gram negative genera, and the greatest number of strains are members of the fluorescent *Pseudomonas* spp. (Kloepper, 1993). Therefore, considerable research efforts are underway globally to exploit the potential of fluorescent pseudomonad bacteria (FPB) as PGPR inoculates, since they represent not only a dominant bacterial group in the rhizosphere ecosystem, but are also metabolically and functionally most versatile (Lugtenberg and Dekkers, 1999).

The main objective of this study was to find strains of fluorescent *Pseudomonas* spp. that have broad spectrum of plant growth-promoting traits and antagonistic potential against phytopathogenic fungi, that could be used as safe alternative for the overuse of harmful agrochemicals.

MATERIALS AND METHODS

- Materials

Plants

Five cultivated and five wild plant species were collected from different areas and fields in Fayoum Governorate, Egypt; the cultivated plants were Onion *(Alium cepa* L.), Maize (*Zea mays* L.), Sugar beet (*Beta vulgaris* L.), Tomato (*Solanium lycopersicum* L.), and Snap bean (*Phasolius vulgaris* L.), and the wild plants were: Homaid (*Rumex vesicarius* L.), Akol (*Alhagi graecorum, boiss*), Rotrait (*Zygophyllum coccineum*), Kokia (*Spergularia marina* L.) and Water hyacinth (*Hyacinthus* sp.).

Fungal cultures

Cultures of five soilborne pathogenic fungi, *Pythium ultimum*, *Rhizoctonia solani, Fusarium oxysporum*, *Phytophthora infestans* and *Sclerotium cepivorum* were kindly provided by the Plant Pathology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

- Methods

Plants collection

For each plant species selected for isolation of root-associated fluorescent pseudomonas bacteria (FPB), roots of ten young and healthy plants were separated from shoots, shaken vigorously to remove loose soil, placed in sterile paper bags, and carried to the laboratory in an ice-box. In the laboratory, the ten roots belong to one plant species were pooled for an average sample. For each plant species, enumeration and isolation of FPB were conducted within 3 days of sampling.

Enumeration and isolation of root-associated fluorescent pseudomonad bacteria (FPB)

Soil adhering to roots was removed and roots were subjected to gentle washing by sterile distilled water (SDW) until clear root surface was exposed and sufficient portion of roots were aseptically sliced into ca. 2cm length segments. To enumerate and isolate the FPB inhabiting the surface-washed roots, sets of fresh, washed root segments equivalent to 10 g dry weights were placed in sterile 250 ml conical flasks and a solution of Tween phosphate buffered saline was added to give 100 ml final volume. The flasks were shaken on rotary shaker at 500 rpm for 10 min. Ten fold serial dilutions were made in sterile 0.1 M $MgSO_4$ (pH 7.0), and 0.1 ml aliquots from appropriate dilutions were spread-plated on King's medium B (KB) (King *et al.,* 1954) plates supplemented with both penicillin (75000 unit l^{-1}) and propyl-parahydroxylbenzoic acid (0.5 g l^{-1}) to avoid growth of G^+ bacteria and fungi, respectively. Two replicated plates were prepared from suitable dilutions and plates were incubated at 28°C for 48h (Kremer et al., 1990).

To enumerate and isolate the FPB from the endorhizosphere, the surface-washed roots were aseptically sliced into *ca.* 6cm length segments which surface disinfected with H_2O_2 (10%, 15 sec), rinsed three times with SDW and dipping them in 1 % sodium hypochlorite for 30 sec followed by washing in SDW, dipping them again in 70% ethanol for 60 sec followed by washing in several changes of SDW. Before disinfection, the two ends of root segments were sealed with paraffin wax to prevent the penetration of the disinfectants to interior root tissues. The surface sterility of the root segments was verified by spread-plating 0.1 ml aliquot from SDW used in the last washing on nutrient agar plates which incubated at 30 °C for 72h, and if bacterial growth occurred, the sample was discarded. After cutting aseptically the two ends of root segments sealed with paraffin wax, portions equivalent to 10 g dry weight were mixed with small amounts of both sterile 0.1% M $MgSO_4$ (pH 7.0) and washed, sterilized quartz sand, and macerated aseptically in sterile mortar (Kremer *et al.,* 1990). The resulting slurry was homogenized and transferred to sterile 250 ml conical flasks and a sterile 0.1 M $MgSO_4$ (pH 7.0) solution was added to give 100 ml final volume. Shaking, preparation of dilutions, and inoculation of KB plates were performed as described in case of the surface-washed roots. After incubation of inoculated plates for 48h, developed colonies which on exposure to UV-light (λ= 356 nm) exhibited fluorescence phenomenon were counted (Kremer et al., 1990). Colonies representing different morphological types were randomly selected, isolated, and further purified on KB plates. Isolates shown to be Gram negative rods and catalase and oxidase positive were designated as FPB with no further characterization attempted. In the present study, the FPB isolated from surface-washed roots (soil-free root surfaces) were designated as "Ectorhizosphere isolates", whereas those isolated from surface-disinfected, macerated roots (interior root tissues) were designated as "Endorhizosphere isolates".

Screening FPB isolates for traits related to plant nutrition and growth stimulation

N_2-fixation was tested by growing them and an *E. coli* culture as control bacteria on plates of N-free agar medium (Haahtela *et al.*, 1983 a,b) for 48h at 28°C. The isolates that grow after being sequentially transferred 10 times to the same medium were considered presumptive positive for N_2 fixation (Cattelan *et al.*, 1999). Phosphate and zinc solubilization was tested by the dissolution of precipitated tricalcium phosphate [Ca_3 $(PO_4)_2$] in an agar medium as described by Rodriguez et al., (2004). Zinc-solubilizing ability of the isolates was tested by the dissolution of precipitated zinc oxide (ZnO) in an agar medium reported by Saravanan et al., (2003). A pinpoint inoculation of the bacterial isolates was made on surface dried plates. The plates were then incubated at 28° C for 7 days. Solubilization index (SI) was calculated according to the ratio of the total diameter (colony + halo zone) to the colony diameter (Edi-Premono *et al.*, 1996). Indoleacetic acid (IAA) production was tested using the procedure described by Loper and Schroth, (1986) using KB medium supplemented with L- tryptophane (2 mg ml^{-1}) and Salkowski's reagent. Development of a pink color indicates IAA production, and absorbance at 530 nm was recorded. The quantity of IAA was determined by comparison with a standard curve using IAA in the concentration range of 0-15 µg/ml. Antagonism towards phytopathogenic fungi (Fig. 1) was tested using five soilborne pathogenic fungi: *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Phytophthora infestans* and *Sclerotium cepivorum* in dual culture plate (Koch, 1997). Five µl of an exponentially growing bacterial culture was streaked along two opposite sides of surface-dried potato dextrose agar (PDA) plates. Plates subsequently incubated at 28°C for 24 h. Following bacterial growth, mycelial agar plug of 5-mm-diameter from a 7-day-old culture from the target fungi grown on PDA plate was placed in the center of the plate between the two parallel streaks of the test bacteria. Plates inoculated with target fungi alone served as control plates, and two replicate plates were used for each bacterial isolate. Plates were then incubated at 25°C for 7 days. Antagonistic activity was assessed by relating mycelial diameter on plates inoculated with bacteria to mycelial diameter on control plates and computing percentage growth inhibition (GI%).

Screening fluorescent pseudomonad isolates for antifungal triats

Siderophores production was detected as described by Schwyn and Neilands, (1987) modified by (Palli, 2005). The assay was performed in 6-well plates and utilized the ternary complex chrome azurol S/ iron (III) / hexadecyl-trimethylammonium bromide as an indicator. Change of the dye color from blue to orange was scored as positive for siderophores production. Hydrogen cyanide (HCN) production was detected by the method of Castric (1974) in which HCN was detected by color shift from yellow to orange in the filter paper strip saturated with alkaline picrate reagent .Chitinase production was assessed qualitatively by a microbiological method based on spoting of isolates on chitinase medium amended with colloidal chitin (Frändberg and Schnürer, 1998). Isolates exhibiting a transparent halo around the colony were considered positive for production of chitinase. Cellulase production was visualized by flooding the cellulose decomposition medium plates

previously inoculated and incubated at 30 min for 8 days with 0.1% (w/v) Congo red for 15 to 30 min followed by bleaching the plates with 1M NaCl , according to the method of Andro *et al.,* (1984). Protease activity was indicated by casein degradation and formation of clearing zones in skim milk agar (Krechel *et al.,* 2002). Salicylic acid (SA) production was assessed by the method of Leeman et al., (1996). Isolates were grown at 28°C for 48h on a rotary shaker at 200 rpm in flasks containing 25 ml of the standard succinate medium (Meyer and Abdallah, 1987). Four ml of cell free culture filtrate was acidified with 1N HCl to pH 2 and SA were extracted in $CHCl_3$ by vigorously shake by hand for 30 seconds. After that, the tubes were centrifuged for 5 min at 3500 rpm. To the pooled $CHCl_3$ phase, 4 ml of distilled water and 5 µl of 2M $FeCl_3.6H_2O$ were added. The absorbance of the purple iron-SA complex developed in the aqueous phase was measured at 527 nm.

RESULTS AND DISCUSSION

In order to achieve the objective of this study, the fluorescent pseudomonad bacteria (FPB) were enumerated and isolated from ectorhizosphere (Ecto) and endorhizosphere (Endo) of different cultivated (CP) and wild plants (WP). The FPB isolates were *in vitro* screened for traits that might be associated with ability to function as PGPR.

Involvement of wild plants in this study is based on the speculate that wild plants are likely to harbor unique rhizobacterial populations that differ from those of cultivated plants which extensively bred and subjected to intensive applications of various agrochemicals (Elbeltagy et al., 2000 and Engelhand et al., 2000).In addition, the endophytic FPB inhabiting endorhizosphere were isolated in addition to FPB inhabiting ectorhizosphere. Endophytic bacteria have defined by Hallmann *et al.,* (1997) as those bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plant and don't visibly harm the plant. It has been demonstrated that endophytic bacteria may have beneficial effects on host plants, such as growth promotion and biological control of pathogens (Sturz, *et al.,* 2000).

Enumeration and isolation of FPB

Data presented in Table (1) shows that the FPB population density inhabiting rhizospheres of wild plants was higher and ranged from 3.5 $x10^3$ to 22.5 x 10^3 CFU g^{-1} roots, compared to that of cultivated plants which ranged form $4.0x10^3$ to $11x10^3$ CFU g^{-1} roots. On the other hand, population density was considerably higher in ectorhizosphere than in the endorhizosphere, and FPB were not detected in endorhizosphere of seven plants species out of the ten tested. In addition, the effects of plant species and root microhabitat were obvious where roots of wild plants and ectorhizosphere harbored higher FPB population density compared with roots of cultivated plants and endorhizosphere, respectively.

Table 1: Counts of fluorescent pseudomonas bacteria (FPB) associated with rhizospheres of different plant species and number of isolates obtained.

Plant species	Count (CFU g^{-1} dry roots)		No. of isolates		Total isolates
	Ecto	Endo	Ecto	Endo	
(a) Cultivated plants					
Onion(*Alium cepa* L.)	4×10^3	NCD	3	0	3
Maize (*Zea mays* L.)	1.1×10^4	NCD	9	0	9
Sugar beet (*Beta vulgaris* L.)	8×10^3	NCD	6	0	6
Tomato(*Solanium lycopersicum* L.)	4×10^3	NCD	3	1	4
Snap bean (*Phasolius vulgaris* L.)	6×10^3	2×10^1	5	1	6
(b) Wild plants					
Homaid (*Rumex vesicarius* L.)	3.5×10^3	NCD	2	0	2
Akol (*Alhagi graecorum, boiss*)	2.2×10^4	4.5×10^2	10	9	19
Rotrait (*Zygophyllum coccineum*)	1.35×10^4	NCD	12	0	12
Kokia (*Spergularia marina* L.)	2.25×10^4	6×10^2	14	9	23
Water hyacinth (*Hyacinthus* sp.)	1.8×10^4	NCD	11	0	11
Total			**75**	**20**	**95**
NCD: No colonies detected					

Notable, was the observation that no FPB were detected in the endorhizosphere samples of 7 plant species out of the 10 tested. This may indicate that FPB did not colonize these endorhizosphere or FPB were present in much low numbers that counting method and /or medium used were not appropriate to recover them. Also, it must be taken into consideration that the lethal effect of the root surface disinfectants may extended to the interior root tissues and killed endophytic bacteria. However, it was reported that some plants are uncolonized by endophytic bacteria. The apparent absence of internal populations of bacteria was detected in 6% of alfalfa plants (Gagne et al., 1987), in 16% of pear trees (Whiteside and Spotts, 1991) and in 31% to 49% of cotton plants (Misaghi and Donndelinger, 1990).

A total of 95 FPB were isolated, the lower proportion (29.5%) was isolated from roots of cultivated plants and the higher proportion (70.5%) was isolated from roots of wild plants. Taken into consideration that these isolates were taken from colonies representing different morphological types, the above finding may suggest that diversity of FPB inhabiting roots of the wild plants is much wider than that of FPB inhabiting roots of cultivated plants. Differences in numbers and composition of microorganisms in rhizospheres of different plant species and even different varieties within species have been reported. This may influenced by specific root exudates or other factors possibly controlled by specific genes in the plant (Kremer *et al.*, 1990).

In this regard, Germida *et al.*, (1998) found a more diverse flora of endophytic and rhizosphere isolates in oilseed rape than in wheat. Moreover, Hallmann et al., (1997) studied the endophytic bacteria of cotton and found that in some cases, individual cotton plants were colonized by only one or two

species, while in other cases; up to 12 species of bacteria were recovered per plant.

Screening FPB isolates for plant growth-promoting traits

Although many studies have been conducted to identifying the specific traits by which PGPR promote plant growth, usually they were limited to studying just one or two of these traits (Cattelan *et al.*, 1999). In the present work, the 95 FPB isolates were *in vitro* screened for a wide array of traits that might be associated with ability to function as PGPR.

Screening FPB isolates for traits related to plant nutrition and plant growth regulation

Several *Pseudomonas* spp. have been reported to fix N_2 (Cattelan *et al.*, 1999). Among the total 95 FPB isolates, (31.6%) were found putative nitrogen fixers (PNF). The percentages of PNF isolates among cultivated plants, wild plants, ectorhizosphere and endorhizosphere isolates were 25.0%, 34.3%, 34.7% and 20.0%, respectively (Table 2). However, testing for ability to reduce acetylene to ethylene should be preformed to confirm N_2-fixation ability of these isolates.

In soil, both macro and micronutrients undergo a complex dynamic equilibrium of solubilization and insolubilization that is greatly influenced by the soil pH and microflora, and that ultimately affects their accessibility to plant roots for absorption (Saravanan *et al.*, 2003)..

The FPB isolates were screened for their abilities to solubilize mineral phosphate (P) and zinc (Zn). The results presented in Table 2 revealed the following points:

1-Among the total 95 FPB isolates, 75(78.9%) and 57(60.0%) isolates found to have the ability to solubilize mineral P and Zn, respectively.

2-The isolates varied in their abilities to solubilize P and Zn as indicated by differences in solubilization index.

3-Proportions of WP and End isolates solubilizing P were higher (89.6% and 95.0%, respectively) as compared with those of CP and Ecto isolates (53.6% and 74.7%, respectively).

4-On the other hand, proportions of CP, WP, Ecto and Endo isolates that able to solubilize Zn are approximately equal (about 60%).

However, the occurrence and role of FPB able to solubilize mineral P and Zn in the endorhizosphere are questionable.

Table 2: Numerical data concerning fluorescent *Pseudomonas* isolates showed positive results in screening *in vitro* for plant growth promoting traits.

Traits			Cultivated plants						Wild plants						All plants					
			Number of Ecto isolates	(1) %	Number of Endo isolates	(1) %	Number of total isolates	(2) %	Number of Ecto isolates	(1) %	Number of Endo isolates	(1) %	Number of total isolates	(2) %	Number of Ecto isolates	(1) %	Number of Endo isolates	(1) %	Number of total isolates	(3) %
			26	-	02	-	28	-	49	-	18	-	67	-	75	-	20	-	95	-
Plant nutrition and growth regulation traits	PNF		07	26.9	-	-	07	25.0	19	38.8	04	22.2	23	34.3	26	34.7	04	20	30	31.6
	PS		13	50.0	02	100	15	53.6	43	87.8	17	54.4	60	89.6	56	74.7	19	95	75	78.9
	ZnS		15	57.7	02	100	17	60.7	30	61.2	10	55.4	40	59.7	45	60.0	12	60	57	60.0
	Sid		02	07.7	-	-	02	07.1	09	18.4	08	44.4	17	25.4	11	14.7	08	40	19	20.0
	IAA		02	07.7	-	-	02	07.1	24	49.0	07	32.9	31	46.3	26	34.7	07	35	33	34.7
Antifungal traits	Chitinase		04	15.4	-	-	04	14.3	04	08.2	-	-	04	06.0	10	10.7	-	-	08	8.4
	Cellulase		04	15.4	-	-	04	14.3	14	28.6	02	11.1	16	23.8	18	24.0	02	10	20	21.1
	Protease		01	03.8	01	50	02		25	51.0	02	11.1	27	40.2	26	34.7	03	15	29	30.5
	Sid		02	07.7	-	-	02	07.1	09	18.4	08	44.4	17	25.3	11	14.7	08	40	19	20.0
	SA		07	26.9	-	-	07	25.0	33	67.3	14	20.9	47	70.0	40	53.3	14	70	54	56.8
	HCN		07	26.9	01	50	08	28.6	24	49.0	02	77.7	26	38.8	31	41.3	03	15	34	35.8
Antifungal activity	Antagonistic isolates		21	80.8	01	50	22	78.6	46	93.9	18	100	64	95.5	67	89.3	19	95	86	90.5
	(4) Against	F_1	09	(5) 42.9	-	(5) -	09	(5) 40.9	27	(5) 58.7	10	(5) 55.5	37	(5) 57.8	36	(5) 53.7	10	(5) 52.6		(5) 53.5
		F_2	11	52.4	-	-	11	50.0	45	97.7	18	100	63	98.4	56	83.5	18	94.7	74	86.0
		F_3	14	60.7	01	100	15	68.2	42	91.3	18	100	60	93.7	56	83.5	19	100	75	87.2
		F_4	15	71.4	01	100	16	72.7	45	97.8	18	100	63	98.4	60	89.5	19	100	79	91.9
		F_5	15	71.4	01	100	16	72.7	45	97.8	18	100	63	98.4	60	89.5	19	100	79	91.9
	(6) Spectrum	1F	04	19.9	-	-	04	18.2	-	-	-	-	-	-	04	06.0	-	-	04	04.7
		2F	06	28.6	-	-	06	27.3	02	04.3	-	-	02	03.1	08	11.9	-	-	08	09.3
		3F	02	09.5	01	100	03	13.6	02	04.3	-	-	02	03.1	04	06.0	01	05.3	05	05.8
		4F	04	19.5	-	-	04	18.2	23	50.0	07	38.9	30	46.8	27	40.3	07	36.8	34	39.5
		5F	05	23.8	-	-	05	22.7	26	56.5	11	61.1	37	57.8	31	46.3	11	57.9	42	48.8

(1) Related to number of ectorhizosphere or endorhizosphere isolates

(2) Related to number of cultivated or wild plant isolates

(3) Related to the total 95 isolates

(4) F_1= (*P. ultimum*), F_2 =(*R. solani*), F_3= (*F. oxysporum*), F_4= (*P. infestans*), F_5= (*S. cepivorum*)

(5) Related to number of antagonistic isolates

(6) 1F (antagonistic against one fungs) ....etc.

Indoleacetic acid production

Indoleacetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophane metabolism by several microorganisms including PGPR (Arshad and Frankenberger, 1991). It is presumed that PGPR producing plant growth promoting agents play a critical role in plant growth promotion. It was found that inoculation of canola seeds with a PGPR isolate (*Pseudomonas putida* GR-122), which produces IAA resulted in 2-3 fold increases in the length of seedling roots (Caron *et al.*, 1995). In the present work, the 95 FPB isolates were screened for their ability to produce IAA. Results presented in Table 2, show that 34.7% of the total 95 FPB isolates produced detectable levels of IAA in culture supernatants. These isolates varied greatly in their ability to produce IAA. Wild plant isolates showed very higher proportion (46.3%) of IAA producers as compared with cultivated plant isolates (7.1%). On the other hand, proportions of IAA producers were approximately equal in both ectorhizosphere and endorhizosphere isolates.

Screening FPB isolates for antifungal traits

The traits tested were production of siderophores (sid), salicylic acid (SA), hydrogen cyanide (HCN) and hydrolytic enzymes: cellulase, chitinase and protease, and the results are presented in Table 2. It was found that a 20 % of the total FPB isolates is able to produce Sid. The results also show that percentage of Sid -producers among WP (25.3 %), Endo isolates (40.0%) was higher than that for CP (7.1%) and ectorhizosphere (Ecto) (14.7%) isolates. It was reported that the ability of FPB to antagonize *pathogenic* fungi was related to the production of extracellular Sid which deprive phytopathogenic microflora of iron, thus limiting their growth (Leong , 1986). Also it was reported that plants use microbial Sid for iron acquisition (Yehuda et al., 1996), and Sid is among factors involved in induced systematic resistance (ISR) (de Mayer and Höfte, 1997). The data showed that the ability to produce SA appears to be widespread among the FPB isolates .More than half of the 95 FPB isolates (54, 56.8%) were able to produce SA. The highest percentage of SA-producers (70.0%) was recorded for both WP and Endo isolates, whereas the lowest one (25.0%) was recorded for isolates of CP. Many studies indicated that SA plays an important role in plant defense response against pathogen attack and is essential for development of both systematic acquired resistance (SAR) and ISR in plants (van Loon *et al.*, 1998 and Zang *et al.*, 2002).

The results (Table 2) revealed that the proportion of HCN-producers varied among the isolates of different plants species and rhizosphere microhabitats. Of the total 95 isolates, 34 (35.8%) were able to produce HCN which ranking the second higher proportion after SA producers. The Ecto-isolates showed the highest proportion (41.3%) followed by WP isolates (38.8%), whereas the lowest proportion was recorded for Endo-isolates (15.0%). However, it was reported that cyanide production is an ambiguous trait and is sometimes associated with deleterious as well as beneficial rhizobacteria (Alström and Burns, 1989). Therefore, the high percentage of cyanogenic bacteria in the bacterial population of plant roots may possibly adversely affect plant growth, but may also inhibit the growth of fungal root

pathogens. The results (Table 2) show that among the enzymes tested number of protease- producers is the highest (29, 30.5%) followed by cellulase-producers (20, 21.1%), whereas chitinase-producers recorded the lowest number (8, 8.4%). Compared to WP isolates, CP isolates showed high proportion of chitinase-producers. It is of interest that none of the total 20 Endo-isolates was able to produce chitinase, whereas among Ecto-isolates the proportion was 10.7%. *In vitro* studies have demonstrated that the exposure of selected phytopathogenic fungi to lytic enzymes such chitinase, protease, gluconase or cellulase can result in degradation of the structural matrix of fungal cell wall (Dunne *et al.*, 1998).

Screening FPB isolates for antagonism towards plant pathogenic fungi

Biological control of plant pathogens has been the focus of many studies in plant protection that search for alternative or complementary methods to the use of chemical pesticides. PGPR including FPB have captured the attention of many researchers because of the potential for developing these bacteria as inocula for plant disease control.

The 95 FPB isolates were *in vitro* screened for their ability to inhibit growth of five different soilborne plant pathogenic fungi: *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Phytophthora infestans* and *Sclerotium cepivorum*. Results presented in Table 2, show that 86 (90.5%) of the 95 isolates screened were shown to exhibit a wide range of antagonistic activity against one or more of the five phytopathogenic fungi used in the screening. It was also observed that there are differences in proportion of CP and WP isolates that antagonize at least one of the 5 fungi tested which were 78.6% and 95.5%, respectively.

Berg *et al.*, (2002) found more bacterial isolates antagonistic to plant-pathogenic fungi in the rhizosphere of strawberry than in the rhizosphere of oilseed rape and potato. In the present study, among the 75 Ecto and the 20 Endo isolates, 89.3% and 95.0%, respectively were found to have antifungal activity. Chen *et al.*, (1995) showed that of 170 bacterial strains isolated from internal tissues of cotton, 40 (23.5%) possessed biological control against *Rhizoctonia solani* in cotton, and 25 (14.7%) induced systemic resistance to *Colletotrichum orbiculare* in cucumber. In the present work, of the 86 isolates showed antifungal activity, 46 (53.5%), 74 (86.0%), 75 (87.2%), 79 (91.9%) and 79 (91.9%) were antagonistic against *P. ultimum*, *R. solani*, *F. oxysporum*, *P. infestans* or *S. cepivorum*, respectively.

Regarding the antagonistic spectrum of the antagonistic FPB isolates (Table 2), antagonistic isolates of WP showed broader spectrum as compared with antagonistic isolates of CP where 57.8% and 22.7%, respectively were found to antagonize all the five fungi used. The proportions were 46.3% and 57.9% among antagonistic isolates of Ecto and Endo, respectively.

On the basis of the results obtained, FPB isolates belonging to WP or Endo seem to be highly efficient against fungal pathogens tested as compared to CP or Ecto isolates. It was reported that the plant species or cultivar and especially the composition of root exudates plays a key role in the diversity of rhizobacterial populations colonizing the roots (Siciliana et al.,

1998) and can influence the frequency of antagonistic bacteria (Berg *et al.*, 2000).

Assessment of the FPB isolates according to their *in vitro* plant growth-promoting traits

In an attempt to better select FPB isolates with high plant growth promotion potential , and to compare the potential of the FPB isolates from different plant species and two different rhizosphere microhabitats, a bonitur scale similar to that described by Krechel et al., (2002) was generated (Fig. 2) and used for assessment of FPB isolates. In this scale, points were given to each bacterial trait *in vitro* determined within this study. Up to three points each were given for antagonistic activity towards each of the five indicator fungi, one point for each of the three hydrolytic enzymes, HCN and PNF, three points for phosphate solubilization, and IAA production, and two points for zinc solubilization. Siderophores were given two points, one as antifungal trait and one for their use by plants for iron acquisition. The point given to HCN production was included in the assessment points of antifungal traits, whereas excluded from the sum assessment points because HCN can adversely affect plant growth. This generated a bonitur scale of 32 points.

Results of the assessment revealed the following points:

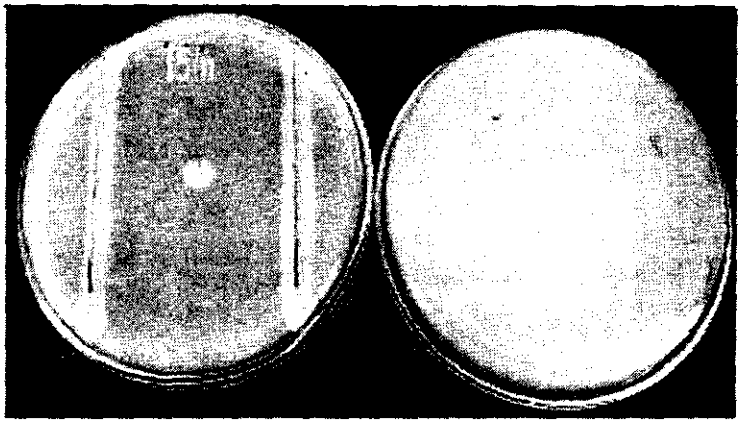
- For all the 95 FPB isolates, Σ assessment values varied between 1 point and 27 points.
- Isolates of the wild plant Rotrait showed the highest Σ assessment values ranged from 17 to 27 points, while the lowest values (1 to 5 points) were for FPB isolated from roots of onion plants.
- Compared with isolates of wild plants and ectorhizosphere, cultivated plants and endorhizosphere isolates showed lower Σ assessment values. This mainly caused by low or no antifungal activity and /or absence of lytic enzymes.

Table 3: Top 20 fluorescent *Pseudomonas* isolates and their *in vitro* plant nutrition, plant growth regulation; antifungal traits; in addition to their antifungal activity

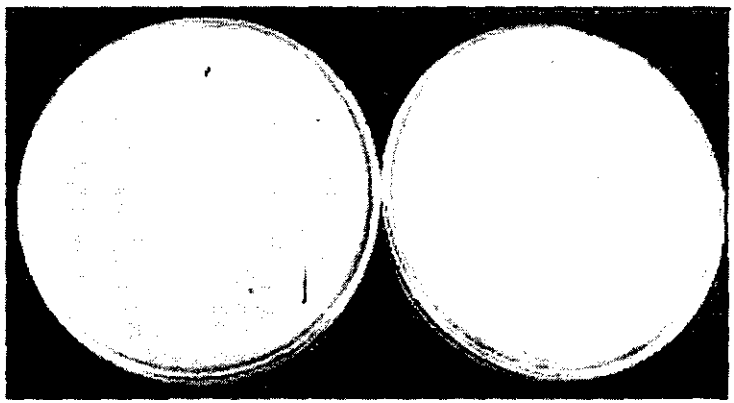
Isolates			Plant nutrition and growth regulation traits					Ass. (10)**	Rank	Antifungal traits						Ass. (7)**	Rank	Antifungal activity against:					Ass. (15)**	Rank	Total Ass. (32)**	Rank
No.	Plant	Habitat and Code	PNF[2]	P-SI[3]	Z-SI[4]	Sid[5]	IAA[6]			Sid	Chitinase	Cellulase	Protease	SA[9]	HCN[10]			F1[11] GI[12] (%)	F2 GI (%)	F3 GI (%)	F4 GI (%)	F5 GI (%)				
01	Zc[1]	[1]Ecto5N	+[8]	3.00	1.80	+	4.2	8	1	+	+	+	+	250	+	7	2	86	75	73	100	64	13	3	27*	1
02	Ag	Ecto112	-*	1.88	1.80	-	3.8	5	4	+	+	+	+	170	+	6	2	100	75	60	100	45	14	2	24*	2
03	Zm	Ecto108A	+	2.60	1.50	+	2.7	6	3	+	-	+	-	113	+	4	4	71	58	64	100	72	15	1	24*	2
04	Zc	Ecto3N	+	3.00	1.40	+	2.9	7	2	-	-	+	+	237	+	5	3	71	50	67	100	47	11	5	22*	4
05	Zm	Ecto105B	+	2.00	1.20	+	2.5	6	3	+	-	+	+	175	+	5	4	79	50	82	100	55	13	3	23*	3
06	Zc	Ecto1N	-	2.86	1.50	+	2.5	4	5	+	-	+	-	182	+	4	3	84	72	58	100	72	14	2	21*	5
07	Zc	Ecto311	+	4.40	2.00	-	2.6	7	2	-	-	-	+	170	-	2	6	50	55	73	100	65	12	4	21	5
08	Zc	Ecto312	+	3.33	2.00	+	2.1	6	3	+	-	-	-	135	-	2	7	76	78	62	100	80	14	2	22	4
09	Sm	Ecto5N	+	4.00	2.60	-	2.8	7	2	-	-	+	+	149	+	4	4	41	65	55	100	69	10	6	20*	6
10	Zm	Ecto105	+	2.40	1.12		2.6	5	4	-	-	+	-	170	+	3	5	100	75	91	100	47	13	3	20*	6
11	Sm	Ecto2N	-	3.60	NSZ[7]	+	3.5	6	3	+	-	+	+	175	-	4	6	43	47	64	100	36	11	5	21	5
12	Sm	Ecto7	-	5.00	1.30	-	2.6	6	3	-	-	+	+	102	-	3	1	30	53	73	100	67	11	5	20	6
13	Sm	[2]Endo1N	+	1.83	2.20	-	3.7	7	2	-	-	+	-	180	+	3	5	NI[13]	68	84	100	62	10	6	19*	7
14	Sm	Endo3	+	3.67	1.70	-	2.9	6	3	-	-	+	+	145	-	3	6	59	58	73	100	62	12	4	21	5
15	Zc	Ecto303	+	2.40	1.80	-	2.6	7	2	-	-	+	+	145	+	4	4	NI	55	58	100	55	09	7	19*	7
16	Sm	Endo2	+	2.80	2.20	-	2.2	6	3	-	-	-	+	175	+	3	6	NI	52	73	100	58	13	3	21	5
17	Zc	Ecto4N	+	2.40	1.80	-	2.2	5	4	-	-	-	+	0	+	2	6	59	62	73	100	51	12	4	18*	8
18	Zc	Ecto313	+	3.25	1.80	-	1.0	7	2	-	-	-	+	170	-	2	5	46	35	73	100	33	10	6	19	7
19	Ag	Ecto112b	-	2.60	1.60	-	3.2	4	5	-	-	-	+	0	+	2	6	100	63	69	100	58	13	3	16*	8
20	Ag	Ecto113C	-	3.00	NSZ	-	0.0	2	6	-	-	-	+	120	+	3	5	100	75	100	100	58	14	2	18*	8

1. Zc = *Zygophyllum coccineum* L., Ag = *Alhagi graecorum*, *boiss*, Zm = *Zea mays* L., Sm = *Spergularia marina* L.

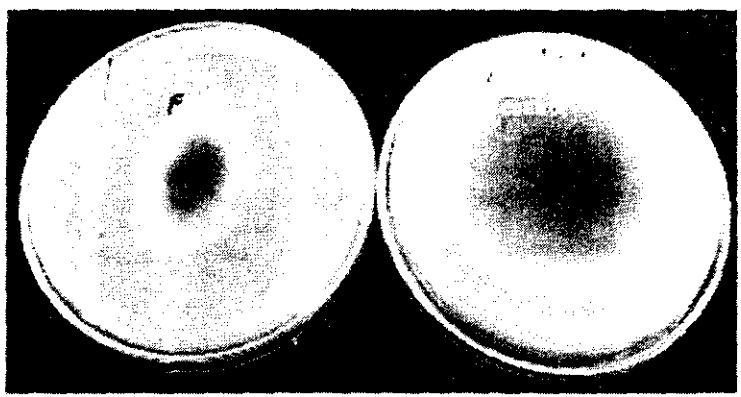
2. Ecto, Ectorhizosphere; Endo,Endorhizosphere;3. PNF = Putative N_2-fixation; 4.SI = Solubilization index; 5. Sid= Siderophores production; 6. IAA= Indoleacetic acid production (μg ml^{-1}); 7. NSZ= No solubilization zone; 8. +,-= Positive and negative result; 9. SA = Salicylic acid production (absorbance value at 527 nm); 10. HCN = Hydrogen cyanide; 11.F1= *P. ultimum*, F2= *R. Solani*, F3= *F. Oxysporum*, F4= *P. Infesians*, F5= *S. cepivorum* ; 12. GI (%) =Growth inhibition percentage; 13. NI= No inhibition; *the point given for HCN production was excluded; **Assessment points.



(A)

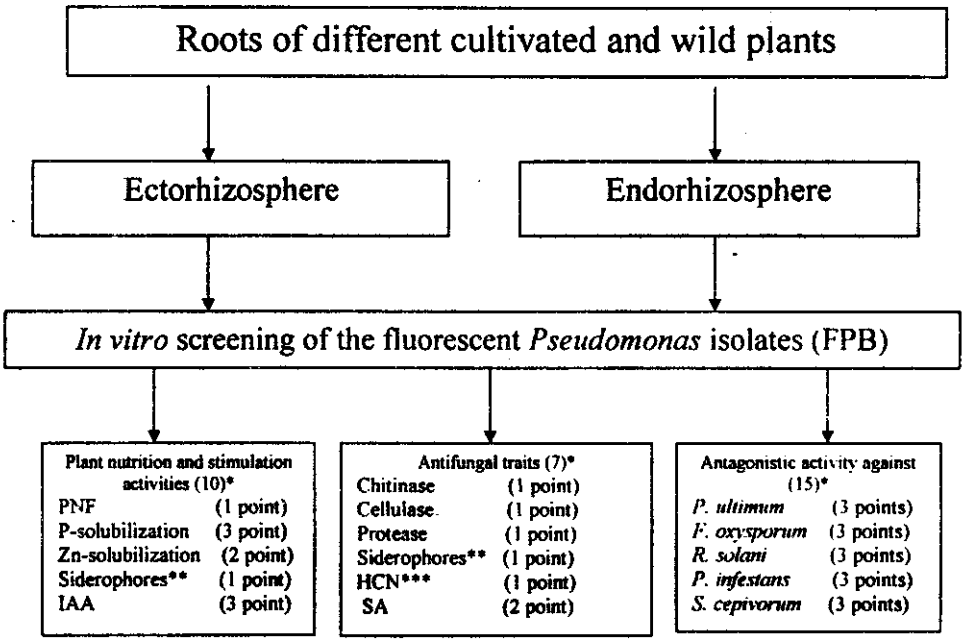


(B)



(C)

Fig.1: Dual assay plates showing antifungal activity of FPB isolates against *S. cepivorum* (A) *P. Infestans* (B) and *F. oxysporum* (C).



PNF = Putative N_2-fixation, - = 0, + = 1
Solubilization Index, Phosphorus, 1= > 2.99; 2=3-4.99 ; 3 = <5
Zinc, 1= <1.99 ; 2= >2
IAA, 1= <1.99 ; 2= 2 -2.99 ; 3 = >3 $\mu g\ ml^{-1}$
SA, 1= 100-199; 2=200-300 $\mu g\ ml^{-1}$
Fungi Growth inhibition % , 1= 30 – 49.9 % ; 2 = 50 – 69.9% ; 3= > 70 %
*Assessment points
** Two points were given to siderophores production, one as antifungal traits and one for facilitating iron uptake by plants.
***The point given to HCN production, if positive, was excluded from $\sum$ assessment points because it consider as antifungal traits and in the same time can inhibited plant growth.

Fig. 2: A bonitur scale of 32 points used for assessment the FPB isolates based on their results *in vitro* screening for PGPR traits.

- Based on the Σ assessment values of all the 95 FPB isolates, the top 20 isolates and their traits are listed in Table 3. It is of interest that among those 20 isolates, 17 (85%) were isolated from WP roots, and the remaining 3 (15%) were isolated from roots of the cultivated maize plants. Regarding rhizosphere microhabitats, numbers of Ecto-isolates was the higher (17, 85 %) compared to Endo-isolates (3, 15 %).

Collectively, the results suggest that compared with cultivated plants, rhizospheres of wild plants are very good source for FPB with multiple plant growth promoting traits.. Moreover, FPB inhabiting rhizospheres of wild plants showed higher density, wider diversity, and higher proportion of isolates possess high number of PGP traits with high values, and higher proportion of antifungal isolates with broader antifungal spectrum. Taking into consideration that the presence of plant growth promoting traits *in vitro* does not guarantee that a particular isolate is a PGPR, further studies on the performance of these isolates *in vivo* are needed to uncover their efficacy as effective plant growth promoting rhizobacteria.

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تقييم معملى لبعض من بكتريا السيدوموناس الفلورسنتية المعزولة من ريزوسفير نباتات زراعيـــة وأخرى بريـــة للسمات المشجعة لنمو النبات

حسنى جمال الدين، مدحت البدرى ، ســـالم محفوظ و سيد عبد العظيم عبد العزيز
قســـم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة الفيوم– مصر

هدف هذا البحث هو الحصول على عزلات من بكتريا السيدوموناس الفلور سنتية (FPB) تتميز بمدى واسع من السمات المشجعة لنمو النبات يمكن استخدامها كبديل أمن للكيماويـــات الزراعيـــة الضـــارة. ويتضمن البحث تقدير أعداد وعزل FPB التى تستوطن ريزوسفير خمس نباتـــات زراعيـــة (بصـــل – ذرة شامية – بنجر السكر – طماطم– فاصوليا خضراء)، وخمس نباتات برية (حميض –عاقول – روطـــريط – كوكيا– ورد النيل) وظهر من النتائج أن أعداد FPB فى ريزوسفير النباتات البرية كانت أكبر من أعـــدادها فى ريزوسفير النباتات الزراعية ، وأن الأعداد فى الريزوسفير الخارجى (سطح الجذر الخالى مـــن التربـــة) كانت أكبر من أعدادها فى الريزوسفير الداخلى (أنسجة الجذر المعقم سطحه) وقد تـــم الحصـــول علـــى ٩٥ عزلة FPB منها ٢٨ عزلت من ريزوسفير النباتات الزراعية و ٦٧ عزلت من ريزوسفير النباتات البرية ، وقد أجريت عدة دراسات معملية لمعرفة مدى تميز هذه العزلات بالسمات المشجعة لنمو النباتات وأوضـــحت النتائج أن من بين العزلات الـ ٩٥ وجد أن ٢٧,٣% منه أستطاعت النمو على بيئة خالية مـــن النيتـــروجين ،٥٨,٩% مذيبة للفوسفات ، ٤٧,٣% مذيبة للزنك، ١٦,٨ % منتجة للسيديروفورز، ٤,٣١% منتجة لحمـــض الأندول أسيتيك، وأظهراختبار العزلات للسمات التى تساهم فى تضاد الفطريـــات أن نســـبة ٥٨,٨% مـــن العزلات تنتج حامض السيلسيليك ٣٥,٨% تنـــتج ســـيانيد الهيـــدروجين، ٣٠,٥%، ٢١,١% ، ٨,٤% مـــن العزلات تنتج أنزيمات البروتييز والسليوليز والشيتينيز، على التوالى. وعندما أختبرت عزلات FPB معمليـــا لنشاطها المضاد لنمو خمسة فطريات ممرضة للنبات وهى:

Pythium ultimum, Rhizoctonia solani, Fusarium oxysporum, Phytophthora infestans and *Sclerotium cepivorum*

أظهرت النتائج أن ٩٠,٥% من العزلات أظهرت تضاد لفطر واحد على الأقل من بين الفطريات الخمسة وكانت النسبة فى حالة عزلات النباتات البرية ٩٠,٥% بينما كانت ٧٦,٦% فقط فى حالـــة عـــزلات النباتات الزراعية. وقد وجد أيضا أن ٩٥% من عـــزلات الريزوســـفير الـــداخلى، ٨٩,٣% مـــن عـــزلات الريزوسفير الخارجى تضاد الفطريات بمعدل فطر واحد على الأقل وتبين النتائج أيضا أن نسبة كبيـــرة مـــن عزلات النباتات البرية (٥٧,٨%) أظهرت تضادا للخمسة فطريات المستخدمة فى الدراسة.بينما كانت النســـبة ٢٢,٧% فقط فى حالة عزلات النباتات الزراعية. ولتحديد العزلات المتميزة من بين عزلات FPB الـــ ٩٥ فقد تم أستخدام مقياس رقمى مكون من ٣٢ نقطة نتجت من جمع النقاط التى أعطيت لكل نتيجة موجبـــة فـــى الاختبارات المعملية السابق ذكرها وبتطبيق هذا المقياس تبين أن أفضل ٢٠ عزلة من بـــين عـــزلات FPB الـــ ٩٥ حازت مابين ١٨ و ٢٧ نقطة ، واتضح أنه من بين هذه العزلات ١٧عزلة من ريزوسفير النباتـــات البرية (ثمانية من نبات الروطريط ، وثلاث من نبات العاقول، وستة من نبـــات الكوكيـــا) وثلاثـــة فقـــط معزولة من ريزوسفير النباتات الزراعية (نبات الذرة الشامية) ، وكانت الـــ ١٧ عزلة من العزلات العشرون معزولة من الريزوسفير الخارجى وثلاثة فقط معزولة من الريزوسفير الداخلى.