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In vitro Control of *Fusarium oxysporum* ATCC-48112™ and *Aspergillus flavus* ATCC-9643™ by Wheat Rhizospheric *Pseudomonas fluorescens*

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There 70 bacterial strains were isolated from the rhizosphere zone of wheat. All of them subjected to screening to exhibit an antifungal activity against two main phytopathogenic fungi; *F. oxysporum* ATCC-48112™ and *A. flavus* ATCC-9643™. The screening test revealed that, 32 bacterial strains were found have an antifungal activity against both fungi mentioned before. There were 7 fluorescent strains observed under UV light. These fluorescent strains exhibited more antifungal activity than others. Nevertheless, one of them was the most potent antifungal fluorescent strain. Seven fluorescent strains were identified as *P. fluorescens*, which subjected to PCR to detect *PhlD* gene which responsible for 2,4-diacetylphloroglucinol (DAPG) production. *P. fluorescens* produced DNA fragments corresponding to approximately 745-bp size that corroborated with the predicted known *PhlD* sequence of Pf-5(NRRL B-23932) used as positive control in the current study. The antifungal compound was extracted from the suspension of *P. fluorescens* and then identified by using NMR and HPLC techniques. Eventually, there some physiological conditions were studied which impact on DAPG production such as oxygen percentage, temperature, sugars, amino acids.

Key words: *Aspergillus* species, *Fusarium oxysporum*, King's-B, *Pseudomonas fluorescens* and wheat rhizosphere.

There is a promising research area to control different phytopathogenic agents, which is the use of biocontrol approach mainly represented in plant growth promoting rhizobacteria (PGPR). These bacteria are capable of phytopathogens damage[1]. One of the most potent rhizobacteria using in this field is *P. fluorescens* which promote growth and at the same time suppress plant pathogens by multiple mechanisms. *P. fluorescens* particularly has been widely used as biocontrol agent due to it's highly production of secondary metabolites such as siderophores, antibiotics, volatile compounds, HCN, enzymes and phytohormones[2]. Many researches proved that *P. fluorescens* is considered one of the most potent biocontrol agents against various plant related diseases including root diseases[3]. *P. fluorescens* have the ability to highly synthesize broad-spectrum antibiotics[4,5]. For example, 2,4-diacetylphloroglucinol (DAPG), phenazine (Phz), pyrrolnitrin, oomycin A, viscosinamide, pyoluteorin and hydrogen cyanide (HCN) are considered more potential produced antibiotics by *P. fluorescens*. Fluorescent pseudomonads are recognized by their ability to produce an antifungal antibiotic called 2,4-diacetylphloroglucinol (DAPG), which plays an important role in the biocontrol of a considerable number of phytopathogens, including *Pythium*

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ultimum, *Gaeumannomyces graminis* var. *trici* and *Thielaviopsis basicola*[6,7,8,9]. Strains of *P. fluorescens* that produce the antibiotic DAPG are responsible for the natural suppression of take-all diseases of wheat[10,11,12]. DAPG has a broad spectrum of antagonism against bacteria, fungi, protists, and nematodes. DAPG targets may include basic cellular processes; in the fungus *Pythium ultimatum* var. *sporangiiferum*, DAPG causes alterations of the plasma membrane, vacuolization, and the disintegration of cell contents[13], suggesting that it impedes the maintenance of membrane integrity. This study aims to *in vitro* control the growth and subsequently the hazardness of *F. oxysporum* ATCC-48112™ and *A. flavus* ATCC-9643™ as phytopathogens by obtaining an antifungal compound such as 2,4-diacetylphloroglucinol (DAPG) from rhizospheric fluorescent pseudomonads strain.

MATERIALS AND METHODS

Test Microorganisms

There are two phytopathogenic fungi used in this study as test microorganisms for antifungal activity assay. These fungi are *F. oxysporum* ATCC-48112™ and *A. flavus* ATCC-9643™. They were obtained as well identified phytopathogenic fungi from American Type Culture Collection Lab.

Soil Sampling

The soil samples were collected from the upper 30 cm of the soil profile, stored in plastic bags on ice prior to use. The top of each plastic bag was closed with a rubber band to prevent drying of soil and then transferred to the laboratory for bacterial isolation.

Isolation of *Pseudomonas fluorescens* from wheat Rhizosphere soil

The rhizosphere soil samples were collected and processed after 22 hours. Loosely adhering soils were shaken and detached from the roots and was discarded. 4.5 gm of root portions by weight with a layer of closely adhering rhizosphere soil was then transferred to 45.5 ml sterilized distilled water and vigorously shaken for 10 minutes. The suspensions from all soil samples were serially diluted up to 10-7 with three replications for each sample. 100 µl of 10-1,10-3,10-5 and 10-7diluted samples were spread on *Pseudomonas* selective medium based on King's medium B (KMB)[14] that was made selective for isolation of *Pseudomonas fluorescens* by adding chloramphenicol (13µg/ml), cyclohexamide (100µg/ml) and Ampicillin (50µg/ml) in them[15]. Three replicate plates were incubated at 27°C for 48 hours. After 48 hours of incubation, all the isolates were checked for fluorescence under UV light[16] and representative types of colonies were selected and further purified on KMB agar medium. Pure isolates were preserved at -80°C after an addition of glycerol to a final concentration of 40 % (v/v).

Morphological and biochemical characterization of isolates

Morphological features including colony type, bacterial shape and growth characteristics of the isolates were determined using King's B agar medium. Catalase test, fluorescent pigment on KB media and siderophore detection were obtained according to previously reported manual[17,18,19]. HI Assorted Biochemical kit (HI Media, Mumbai) containing sterile media for Citrate, ornithine, lysine decarboxylase, urease', phenylalanine deaminase, Nitrate reduction, H_2S production test and 5 different carbohydrates for fermentation test-Glucose, Adonitol, Lactose, Arabinose, and Sorbitol was used for further screening of isolates.

Preparation of inoculum

Single well isolated colonies were picked up with sterile loop and inoculated in 5 ml nutrient broth and incubated at 37°C for 4-6 hours until the inoculums turbidity reached 0.1 OD at 620 nm. The biochemical test kit was opened and used as per vendor's instructions. Each well was inoculated with 50 µl of the overnight grown cultures by surface inoculation method and kept for incubation at 35-37°C for 18-24 hours. At the end of the incubation period, a series of reagents were added to carry out biochemical tests.

In vitro fungal inhibition assay

Seven to ten days old cultures of *F. oxysporum* ATCC-48112™ and *A. flavus* ATCC-9643™ were used in the experiment. Fungal bioassay was performed by using the paper disc method[20]. Fungal suspension was spread over the PDA plates and an overnight grown *P. fluorescens* used as a source of antifungal agent. A fresh colony of *P. fluorescens* isolated from rhizosphere soil was

inoculated in nutrient broth and incubated at 27°C for 24 hrs. Paper discs were soaked in 5 ml of this bacterial culture for 30 seconds and later placed on agar plates. The discs were dried between each application and were applied on agar plates within 15 mints after fungus inoculation, and plates incubated at 27°C for 3-7 days. At the end of incubation period, the plates were checked for clear zones of inhibition formed around the discs. The experiment was done in triplicate for each antifungal *P. fluorescens* isolates.

Isolation of crude antibiotic compound

P. fluorescens was inoculated on SA semisolid agar (0.35 %) plates and incubated at 28°C for 8 days. The total contents of 12 plates were placed in a 1-liter Duran flask (Schott: Mainz, Germany), to which was added 250 ml of 80 % aqueous acetone. The flasks were shaken on a gyratory shaker (model GIO; New Brunswick Scientific Co., Inc., Edison, N.J.) at 200 rpm for 24 h at room temperature, and the contents were then centrifuged at 15,000 rpm on a Beckman JA-10 rotor for 20 min at 10°C. The supernatant was decanted and condensed by removal of the acetone in vacuum at 45°C. After the aqueous concentrate was filtered under vacuum through a filter (pore size, 0.45 lxm), 20-ml portions were extracted twice with 2.5 volumes of diethyl ether. The ether extracts were taken to dryness in vacuum, and the residue was extracted with 30 ml of acetone, to yield a white cloudy suspension. Methanol was added drop wise until a white solid precipitated. The liquid was filtered through a 0.45- > m-pore-size filters and condensed in vacuum to approximately 2 ml.

Purification

Two milliliters of the crude isolate was streaked on thin-layer plates coated with silica gel G.F. (Sigma Chemicals Ltd.) and developed in dichloromethanehexane-methanol (50:40:10, v/v). The plates were observed under long and shortwave UV light for fluorescing and absorbing bands. All bands and blank areas were removed separately from the plates and eluted with acetone (50 ml). After the detection of active bands by using the fungal inhibition assay, the acetone elute was taken to dryness in vacuum and taken up in hot hexane (10 ml), and the antibiotic was precipitated from it by slow cooling.

Sample pretreatment method for HPLC assay

Because of the inherently complex nature of the sample, which included medium constituents and a range of secondary metabolites, a sample pretreatment method with solid-phase extraction was developed. Advantage was taken of the retention behavior of the antibiotic on octadecylsilica. The antibiotic was retained on a Sep-Pak C_{18} cartridge when applied in water as the solvent. The cartridge was conditioned by flushing with 10 ml of methanol and then 10 ml of H_2O, and this procedure was repeated three times. To obtain a supernatant from solid agar plates, the agar was first pulverized and then centrifuged at 10,000 rpm for 15 min, and the supernatant was decanted. Then 5 ml of filter-sterilized culture supernatant was injected onto the cartridge, which was washed with 30 ml of H_2O and then with 20 ml of methanol to elute the antibiotic. The methanol elute was taken to dryness in vacuum, the residual material was dissolved in 5 ml of the mobile phase, and 20ul aliquots were injected into the HPLC system. To evaluate the effect of soil on the detection of DAPG via the HPLC assay, purified DAPG (in ether) was mixed into the soil at concentrations of 100, 50, and 25 Vig/g of soil. The soil used (pH 6.9) was taken from the upper 5 cm of the soil profile, sieved through a 0.5 cm-mesh screen, and air dried before use. The ether was removed in vacuum at 35°C, giving a uniform dispersion of DAPG throughout the soil. To measure the amount of recoverable DAPG, the soil was washed repeatedly in diethyl ether. The washings were pooled and dried under vacuum at 30°C, and the residue was reconstituted in the mobile phase and injected into the HPLC system.

PCR amplification of PhlD Gene

PCR amplification of *PhlD* gene was performed using self designed SGF (5'-CCAAGGGCATAGCTCATCAT) forward and SGR (3'-GCTCAAGGAGCAATCGTTTC) reverse primer. PCR amplification was carried out in 25µl reaction mixtures containing 2µl DNA, 1 µl of each forward and reverse primers, 2.5 µl of 1x Taq polymerase buffer with 1.5Mm of $MgCl_2$, 2µl of 25 mM dNTP mix (Fermentas, USA), 1 U of Taq DNA polymerase (Fermentas, USA) and rest 14 µl of sterile water with following cycling conditions: Initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec with a final extension of 72°C for 10 min. Amplification was carried out in Techne cycler (TC-

2295, UK). The amplification products were electrophorised on 1 % (w/v) agarose gel with 0.5x TBE buffer. Before loading, reaction mixtures (8 µl) were properly mixed with 2 µl of gel loading dye and loaded onto the wells of the gel along with DNA ladder. After electrophoresis, the gel stained with ethidium bromide (1.0µg/ml) was visualized using a UV Trans-illuminator and photographed using Gel Documentation system.

Effect of different growth conditions on DAPG production

The amount of surface contact in the growth container was increased by adding different amounts of granite chips 1 to 2 mm in diameter. These were pre-washed twice in alcohol and water and autoclaved before they were added to each of 15 50-ml Erlenmeyer flasks containing 15 ml of SA broth. The effect of agar concentration on DAPG was studied by using SA incorporating different agar concentrations. The agar preparations were sterilized by autoclaving, and 20-ml volumes were poured into sterile Petri dishes. In all cases, the inoculum used was 4 % of an overnight culture that was spread plated for solid media and incubated stationary at 28°C for 8 days. To test the effect of temperature on DAPG production, four 100-ml Erlenmeyer flasks each containing 5 ml of SA broth were inoculated with *P. fluorescens* and incubated with shaking at 28°C for 18 h. The flasks were removed and incubated stationary at the desired temperatures. To investigate the effect of oxygen on DAPG production, 71-ml serum bottles with 2 ml of SA broth were used. For growing cells, each bottle was inoculated with ~10^3 CFU/ml, whereas for stationary-phase cells an inoculum of ~10^9 CFU/ml was added. In each case, the gas phase in the assay flasks was composed of the desired percentage of oxygen and the remainder was made up with argon. To check the carbon source, pH, and iron concentration most suited to antibiotic production, 80 mM solutions of a range of carbon sources were prepared and buffered with inorganic salts K_2HPO_4, KH_2PO_4, and NH_4Cl (0.004, 0.0022, and 0.0049 M, respectively). $MgSO_4$. $7H_2O$ (0.05 %) was added after autoclaving. For pH and Fe analysis, sucrose was the carbon source of choice. The pH was adjusted with 2N HCl and 2N NaOH across the pH range 2 through 10. Iron-rich conditions were obtained by adding 10, 50, 100, and 200 uM $FeCl_3$, and iron-poor conditions were obtained by adding 0, 10, and 100 uM ethylenediamine-di-O-hydroxyphenyl acetic acid. In all cases, approximately 10^9 CFU were added per ml of test solution. The cells were pre-grown to the stationary phase in SA medium, washed three times in Ringer solution (Oxoid Chemicals), and incubated stationary at 12°C. All samples analyzed for antibiotic production were pretreated as outlined above for the solid-phase extraction and HPLC assay procedures, and the antibiotic production profiles were recorded after 4 and 8 days.

RESULTS AND DISCUSSION

Isolation of *Pseudomonas fluorescens*

In the present study, from 70 bacterial isolates only 7 were able to be fluorescent under UV light. At biochemical level all of them showed Catalase and Oxidase activity and also produced yellowish green fluorescens pigment on King's B medium peculiar to *P. fluorescens*. Results were further authenticated by HI Assorted Biochemical Kit (Table 1). Fluorescent pseudomonads, normally present in soil are effective colonizers of the rhizosphere of many crop plants possessing potential to inhibit growth of number of phytopathogenic fungi[21]. Such type of bacteria have been studied mainly because of their widespread distribution in soil, their ability to colonize the rhizosphere of host plants and ability to produce a wide range of compounds inhibitory to a number of serious plant pathogens[22,23,24,25]. They aggressively colonize rhizosphere of various crop plants, and have a broad spectrum antagonistic activity against plant pathogens[26,27,28].

***In vitro* antifungal assay**

It was noteworthy that among the 7 *P. fluorescens* isolates, only one isolate was found to inhibit the growth of *F. oxysporum* ATCC-48112™ and *A. flavus* ATCC-9643™ (Fig. 1 a & b). These results gave us a clue that this isolate posing antifungal activity may be involved in the production of DAPG antifungal metabolite (antibiotic) and could be better exploited as biocontrol agents. The production of DAPG by *P. fluorescens* has been detected by using bioassays[29,30]. However, although these assays indicate the presence of an inhibitory substance, they do not specify the nature of the compound or the amount produced.

PCR-based screening method to detect DAPG production

In order to check the mode of action, *P. fluorescens* isolate posing antifungal activity was subjected to PCR with self designed SGF and SGR forward and reverse primers respectively. This isolate able to produce DNA fragments corresponding to approximately 745-bp size that corroborated with the predicted known *PhlD* sequence of Pf-5(NRRL B-23932) used as positive control in the current study (Fig. 2). The 745-bp internal fragment from the *phlD* gene of *P. fluorescens* has been extensively used to enumerate producers of 2,4-DAPG among fluorescent pseudomonads from the rhizosphere of wheat[31,32] and maize[33].

Antibiotic identification

The mass spectrum of the isolated compound was matched to that of ethanone 1,1-(2,4,6,trihydroxy-1,3-phenylene), the trivial name of which is DAPG. This, in conjunction with ^{1}H and ^{13}C nuclear magnetic resonance, infrared spectra, and melting point and elemental analyses (data not shown), confirmed the structure indicated by the mass spectrum data.

Confirmation that the antibiotic activity of Pseudomonas fluorescens is due to DAPG

The crude extract of *P. fluorescens* was subjected to thin-layer chromatography and HPLC. To develop a rapid method for detection of DAPG production in vivo, a simple HPLC-based method was developed. This assay shows the presence of DAPG both qualitatively and quantitatively in culture supernatants. Phloroglucinols have previously been investigated chromatographically by using gas-liquid chromatography[34] and HPLC[35]. However, in this study, we modified the HPLC procedure for the detection of a particular phloroglucinol (DAPG), incorporating a sample pretreatment step that eliminates non-C_{18}-retained material.

Physiological parameters influencing production of DAPG

The effect of a number of parameters on the production of DAPG by *P. fluorescens* was studied. *P. fluorescens* grown in 1-liter Duran flasks containing large volumes of SA broth (> 600 ml) produced negligible quantities of antibiotic. However, when was grown in volumes of SA broth such that the liquid depth was less than 1 cm, the amount of antibiotic produced was greatly increased (> 500 %). Similar quantities of DAPG were also recorded from *P. fluorescens* grown on semisolid agar. Monitoring antibiotic production by *P. fluorescens* on both semisolid SA agar and in small volumes of SA broth showed that maximum antibiotic production occurred between 4 and 8 days, with negligible increases thereafter. The above observation prompted us to investigate the role of oxygen availability on DAPG production. Lower percentages of oxygen supplied to the growing cells resulted in a significant decrease in the number of *P. fluorescens* cells present and thus in the amount of antibiotic detected. However,

Table 1. Biochemical characteristics of *Pseudomonas fluorescens* isolates according to HI Assorted Biochemical Kit

Well	Test	*P. fluorescens*	*P. fluorescens* strains isolated from soil samples						
		ATCC-13525	1	2	3	4	5	6	7
1	Citrate utilization	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2	Lysine decarboxylase	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
3	Ornithine decarboxylase	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
4	Urease	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
5	Phenylalanine deamination	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
6	Nitrate reduction	v	+ve	-ve	+ve	-ve	-ve	-ve	-ve
7	H2S production	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8	Glucose fermentation	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
9	Adonitol	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10	Lactose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
11	Arabinose	v	-ve	-ve	-ve	-ve	-ve	-ve	-ve
12	Sorbitol	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

+ve = Positive (> 90 %), -ve = Negative (no reaction), V = 11-89 % Positive.

when DAPG concentration was correlated with the number of CFU in the cell suspension, no significant variation was observed with different oxygen levels supplied in the gas phase (Table 2). Similarly, when resting cell suspensions pre-grown at normal aerobic conditions were exposed to various oxygen levels for 8 days, no apparent differences in DAPG production were observed (Table 2). However, when no oxygen was added to the gas phase, a significant reduction in DAPG production occurred. It is also possible that the ratio of the culture volume to the total surface area in a growth container affects the production of DAPG by *P. fluorescens*. This was investigated by adding increasing amounts of agar to a broth culture, thus increasing the viscosity, and monitoring the effect on antibiotic production. When the agar concentration was increased from 0 to 1.5 %, the amount of antibiotic produced per 10^5 CFU increased from 0.46 to 5.21 ppb. However, further increases in agar concentration did not significantly affect DAPG concentration. In addition, increasing the available surface area by sequentially adding sterilized 1 to 2 mm granite chips to a broth culture resulted in a corresponding increase in DAPG production. This effect did not increase indefinitely, suggesting that there is a minimum amount of surface contact required for optimum DAPG production. To test the effect of temperature on DAPG production by *P. fluorescens*, pre-grown cells were incubated at temperatures ranging from 4 to 37°C. Optimum DAPG production occurred at ~12°C. Negligible DAPG production occurred at 37°C, even though all cells remained viable. Other parameters in soil that are known to affect microbial ever, adding iron concentrations in the range 0 to 200 uM $FeCl_3$ did not effect DAPG production by pre-grown cells of *P. fluorescens* under the assay conditions used. Similarly, when pre-grown cells were resuspended in assay solutions ranging in pH from 2 to 10, there was no change in DAPG produced per viable CFU. It was noted that at both extremes of pH no viable cells

Table 2. Effect of oxygen on antibiotic production by *Pseudomonas fluorescens*[a]

O_2 %	DAPG µg/L/10^5 CFU	
	Growing cells[b]	Resulting cells[c]
0	ND	0.03
2	3.61	3.84
3	3.90	4.30
4	4.02	3.84
5	4.13	4.69
6	3.46	5.32
8	3.95	4.36
10	4.21	4.10
15	3.72	4.23
20	4.02	4.16

[a] = the values shown were recorded after 8 days. ND = not detected. [b] = cells were grown under the stated oxygen levels with an initial inoculum of 10^3 CFU/ml. [c] = resting cells (10^9 CFU/ml) pre-grown under the atmospheric oxygen conditions used in each case

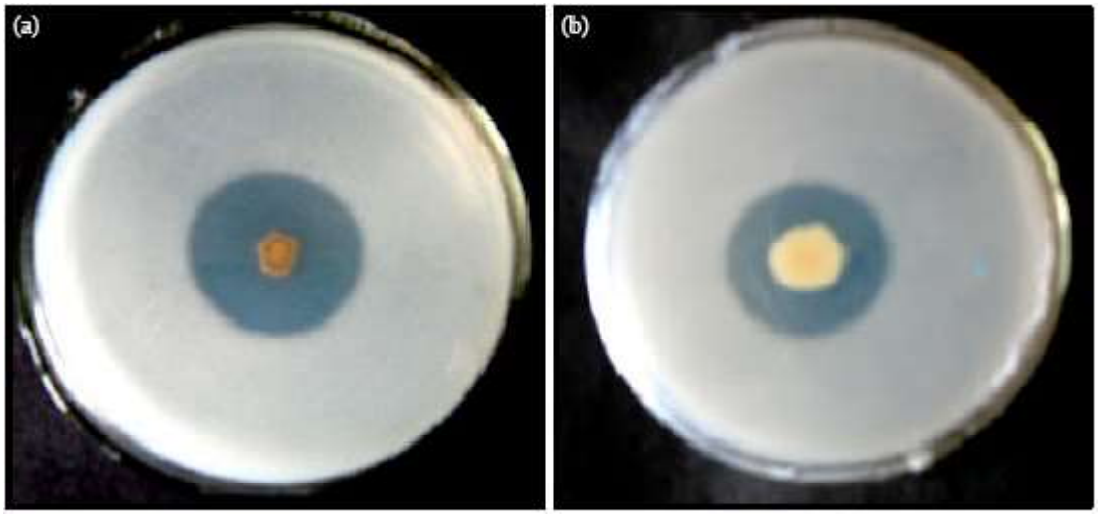

Fig. 1. The antifungal activity of Pseudomonas fluorescens against (a) *F. oxysporum* ATCC-48112™ and (b) *A. flavus* ATCC-9643™

remained after 4 days. Different carbon sources influenced the production of DAPG by *P. fluorescens*. When pre-grown cells were incubated in the presence of sugars and amino acids, fructose, sucrose, and mannitol promoted high yields of DAPG, whereas no DAPG production was observed when cells were incubated in glucose and asparagine, even though all cells remained viable. This indicates that the type of carbon source greatly influences the production of DAPG by *P. fluorescens*. The amount of DAPG produced was affected by different growth conditions employed. However, other conditions examined, such as pH and iron concentration, had no effect on its production under the assay conditions used. The optimum temperature for DAPG production was ~12°C, indicating that soil temperature is conducive to maximum antibiotic production. This differs from the results from the strain studied by Bolton and Elliot[36], who found that toxin production from soil pseudomonads, was not strongly influenced by temperature. *P. fluorescens* produced small quantities of DAPG unless the ratio of surface area to volume was increased. One of the experiments supporting this conclusion involved the inclusion of granite chips in the growth container. In this attempt to simulate natural soil conditions, one cannot eliminate the possibility that certain minerals leached from the chips influenced DAPG production. However, the experiment further suggests that *P. fluorescens* may be required to attach to a solid surface for optimum DAPG production. It is also possible that the bacteria need to form microcolonies to produce significant quantities of DAPG. However, additional work is required to evaluate this aspect. All physical parameters examined (temperature, oxygen, and surface contact) indicate that the physical parameters of soil should allow maximum production of DAPG by *P. fluorescens*. The parameters conducive to antibiotic production in soil have been reviewed[37,38], and the lack of specific nutrients is recognized as a major factor in limiting antibiotic production. Soil treated with various nutrient sources, e.g., fragments of straw, support antibiotic production[39]. However, microorganisms often display a preference for specific carbon sources for the production of particular secondary metabolites[40,41]. In this study, DAPG production by *P. fluorescens* was promoted by some sugars, whereas others were ineffective for this purpose. These results suggest that optimum DAPG production by *P. fluorescens* in soil should be influenced by the availability of sucrose, fructose, or mannitol. In conclusion, antibiotic production by fluorescent Pseudomonads spp. is now recognized as an important feature in plant disease suppression by some strains[42,43]. However, due to the scarcity of nutrients in most soils, antibiotic production is generally restricted. In this study we have demonstrated the selective enhancement of an antibiotic from a fluorescent pseudomonad by certain carbon sources. These results strongly suggest that prior knowledge of plant exudates composition and the ability of different carbon sources to induce a particular antibiotic are essential in selecting suitable bacterium-plant combinations for biocontrol purposes.

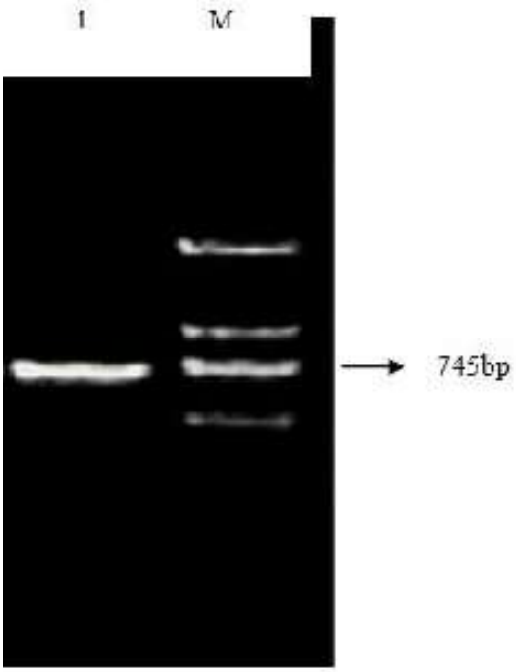

Fig. 2. Photograph showing amplified product of *PhlD* gene

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