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APPLICATION OF SOME STRAINS OF FLUORESCENT PSEUDOMONADS IN MANAGING ROOT-INFECTING PATHOGENS OF MAIZE

[27]

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ABSTRACT

Possibility of manipulating some of the efficient strains of fluorescent pseudomonads to manage the root-infecting pathogens of maize was studied throughout this study. Out of 110 isolates 24 of *Pseudomonas* species, recovered from the plant rhizosphere showed to have inhibitory effect against two major root-infecting pathogens of maize, namely *Cephalosporium maydis* and *Fusarium verticillioides in vitro.* Pot experiment revealed that just 4 isolates could reduce infection with both pathogens and enhance the plant growth as well. Based on the genotypic identifications of these four isolates showed that they were: *Pseudomonas putida* strain Pau9, *P. putida* strain Pau11, *P. putida* strain Psf3 and *P. aeruginosa* strain Psf9.

INTRODUCTION

Root-infecting pathogens can cause serious diseases to field crops under unfavorable conditions. Maize plants are subjected to several soil borne pathogens. In Egypt, late-wilt caused by *Cephalosporium maydis* is the most serious and wide spread fungal pathogen that can infect plants through roots of susceptible varieties and hybrids causing 30-40 % grain losses in infected plants **(Abdel-Rahim, 1971)**. The second serious fungal pathogen is *Fusarium verticillioides* , which infects maize plants at seedling stage, causing wilting and death **(Rheeder et al 2002; Duncan and Howard, 2010)**. These two root-infecting pathogens are of concern in the time being **(El-Assiuty et al 1998; Alakonya et al 2008)**. It is extremely difficult to control soil-borne pathogens by fungicides. Thus, biological control is being considered a supplemental method of reducing the use of chemicals in agriculture to the benefit of manhood **(Kloepper et al 1999 and Widmer et al 1998)**. Plant growth promoting rhizobacteria (PGPR) have been applied to seed and soil successfully for years **(Kloepper et al 1999)**. Fluorescent pseudomonads, an important component of PGPR have been reported by several investigators as efficient bioagents in controlling major diseases of different diseases plants **(Sharma et al 2014 and Minaxi & Saxena, 2010)**.

Therefore study aimed to apply some efficient isolates of fluorescent pseudomonads recovered from plant rhizosphere to decrease infection with major root pathogens of maize.

MATERIALS AND METHODS

Fungal pathogens

Cephalosporium maydis (=*Harpophora maydis*), and *Fusarium verticillioides* (=*F.moniliforme*) were recovered from maize plants showing signs of infection, collected from Etsa, Fayoum governorate. Kokh's postulates were followed and the most efficient pathogenic isolate from each of the target pathogens was chosen in present study. They were compared with the culture collection of Plant Pathology Research Institute, ARC and kept in 15 % glycerol under -80°C for prolonged period.

Isolation of fluorescent pseudomonads

Isolates of fluorescent pseudomonads were recovered from the rhizosphere of maize and sugar beet grown in different geographical locations (governorates) during 2014. Root samples were

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washed vigorously with tap water, shaken to remove excess soil and left to dry. Rhizosphere (0.5g) along with 4.5 ml phosphate buffer were shaken at 120 rpm for 10 min. Serial ten-fold dilutions were prepared, 0.5 ml of suitable diluents were spread onto King's medium **(King et al 1954 and Amkraz et al 2010)** and incubated at 28°C for 1-2 days. Thereafter, isolated single colonies were picked up and restreaked on fresh King's agar plates and incubated similarly. Purified bacterial colonies were examined for fluorescence under UV at 265and 356 nm and Gram staining. Pure isolates were preserved into slants on King's medium at 5ºC. The pure cultures were preserved in 15% glycerol under -80° C until use.

In vitro antagonistic activity

The obtained isolates of *Pseudomonas* spp. were tested for their efficiency for inhibiting the linear growth of both target pathogens by inoculating a (0.5 cm diam.) disc from each of the fungal pathogens at the center of Waksman's plate **(Berg et al 2002)** at 28° C. After 24 h., 4 different bacterial isolates were streaked at the same distance from the fungal disc. Plates were incubated at the same degree of temperature for three days. Three replicate plates were used. Results were recorded as positive or negative effect of the bacterial isolate for antagonizing the target pathogens.

In vivo evaluation of antagonisting bacterial isolates

Fungal inoculants were prepared by inoculating the pathogens individually into autoclaved sorghum grains and incubated at room temperature for a sufficient period (about 2 weeks).Thereafter; potted-soil silt clay soil was infested singly with each of the fungal inoculum at the rate of 2.5 % (w/w). Pots were moistened to permit the pathogens to establish growth for one week before planting. Seed of a sensitive local *Zea mays* variety were coated with each of the bacterial isolate following the method described by Bardin *et al.* (2004). Each set of the bacterial treated seed were planted in the prepared potted soil (no.25) and the experiment was quadruplets. Cultural practices (irrigation fertilization,…*etc.*) were made as usual. Percentage of seedling emergence and plant stand (survival plants) were recorded after 15 and 45 days of planting, respectively.

Biochemical characterization of *Pseudomonas* spp.

The bacterial isolates were characterized biochemically as described by **Buchanan and Gibbson (1974)**.

Molecular identification of *Pseudomonas* spp.

Sequence analysis of 16S rRAN gene

Isolation of cellular DNA was performed as described by **Ausubell et al (1987)**. DNA was extracted by thermo scientific genejet gel extraction kit. Relevant16S rDNA sequences are available in Gene Bank and done by Bio Basic, Canada Inc. Sequence of the used primer described by **Spilker et al (2004)** is shown in **Table (1)**. PCR **a**mplification of targeted DNA was carried out in 50µl reaction volume (Master mix Bioline, 25µl; primer F2µl; primer R, 2 µl; DNA 5µl; 16 µl). DNA was amplified over 25 cycles of denaturation for 2 min at 95°C, each consisting of 20 s at 94°C, 20 s at the annealing temperature of 54°C and extension for 40 s at 72°C. Thereafter, a final extension was applied for 1 min at 72°C **(Spilker et al 2004)**. Amplification of DNA product was run on 1 % agarose gel (ROKO Poligono De Silvota, Spain) and separated by electrophoresis in 1x TBE buffer. DNA gel was photographed with ethidium bromide under UV by transilluminator. PCR product was purified and sequenced by Sigma Company.

Phylogenetic analysis

Sequences were compared with sequences of *Pseudomonas* spp. available in the Gene Bank NCBI database (National Center for Biotechnology Information) using BLAST search network services for similarities present in Gene Bank database. Multiple sequence alignments from all sequences of each gene were performed using Clustal W version 2.0 **(Larkin et al 2007)**. Phylogenetic analyses were constructed by the maximum likelihood (ML) method with maximum parsimony (MP) using MEGA version 7 **(Kumar et al 2016)**. The bootstrap values illustrated on the phylogenetic trees were generated with 1000 replicate heuristic searches.

Table 1. Sequence of specific primer used in identifying *Pseudomonas* spp.

Primer	Sequence (5\-3\)	Target	Annealing temp.(°C)	Location[a]	Product size (bp)
PA-GS-F	GACGGGTGAGTAATGCCTA	*Pseudomonas* sp.	54	95–113	618
PA-GS-R	CACTGGTGTTCCTTCCTATA			693–712	

Statistical analysis

Data were subjected to analysis of variance using SAS-9.1 software **(SAS Institute, 2003)**. Mean values among treatments were compared by L.S.D P <0.05.

RESULTS

Isolation of the fluorescent pseudomonads

Fluorescent pseudomonads were isolated from rhizosphere of maize and sugar beet plants grown in different governorates and its numbers were presented in **Table (2)**.

Table 2. Number of fluorescent pseudomonad isolates recovered from maize and sugar beet rhizosphere grown at different governorates

Governorate	No. isolates	Source	
		Maize	Sugar beet
Fayioum	33	9	24
Sharqiya	32	10	22
Beheira	15	5	10
Gharbiya	15	-	15
Giza	15	10	5
Total isolates	**110**	**34**	**76**

Data in **Table (2)** show that 110 isolates of fluorescent pseudomonads were recovered from different governorates and the most isolates were collected from maize and sugar beet plants grown in Fayoum and Sharqiya governorates being 33 and 32 isolates, respectively.

Antifungal activity

All of the recovered isolates of fluorescent pseudomonads were assayed in vitro for antagonism against both of the target pathogens. Each pathogen showed different levels of sensitivity toward the bacterial isolates. Positive effect was clearly recognized by limiting the fungal growth or by complete inhibition of fungal mycelia. Results showed that 30 bacterial isolates having the potential effect against C*. maydis,* while, 27 isolates affected the linear growth of *F.verticillioides*. About 50% of bacterial isolates under study were unable to inhibit the growth of any of the target pathogens. Out of these, 24 isolates were showed to have high antagonistic effect against both pathogens. Accordingly, they were selected for further study.

***In vivo* evaluation**

Data presented in **Table (3)** explain the effect of coating maize seeds with each of the 24 bacterial isolates on seedling emergence (after 15 days of planting) and plant stand (after 45 days of planting) along with infection percentage at each of the plant growth in soil infested, separately with each of *C. maydis* and *F. verticillioides.* As regards to potted-soil infested with *C. maydis*, significant differences were found between percentage of seedlings emerged from seeds treated with any of the bacterial isolates comparable to the infested control, except four isolates no. Psf 2, Pmf 17, Psf 30 and Psf 31, where no increase in the emergence was found. In contrast, in potted-soil infested with *F. verticillioides*, seedling emergence was non-significant differences by treating with the majority of bacterial isolates, while isolates no. Psf 33, Pssh 39, Pssh 50, Pau9, Pau10, Pau11 and Pau12 were the most effective in increasing seedling emergence comparable to the infested control.

After 45 days of planting, however, data of plant stand were taken and recorded as percentage of the survived plants from emerged seedling. Infection was calculated as the percentage of the diseased plants at this stage of plant growth. Data presented in **Table (3)** show that most of isolates significantly reduced infection in soil infested with any of the target pathogens comparable with the non-treated-infested control, generally. But, isolate no. Pssh 48 failed in decreasing infection in *C. maydis*-infested soil. Also, isolates no. Pssh 48 and Psf11could not significantly affect the plant stand or infection percent with *F. verticillioides.*

Table 3. Efficiency of coating maize seed with fluorescent pseudomonad isolates in managing root-infecting pathogens of maize under greenhouse conditions, 2015

Isolate No.	*C.maydis*-infested-soil				*F.verticillioides*-infested-soil			
	% Seedling emergence	%Seedling infection	%Plant stand	%Plant infection	%Seedling emergence	% Seedling infection	%Plant stand	%Plant infection
Psf 1	72.50	27.50	48.27	51.73	60.00	40.00	79.16	20.84
Psf 2	57.50	42.50	47.82	52.18	72.50	27.50	75.86	24.14
Psf3	65.00	35.00	61.53	38.47	55.00	45.00	90.90	9.10
Psf 4	67.50	32.50	59.25	40.75	65.00	35.00	65.38	34.62
Psf9	62.50	37.50	84.00	16.00	57.50	42.50	91.30	8.70
Psf 10	62.50	37.50	60.00	40.00	57.50	42.50	78.26	21.58
Psf 11	77.50	22.50	64.51	35.49	67.50	32.50	37.04	62.96
Pmf 17	52.50	47.50	66.66	33.34	70.00	30.00	46.42	53.58
Psf 27	70.00	30.00	75.00	25.00	75.00	25.00	46.66	53.34
Psf 30	55.00	45.00	68.18	31.82	75.00	25.00	60.00	40.00
Psf 31	55.00	45.00	54.54	45.46	62.50	37.50	40.00	60.00
Psf 32	72.50	27.50	62.06	37.94	65.00	35.00	50.00	50.00
Psf 33	67.50	32.50	70.37	29.63	80.00	20.00	56.25	43.75
Pssh34	70.00	30.00	75.00	25.00	72.50	27.50	41.37	58.63
Pssh 38	75.00	25.00	62.06	37.94	72.50	27.50	41.37	58.63
Pssh 39	75.00	25.00	73.33	26.67	77.50	22.50	61.29	38.71
Pssh 48	67.50	32.5	33.33	66.67	65	35	30.76	69.24
Pssh 50	72.50	27.50	55.17	44.83	82.50	17.50	54.54	45.46
Pah3	80.00	20.00	62.06	37.94	62.50	37.50	75.67	24.33
Pah5	75.00	25.00	70.00	30.00	90.00	10.00	72.22	27.78
Pau9	80.00	20.00	84.37	15.63	95.00	5.00	84.21	15.79
Pau 10	75.00	25.00	66.66	33.34	87.50	12.50	65.71	34.29
Pau 11	77.50	22.50	87.09	12.91	97.50	2.50	76.92	23.08
Pau 12	92.50	7.50	64.86	35.14	85.00	15.00	61.76	38.24
Cont. (infected)	**37.50**	**62.50**	**33.33**	**66.67**	**57.50**	**42.50**	**38.38**	**61.62**
Cont. (non- infected)	**98.50**	**1.50**	**98.00**	**2.00**	**99.00**	**1.00**	**97.50**	**2.50**
LSD	**23.84**	**--**	**24.54**	**--**	**19.45**	**--**	**25.89**	**--**

Data in **Table (3)** also indicat that Psf9, Pau9 and Pau11 showed to be highly effective in reducing infection with *C. maydis* and Psf3, Psf9 and Pau9 where they were high efficient in controlling infection with *F. verticillioides.* Therefore, isolates no. Psf9, Pau9, Pau11and psf3 were selected for further study in the present work.

Biochemical characterization of fluorescent pseudomonads

The most potent isolates of fluorescent pseudomonads under study were tentatively characterized following Bergy's manual **(Buchanan and Gibbson 1974)**. Results of characterization are presented in **Table (4)**.

Table 4. Biochemical characterization for tentative identification of isolated *Pseudomonas* spp.

Isolate	Gram stain	King's medium using UV	Catalase	KOH 3%	Motility	Aerobic assimilation of glucose	Fermentation of glucose	Pigment production	Growth at 41°C	Gelatinase	Oxidative test	Fluorescene	pyocine	Tentative species
Psf3	-	+b	+	+	+	+	-	+	-	-	+	+	-	*Pseudomonas* sp.
Psf9	-	+b	+	+	+	+	-	+	+	-	+	-	+	*Pseudomonas* sp.
Pau9	-	+b	+	+	+	+	-	+	-	-	+	+	-	*Pseudomonas* sp.
Pau 11	-	+b	+	+	+	+	-	-	-	-	+	+	-	*Pseudomonas* sp.

b: blue color

Biochemical analysis of the four isolates under study shown in Table (4) confirmed that they are related to *Pseudomonas* spp.

Results of classical identification for isolates of fluorescent pseudomonads under study needed to be identified up to species following genetic identification. 16S rDNA- based primer sets were used for sequencing the *Pseudomonas* spp. **(Spilker et al 2004).**

Genotypic identification for the most potent antagonistic bacterial isolates

Four rhizobacterial isolates which gave the best results in pot experiment against the target pathogens (*C. maydis* and *F. verticillioides*) were molecular identified. The phylogenetic analyses according to the BLAST search are shown in **Fig. (2)**. The four isolates were subjected to molecular characterization using the pseudomonas specific primer. A single distinct fragment of approximately 618bp was observed with the four strains as shown in **Fig. (1)**. Sequencing of 16S rDNA from strains, namely Psf 9, Psf3, Pau 9& Pau11was performed .In order to find out the most similar available sequences, a BlAST search of 16S rDNA was done at the NCBI database. Accordingly, the phylogenetic analyses of these pseudomonads based on the maximum likelihood method were identified as follows: Psf3= *Pseudomonas putida KX786158* Psf 9= *Pseudomonas aeruginosa* ss12 GU451299 Pau9=*Pseudomonas putida KX786158* Pau11= *Pseudomonas putida* HF3-27FJ405891 **(Fig. 2)**.

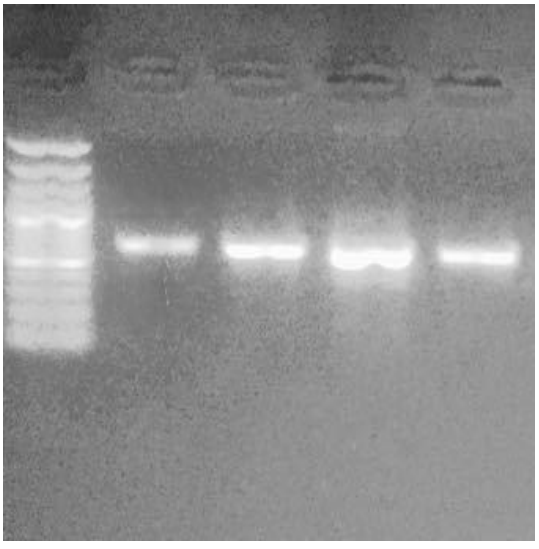

Fig. 1. Agarose gel electrophoresis of the PCR–product of 16S rDNA sequences from the most potent *Pseudomonas* strains along with the target primer. Lane1: M1500bp DNA ladder, Lane2:Psf3, Lane3: Psf9, Lane4: Pau9, Lane5: Pau11.

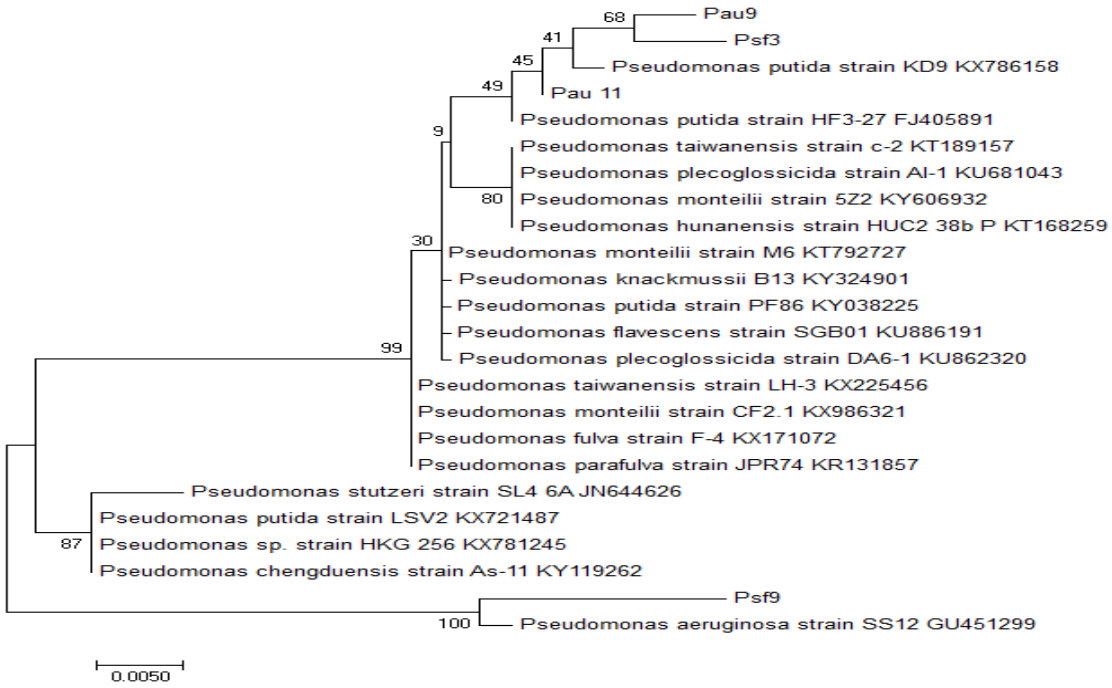

Fig. 2. Phylogenetic tree of tested bacterial strains

DISCUSSION

Increasing use of chemical inputs causes several negative effects, such as evolving of pathogen resistance to the applied pesticides and their serious environmental impacts. Thus, biological control is being considered a supplemental method for reducing the use of the hazardous chemicals in agriculture. Therefore, current study dealt with managing or decreasing major pathogens of maize plant roots by some efficient fluorescent pseudomonad isolates.

Several isolates of these bacterial group recovered from maize and sugar beet rhizosphere were screened for their efficiency towards the target pathogens, namely *Cephalosporium maydis* and *Fusarium verticillioides*. Twenty four isolates were shown to have the potency to antagonize both of the target pathogens in the laboratory. and reduce root infection in pot experiment. Obtained results showed that enhancing plant stands was obviously coincided with the effectiveness in reducing infection with the target pathogens. Inhibitory effect of several strains of this bacterial group were recorded by previous investigators **(Minaxi and Saxena, 2010; Noori and Saud, 2012). Altinok et al (2013)**, reported that *Fusarium* wilt of eggplant could be reduced up to 85 %. by drenching the seedlings in pot experiment with suspensions from one strain of *P. putida* and two strains of *P. aeruginа.* **El-Assiuty et al (2010)** reported that infection of sugar beet with root-rot could be reduced by coating seed with some strains of fluorescent pseudomonads. This application caused an increase in shoot and root biomass and no signs of abnormalities were observed on applied plants. The antagonistic effect and controlling of root-infecting diseases could be attributed to the potential of the applied organisms to produce some metabolites such as antibiotics, siderophores, hydrogen cyanide (HCN), …*etc.* **(Minaxi, and Saxena, 2010; Noori & Saud, 2012 and Sharma et al 2014)**. The production of enzymes related to pathogenesis is also, considered the largest property of the antagonistic strains **(Saikia et al 2004)**. Moreover, novel proteins that exhibit antimicrobial activity were stimulated in leaves after foliar application with some fluorescent psudomonads as, recently reported by **El-Assiuty et al (2010)**. Chitinase glucanase were also, found to be substantially increased in leaves of treated plants. Other investigators **(Meena et al 2000)** who emphasized that

activities of chitinase and phenylamonia lyase were increased after foliar application of rice with *P. fluorescens.* These hypothesized practical considerations involved in the mode of action of the *Pseudomonas* strains used in this study support the recommendation of using them to control such diseases biologically.

Biochemical and genotypic identifications were done to the four most potent isolates. According to the phylogenetic analyses based on the maximum likelihood method, they were identified as follows: Psf3= *P. putida* strain Psf3, Psf9=*P.aeruginosa* strain Psf9, Pau9=*P.putida* strain Pau9 and Pau11= *P.putida* strain Pau11.

Among these identified strains, *P.putida* and *P.aeruginosa* were reported by several investigators as biocontrol agents **(Minaxi &Saxena, 2010 and Sharma *et al*., 2014)**. Furthermore, some strains of these two species were abundantly reported to have the potential to produce some antagonistic metabolites that inhibit the pathogens, in addition to their ability to release of plant growth factors as reported by **Altinok et al (2013)**.The *Pseudomonas* strains in the current study were found to have the potency to produce some metabolic products such as 4-hydroxybenzoic acid, 2,6-di-hydroxyl benzoic acid and hydroquinone (unpublished data). Alleviating infection with plant diseases is hypothesized to be accomplished by these products and others **(Smith-Becker et al 1998 and De Werra et al 2011)**.

Also, **Harwood et al (1984)** reported that the plant derived aromatic metabolites as Benzoxazinoids (BXs) can act as chemo-attractants for *P. putida.* It was hypothesized by **Neal et al (2012)** that these compounds are exudated from roots of maize and they may be attractive and supportive to *P. putida* cells. These metabolites were concluded by the same authors to be responsible for mechanism of tolerance provides *P. putida* with a competitive advantage over other microorganisms in maize rhizosphere.

In addition to the efficacy of these strains in managing the root-infecting pathogens, many of these strains having the potency to secrete plant hormones that accelerate and enhance plant growth as stated by **Noori and Saud (2012)**.

In conclusion, present investigation threw the light on the possibility of applying such of these strains to control root-infecting pathogens of maize.

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