Characterization of Antifungal Metabolites from Antagonistic Fluorescent Pseudomonads

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Received: *1/9/2015*

Abstract: Many *Pseudomonas* species produce antimicrobial metabolites for pathogen suppression and stimulate the plant growth. So twenty fluorescent pseudomonas strains showed highest antagonism efficiency to soilborne phytopathogens which used throughout the present investigation. These bacteria included five strains *P. putida*, 3/ *P. plecoglossicida, 3/ P. palleroniana, 2/ P. corrugate, 2/ P. entomophila* and one from each of *P. moraviensis*, *P. parafulva*, *P. mosselii*, *P. anguilliseptica*, *P. argentinensis*. Detection of the genes that encode for the production of antibiotics by these strains such as phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), pyrrolnitrin (PRN), and pyoluteorin (PLT) was done by polymerase chain reaction (PCR) using gene-specific primers. Results clearly showed that eight *Pseudomonas* strains produced (PCA), twelve strains produced (PCN), nine strains produced (PLT) and two strains produced (PRN). Polymerase chain reaction (PCR) confirmed the obtained result by using TLC. *P. putida* (SAW19) gave the highest antagonistic potential against tested phytopathogenic fungi and formulated using different carriers to test for its viability on different carriers. The populations of *P. putida* were markedly higher in vermiculite compared with the carriers under six months incubation period.

Keywords: fluorescent pseudomonas-PCA-PLT-PRN-PCN-TLC

INTRODUCTION

A huge number of fungal diseases plague the crop plants throughout the year when a farmer fails to take proper preventative measures. Plant disease control has become heavily dependent on fungicides to combat the wide variety of fungal diseases. Beneficial bacteria have been intensively studied as biocontrol agents against soilborne diseases (Weller, 2007; Moeinzadeh *et al.*, 2010; Saber *et al*., 2015). Good results have been obtained with Gram-negative *bacteria Pseudomonas* spp. in the control of several plant pathogens, including *Gaeumannomyces graminis* var. tritici, *Fusarium spp*., and *Pythium spp*. (Raaijmakers *et al*., 2006; Mavrodi *et al*., 2012). Among the great variety of beneficial bacteria and antibiotic metabolites already discovered, our attention has been focused on phenazines, pyrrol-type antibiotics and pyo-compounds. Antibiotics and some strains, e.g. *P. fluorescens* strains CHA0 and Pf-5, produce multiple antibiotics. In general, these antibiotics have broad-spectrum toxic activity against fungi, bacteria, protozoa, nematodes, and sometimes also against plants or even viruses (Raaijmakers *et al*., 2006).

Various types of material can be used as carrier for seed or soil inoculation. A suitable biofertilizer carrier should meet the following criteria: (1) it should be available in powder or granule forms; (2) it should be able to support microorganism growth and survival, and easily release functional microorganisms into the soil; (3) it should have a strong moisture absorption capability, good aeration characteristics, and excellent pH buffering capacity; (4) it should be non-toxic and environmentally friendly; (5) it should be easily sterilized, manufactured, and handled in the field, and have good storage qualities; and (6) it should be cheap (Stephens and Rask, 2000; Rebah *et al*., 2002; Rivera-Cruz *et al*., 2008). Several alternative solid materials have been evaluated as inoculant carriers, such as: soil, vermiculite, perlite, ground rock phosphate, polyacrylamide gel, alginate, decomposed sawdust, coal, compost made from bagasse, sawdust, plant residues compost, farmyard manure and volcanic pumice (Strijdom and Deschodt, 1976; Einnarsson *et al*., 1993; Brockwell and Bottomley, 1995; Stephens and Rask, 2000; Hungria *et al*., 2005). Vermiculite and perlite are cheap and readily available components, both are light weight and pH-neutral (Goh and Haynes, 1977; Atiyeh *et al*., 2000). Perlite was a good carrier material because of its light weight, its porosity, and its environmental-friendliness (Khavazi *et al*., 2007).

MATERIALS AND METHODS

Source of fluorescent pseudomonads

In the present investigation, twenty fluorescent pseudomonads strains were obtained from Botany Department, Fac. Agric., Suez Canal Univ. These strains which showed the highest efficiency to antagonize soilborne phytopathogens were isolated and identified (using gene sequencing) by Fthalla (Alaa) *et al*. (2015). The strains are *Pseudomonas entomophila* SAW7, *Pseudomonas plecoglossicida* SAB1, *Pseudomonas mosselii* SAW1, *Pseudomonas corrugata* SAC1, *Pseudomonas moraviensis* SAw15, *Pseudomonas palleroniana* SAB17, *Pseudomonas palleroniana* SAB15, *Pseudomonas parafulva* SAB14, *Pseudomonas putida* SAB12, *Pseudomonas plecoglossicida* SAB8, *Pseudomonas putida* SAB10, *Pseudomonas putida* SAW3, *Pseudomonas corrugata* SAW23, *Pseudomonas anguilliseptica* SAW24, *Pseudomonas palleroniana* SAW21, *Pseudomonas putida* SAW22, *Pseudomonas putida* SAW19, *Pseudomonas plecoglossicida* SAW10, *Pseudomonas argentinensis* SAW9 and *Pseudomonas entomophila* SAW5.

Detection of *Pseudomonas* strains antimetabolites

1-Phenazine production

Phenazine compounds were extracted from KB plates with fully grown selected bacteria. The plates were washed twice with sterile water to remove bacterial colonies. Agar was excised in small pieces and pooled in Erlenmeyer flasks. Twelve milliliters of chloroform was added and the suspension was shaken at 37°C for 2 hr. The chloroform was air-dried and the extract was dissolved in 60 µl methanol. The 30 µl of methanol extracts was spotted on silica gel plates (Kieselgel 60 F254; Merck, VWR, Leuven, Belgium). The plates were developed in a solvent mixture of toluene/acetone (3:1 v/v) and visualized under UV light. *Pseudomonas fluorescens* CMR12a was included as positive control (Perneel *et al*., 2007).

2- Detection and Extraction Pyrrolnitrin and Pyoluteorin

Detection of Pyoluteorin (PLT) was made by thin-layer chromatography (TLC), bacterial strains were grown in 25 ml of KB broth in 50-ml screw cap vials for 14 days without shaking at 25 °C. Bacterial strains were grown in 25 ml of a minimal medium as described by De Souza and Raaijmakers (2003). Bacterial isolates were shaken at 180 rpm for 24 h at 25 °C and subsequently incubated at 25 °C in the dark for 4 additional days without shaking. Extraction of pyrrolnitrin and pyoluteorin from bacterial cultures was carried out as described by De Souza and Raaijmakers, (2003). To detect PRN by TLC, 15 ml of bacterial culture was centrifuged for 15 min at 8700 x *g*, supernatant was discarded and the cells were extracted twice with 5 ml of ethylacetate by sonicating the mixture for 3 min. For PLT, 15 ml of bacterial culture broth was extracted twice with 5 ml of ethylacetate. The organic phase was evaporated to dryness and the residue was resuspended in 150 µl ethylacetate. A volume of 50 µl was spotted on 0.25 mm silica gel plate (20 x 20 cm, aluminum oxide 60 F254). Separation was performed with chloroform: acetone (9:1 v/v) as the solvent system. The corresponding PRN spots were detected by spraying with 2% *p*-dimethyl amino benzaldehyde (Ehrlich's reagent, Sigma Aldrich Chemie B. V., Bornem, Belgium). Pyoluteorin spots were detected by spraying with an aqueous 0.5% fast blue RR salt solution (Sigma Aldrich Chemie B. V., Bornem, Belgium). The retardation factor (Rf) values were 0.80 for PRN and 0.26 for PLT, as determined by migration of pure PRN, and confirmed by identical color) crimson spot) and Rf values reported for PLT.

Screening of antimetabolites by polymerase chain reaction

Detection of the genes that encode for the production of antibiotics such as phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), pyrrolnitrin (PRN), and pyoluteorin (PLT) was done by PCR using gene-specific primers. The primer sets and the amplification conditions for the screening of gene encoding antibiotics are listed in Table (1). The PCR reaction mixture consisted of 1 µl template DNA, 0.25 µl Taq DNA polymerase, 10 µl 5x reaction buffer, 2.5 µl of each primer, 1µl dNTPs and add distilled water to a final volume of 50µl**.**

Table (1): Primer and amplification conditions for the different PCR based screenings of genes that encode for antibiotics

Primers	Primer sequence	Reference	Amplification conditions
PCA **PCA2a** **PCA3b**	5'-TTGCCAAGCCTCGCTCCAAC 3'; 5'-CCGCGTTGTTCCTCGTTCAT 3'	(Raaijmakers *et al*.,1997)	Initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 60 s, 58°C for 45 s, and 72°C for 60 s; final extension at 72°C for 10 min.
PCN **PhzH-up** **PhzH-low**	5'-CGCACGGATCCTTTCAGAATGTTC-3' 5'-GCCACGCCAAGCTTCACGCTCA-3'	(Mavrodi *et al*., 2001)	Initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 60 s, 67.2°C for 45 s, and 72°C for 60 s; final extension at 72°C for 10 min.
PRN **Prncf** **Prncr**	5'-CCACAAGCCCGGCCAGGAGC-3' 5'-GAGAAGAGCGGGTCGATGAAGCC-3'	(Mavrodi *et al*., 2001)	Initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min; final extension at 72°C for 10 min
PLT **PLTC1** **PLTC2**	5'-AACAGATCGCCCCGGTACAGAACG-3' 5'-AGGCCCGGACACTCAAGAAAACTCG-3'	(De Souza *et al*., 2003)	Initial denaturation at 95°C for 2 min; 30 cycles of 95°C for 2 min, 67°C for 1 min, and 72°C for 1 min; final extension at 72°C for 10 min

A comparative study of different carriers on fluorescent pseudomonads

The objective of this experiment was selecting the suitable carrier for the *P. putida* (SAW19) that gave the highest results for antagonistic activity against phytopathogenic fungi.

The experiment consisted of six materials as carrier including rock phosphate, perlite, bentonite, rock phosphate + perlite + bentonite, vermuclite and vermuclite + rock phosphate + perlite + bentonite.

Fifty grams of tested carriers were dried at 70°C for 3 h to material dryness and the carriers were sterilized in an autoclave at 121°C for one hour (Pesenti *et al*., 1991). Then, they were placed in oven at 70°C for 3 h to dry out the material. The pH of all of the materials was adjusted to 7.0 thoroughly mixing by $CaCO_3$ powder. Different combinations of these materials were prepared as described in Table (2) and packed in polyethylene bags.

Table (2): Different formulations of carrier materials and their field capacity

No.	Carrier material type	Added Bacteria suspension (ml) as water holding capacity to 50 g carrier material
1	Perlite	77
2	Bentonite	35
3	Rock phosphate	9
4	Vermiculite	62
5	Rock phosphate + Perlite + Bentonite	33
6	Vermiculite + Rock phosphate + Perlite + Bentonite	34

Inoculant preparation and incubation

Fluorescent pseudomonads suspension was prepared in 1 L nutrient broth culture. For this purpose, proliferation was performed using a rotary shaker incubated at 28°C at 100 rpm. Then, suspension population was set at $3x10^9$ CFU ml^{-1}. Due to the limited water holding capacity of applied carrier materials, new suspension cultures of bacteria according to the FC moisture content of carrier materials (Table 3) were added to each package and they were heat-sealed. Then, for uniform distribution of bacteria on each carrier, any package containing materials was well mixed and depending on the carrier material was kept for bacterial growth in the incubator at 28°C to the end of the 180 days (Shariati *et al*., 2013).

Bacterial population in the carriers treatments

Bacterial populations were counted at 0, 15, 30, 60, 90, 120, 150 and 180 days, with three replicates. For this purpose, 1 g of carriers was removed in sterile conditions; 10-fold serial dilution (reached to 10^{-10}) was prepared. After that resulting suspensions were cultured on KB agar medium. After one day incubation at 28°C, plates containing 3-300 positive control plates (Pure culture fluorescent pseudomonads).

Table (3): Antagonistic metabolite production among the antagonistic *Pseudomonas* strains

***Pseudomonas* strains**	**PCA**	**PCN**	**PLT**	**PRN**
***P. putida* SAB12**	-	-	+	-
***P. putida* SAB10**	-	+	-	-
***P. putida* SAW3**	+	+	-	-
***P. putida* SAW22**	-	-	+	-
***P. putida* SAW19**	+	+	-	-
***P. plecoglossicida* SAB1**	-	-	+	-
***P. plecoglossicida* SAB8**	+	-	-	-
***P. plecoglossicida* SAW10**	+	+	+	-
***P. palleroniana* SAB15**	-	+	-	-
***P.palleroniana* SAB17**	-	-	+	-
***P. palleroniana* SAW21**	-	+	+	-
***P. corrugata* SAC1**	-	-	-	-
***P. corrugata* SAW23**	-	-	+	-
***P. entomophila* SAW7**	-	+	+	-
***P. entomophila* SAW5**	-	+	-	-
***P. moraviensis* SAw15**	+	+	-	-
***P. parafulva* SAB14**	+	+	-	-
***P. mosselii* SAW1**	+	+	-	-
***P. anguilliseptica* SAW24**	+	-	-	+
***P.rgentinensis* SAW9**	-	+	+	-

+ present - absent

phenazine-1-carboxylic acid (PCA), pyrrolnitrin (PRN), phenazine-1-carboxamide (PCN) and pyoluteorin (PLT)

RESULTS AND DISCUSSION

Production of antifungal metabolites

Antifungal metabolites, phenazine-1-carboxylic acid (PCA), pyrrolnitrin (PRN), phenazine-1-carboxamide (PCN) and pyoluteorin (PLT), were extracted from the fermentation cultures of all tested strains. Thin layer chromatography (TLC) confirmed the production of PCA by the eight *pseudomonas* strains (*Pseudomonas mosselii* SAW1, *Pseudomonas moraviensis* SAW15, *Pseudomonas parafulva* SAB14, *Pseudomonas plecoglossicida* SAB8, *Pseudomonas putida* SAW3, *Pseudomonas anguilliseptica* SAW24, *Pseudomonas putida* SAW19 and *Pseudomonas plecoglossicida* SAW10), PCN was confirmed by twelve strains (*Pseudomonas entomophila* SAW7, *Pseudomonas mosselii* SAW1, *Pseudomonas moraviensis* SAW15, *Pseudomonas palleroniana* SAB15, *Pseudomonas parafulva* SAB14, *Pseudomonas putida* SAB10, *Pseudomonas putida* SAW3, *Pseudomonas palleroniana* SAW21, *Pseudomonas*

putida SAW19, *Pseudomonas plecoglossicida* SAW10, *Pseudomonas argentinensis* SAW9 and *Pseudomonas entomophila* SAW5), PLT was confirmed by nine strains (*Pseudomonas entomophila* SAW7, *Pseudomonas plecoglossicida* SAB1, *Pseudomonas palleroniana* SAB17, *Pseudomonas putida* SAB12, *Pseudomonas corrugata* SAW23, *Pseudomonas palleroniana* SAW21, *Pseudomonas putida* SAW22, *Pseudomonas plecoglossicida* SAW10 and *Pseudomonas argentinensis* SAW9), and PRN was confirmed by two strains (*Pseudomonas palleroniana* SAB15 and *Pseudomonas anguilliseptica* SAW24) as presented in Table (3). The retardation factor (Rf) values were 0.27, 0.53, 0.80 and 0.26 for PCN, PCA, PRN and PLT, respectively, as determined by co-migration with pure standards as shown in Fig. (1).

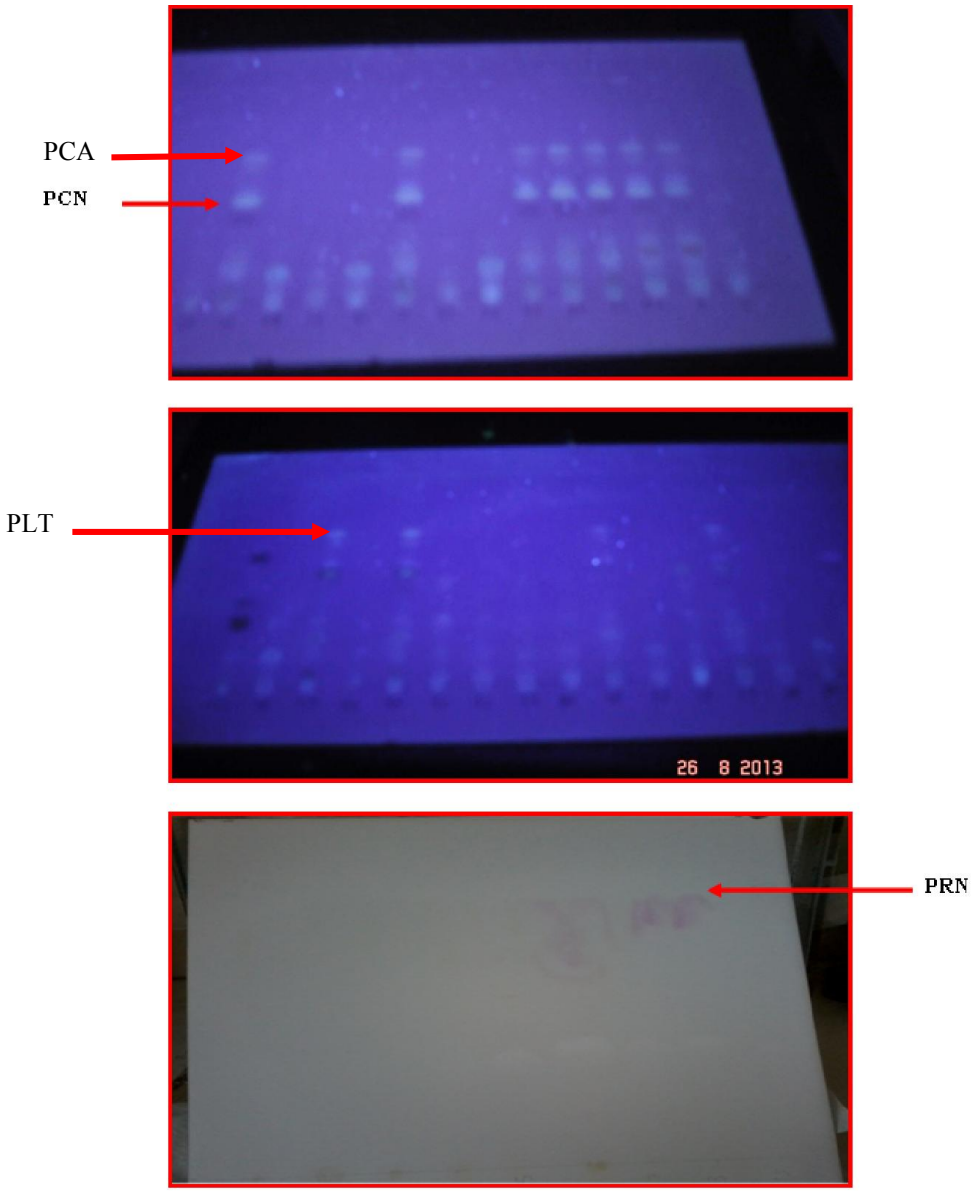

Fig. (1): Thin layer chromatography TLC analysis under ultra violet light of organic solvent extracts of King B agar cultures of *Pseudomonas* strains.
*phenazine-1-carboxylic acid (PCA), pyrrolnitrin (PRN), phenazine-1-carboxamide (PCN) and pyoluteorin (PLT)

Several strains of fluorescent pseudomonads such as *P. fluorescence* produce the broad-spectrum antibiotic of phenazine (Mark *et al*., 1995). Specifically, phenazine-1-carboxylic acid can be produced by fluorescent Pseudomonads such as *P. fluorescens* (Gurusiddaiah *et al*., 1986), *P. chlororaphis* (Pierson and Thomashow, 1992) and *P. aeuroginossa* (Anjaiah *et al*., 1998). Additionally, Production of phenazine-1-carboxamide had been reported in fluorescent pseudomonads such as *P. aeuroginossa* and *P. chlororaphis* (Chin-A-Woeng *et al*., 1998; Mavrodi *et al*., 2001; kumar *et al*., 2005). Pyrrolnitrin can be produced by several strains of *Pseudomonas* and *Burkholderia* (Ligon *et al.,* 2000). Pyoluteorin is produced by several *pseudomonas* sp., including strains that suppress plant diseases caused by phytopathogenic fungi (Bender *et al*., 1999).

Screening of antimetabolites by polymerase chain reaction (PCR)

Total DNA of antagonistic fluorescent pseudomonad strains were tested in PCR using gene-specific primers of PCA, PLT, PRN and PCN. PCA2a and PCA3b amplified the DNA fragment (1100-bp) corresponding to that of PCA which is a key gene in the biosynthesis of PCA in the tested strains and in the reference strain *Pseudomonas CMR5c*. PhzH-up and PhzH-low amplified the DNA fragment (2000-bp) corresponding to that of PhzH, which is a key gene in the biosynthesis of PCN in the tested strains and in the reference strain *Pseudomonas CMR12a*. Prncf and Prncr amplified the DNA fragment (786-bp) corresponding to that of PRN which is a key gene in the biosynthesis of PRN in the tested strains and in the reference strain *Pseudomonas CMR5c*. PLTC1 and PLTC2 amplified the DNA fragment (779-bp) corresponding to that of PLT which is a key gene in the biosynthesis of PLT in the test strains and in the reference strain *Pseudomonas CMR5c*. The presence of different antimetabolites of teted antagonistic *Pseudomonas* strains were observed being eight *Pseudomonas* strains showed the presence of PCA genes (Fig. 2), twelve strains amplified PCN (Fig. 3), two strains amplified PRN (Fig. 4), nine strains amplified PLT (Fig. 5) and one antagonistic strain did not show any amplification of PCA, PLT, PRN and PCN. Detection of genes of specific functions in antagonistic fluorescent pseudomonads by polymerase chain reaction (PCR) confirmed the results obtained by thin layer chromatography (TLC) as shown in Table (3).

It means that the tested *pseudomonas* strains had the ability to produce different antifungal metabolites (PCA, PCN, PLT, and PRN). The ability of these organisms to produce these compounds highly varied from one strain to another. *Pseudomonas plecoglossicida* SAW10 showed high efficiency to produce three antimetabolites PCA, PCN and PLT as compared with other tested *Pseudomonas* strains. Consequantly, one tested strain did not show any activity to produce any of the observed antimetabolites (*Pseudomonas corrugata* SAC1). These results are in accordance with many investigators. Gurusiddaiah *et al*. (1986) stated that fluorescent pseudomonas as *P. fluorescence* had the ability to produce phenazine-1-carboxylic acid (PCA). Pierson and Thomashow, (1992) and Anjaiah *et al*. (1998) confirmed that *P. chlororaphis* and *P. aeurogenosa* had the ability to produce PCA. Production of phenazine-1-carboxemide (PCN) had been reported in fluorescent pseudomonads such as *P. aeurogenosa* and *P. chlororaphis* (Chin-A-Woeng *et al*., 1998; Mavrodi *et al*., 2001 and Kumar *et al*., 2005). Pyoluteorin (PLT) and Pyrrolnitrin (PRN) are the broad spectrum antifungal metabolites produced by several *pseudomonas* species (Bender *et al*., 1999; Dwivedi and Johri, 2003).

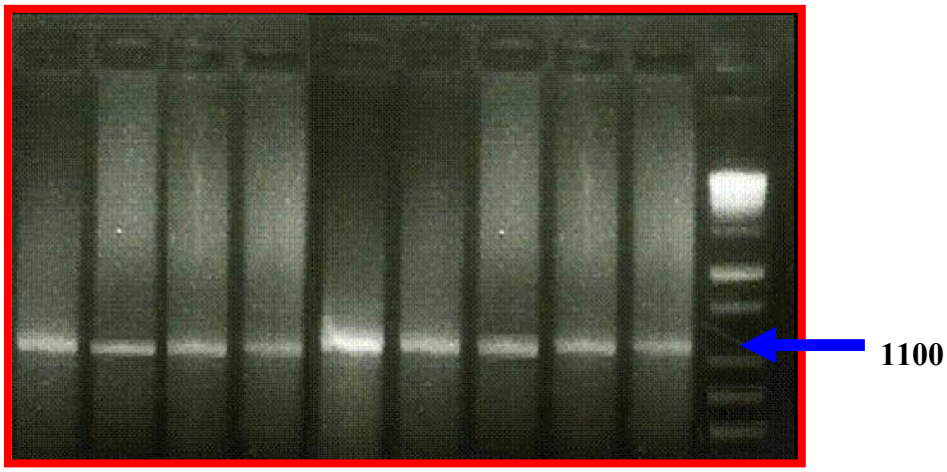

Fig. (2): Detection of phenazine-1-carboxylic acid (PCA) gene by PCR. Positive fluorescent pseudomonad strains showed the amplification of DNA fragment of PCA (1100 bp).

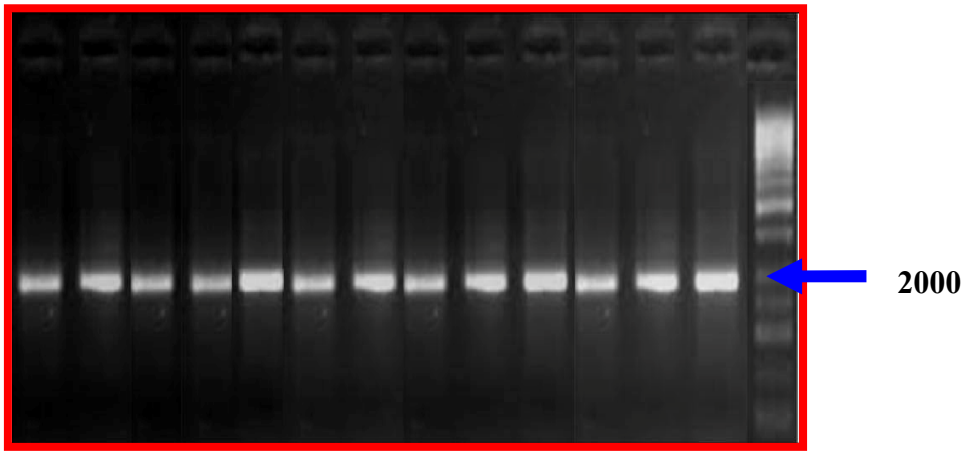

Fig. (3): Detection of phenazine-1-carboxamide (PCN) gene by PCR. Positive fluorescent pseudomonad strains showed the amplification of DNA fragment of PCN (2000 bp).

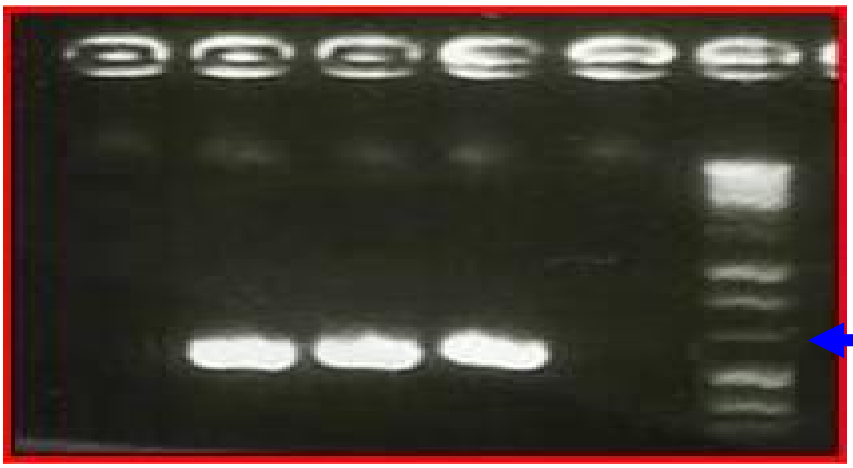

Fig. (4): Detection of pyrrolnitrin (PRN) gene by PCR. Positive fluorescent pseudomonad strains showed the amplification of DNA fragment of PRN (786 bp).

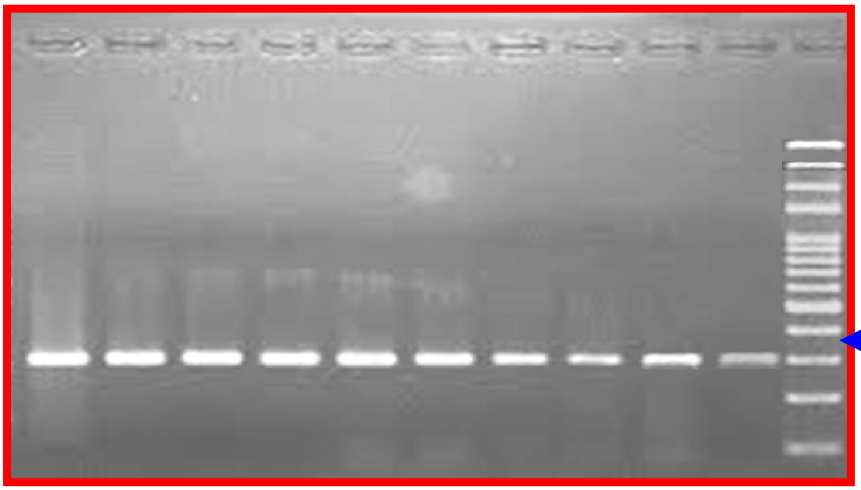

Fig. (5): Detection of pyoluteorin (PLT) gene by PCR. Positive fluorescent pseudomonad strains showed the amplification of DNA fragment of PLT (779 bp).

Survival of *P. putida* on different carriers

Psudomonas putida (SAW19) gave the highest antagonistic potential against tested phytopathogenic fungi by the same author (Mohamed *et al*., 2015). Therefore, the objective of this part was to evaluate the capacity and survival of *P. putida* strain for a long time on different carrier materials individually or mixtures during the incubation period (six months). Six different formulations were prepared using different carrier materials namely, rock phosphate (R), perlite (P), bentonite (B), mixture of R+P+B (1:1:1 *w/w*), vermiculite (V) and mixture of R+P+B+V (1:1:1:1 *w/w*). Survival of *P. putida* strain on different inoculant carriers in logarithmic scale of colony forming units (CFU) was shown in Fig. (6). The obtained results showed that the tested carriers have a different capacity to maintain an adequate survival of the *P. putida* strain. Also, results showed that the initial populations of *P. putida* in all tested carriers are greatly varied and the values ranged approximately from 7.2 to 9.0 $\log_{10}$ CFU g^{-1} and then gradually decreased over time. The count of viable *P. putida* cells after six months was conserved at the average of 5.3-8.0$\log_{10}$ CFU g^{-1} in all the tested carrier materials. Specifically, the populations of *P. putida* were sharply decreased in the inoculant carrier's rock phosphate, perlite and bentonite. However, numbers of *P. putida* slightly decline in the mixture of R+P+B carriers as compared to individually carriers (Fig. 6). The lack of organic matter in these materials leads to decline the populations, indicating that the limitation of nutrients and growth conditions for *P. putida*. Cigdem and Merih (2005) depicted that the drying process and production of toxins mainly deceased the bacterial population during incubation period. Based on the previous results, the current study concluded that perlite is not suitable substrate for survival *P. putida*. On the contrary, perlite is a suitable substrate for bacterial growth and was effective as peat in maintaining high populations of rhizobia and *B. megaterium* (Daza *et al*., 2000; Khavazi *et al*., 2007). Similarly, previous study has also shown that perlite was superior for maintaining survival of several rhizobia and plant growth promoting bacteria (Albareda *et al*., 2008). On the hand, bentonite is unsuitable substrate because it became pasty and sticky after water absorption and therefore does not produce proper ventilation conditions for aerobic bacterium *Pseudomonas fluorescens* (Khavazi and Rejali, 2000; Shariati *et al*., 2013). Due to lack of organic matter, low nutrient contents and water holding capacity of rock phosphate, it was not a good carrier for *P. putida*.

Results suggested that the populations of *P. putida* were markedly higher in vermiculate than in other carriers when incubated for six months. Similarly, the populations of *P. putida* were approximately consistent over time in mixture of carrier (R+P+B+V). This result mainly attributed to its ion-exchange capacity that provides a good-buffered zone for *P. putida* during incubation period. Nakkeeran *et al*. (2006) stated that vermiculite has a high water holding capacity that is very an essential characteristic of a good carrier. Other previous studies have also shown that vermiculite is a good carrier for several biological control agents including *Rhizobium* spp. (Weiss-Graham *et al*., 1987), *Azospirillum brasilense* (Elazar and Yaakov, 1996), *Pseudomonas fluorescens* (Loccoz-Moënne *et al*.,1999) and *Pichia anomala* (Melin *et al*., 2006).

CONCLUSION

The present study revealed the biodiversity among antagonistic fluorescent pseudomonads and identified different species such as *P. palleroniana, P. aeruginosa, P. putida, P. entomophila, P. moraviensis* and *P. plecoglossicida*. The current study demonstrates the ability of fluorescent pseudomonad isolates to produce important secondary metabolite such as phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyoluteorin and pyrrolnitrin that known to have antifungal properties. The antagonistic bacteria exhibited the production of one or more fungal cell wall degrading enzymes. Based on the above mentioned result, among the evaluated carriers, vermiculite was found to be the best carrier, acting as both a substrate and food source for *P. putida* (SWA19).

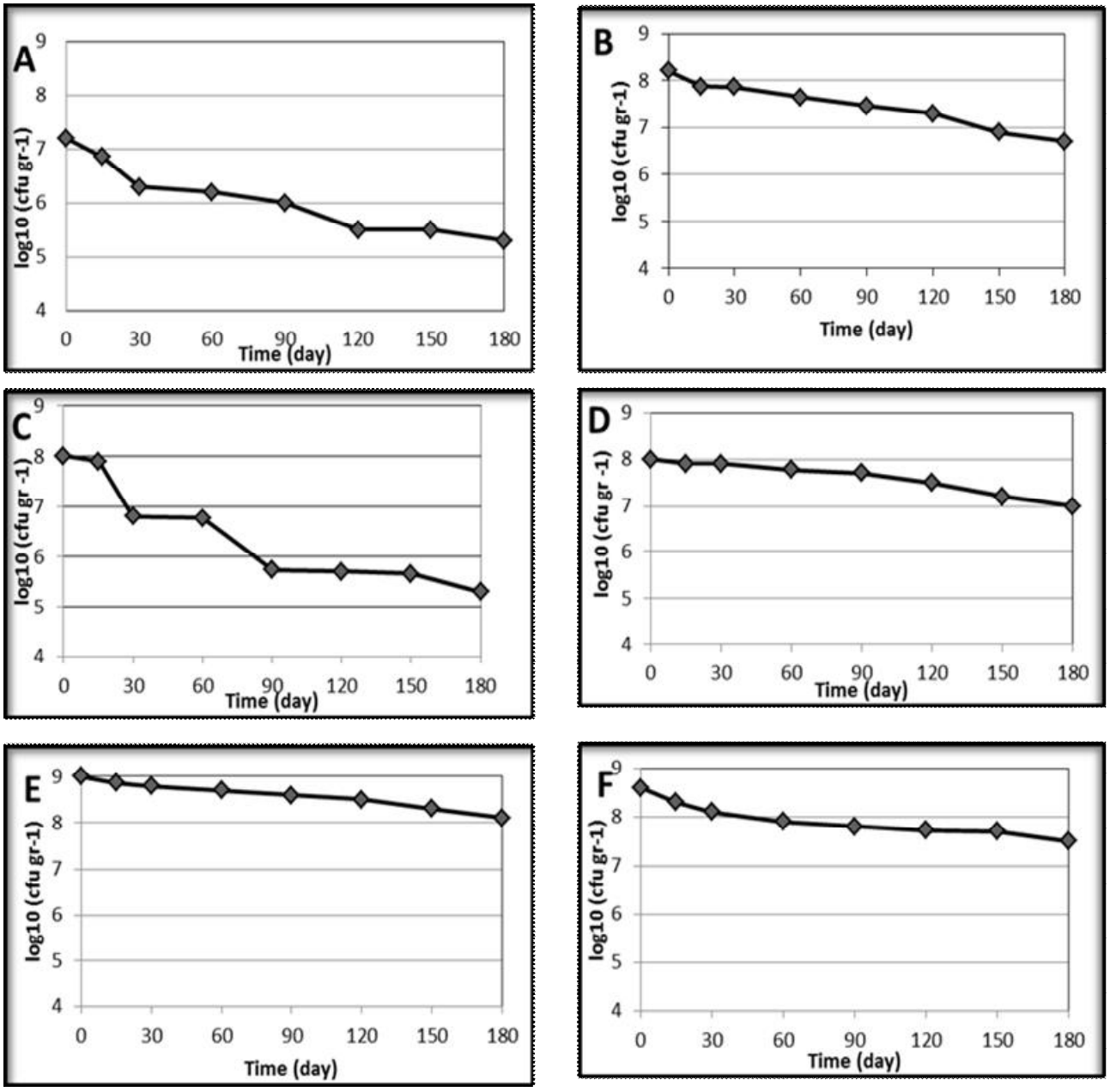

Fig. (6). Survival of *Pseudomonas putida* in different carrier materials at 28°C for six months, A(Rock phosphate), B (Perlite), C (Bentonite), D (Mixture from rock phosphate+ Perlite+ Bentonite), E (Vermiculite) and F (Mixture from rock phosphate+ vermiculite+ Perlite+ Bentonite).

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