Int.J.Curr.Microbiol.App.Sci (2019) *8*(3): 822-835

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 03 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.803.099

Isolation, Cloning and Sequencing of phlACBD Gene Cluster Encoding Antibiotic and Phloroglucinol Derivative 2, 4-diacetylphloroglucinol (2, 4-DAPG) from *Pseudomonas fluorescens*

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Keywords

Pseudomonas fluorescens, Antagonistic, 2, 4-diacetylphloroglucinol, Cloning, Rhizosphere

Article Info

Accepted:
07 February 2019
Available Online:
10 March 2019

A B S T R A C T

Pseudomonas represents as one of the most abundant class of bacterial genus in many antagonistic root-associated communities. *Pseudomonas* spp. residing in the rhizosphere has the ability to produce antimicrobial metabolites including phloroglucinol derivatives 2,4-DAPG, which is a major class of secondary metabolite protecting plants against different types of phytopathogens. The present study was conducted to isolate 2,4-DAPG producing *Pseudomonas fluorescens* isolates from Rhizosphere soil samples of different crops. Among a total of 158 isolates screened for the production of 2,4-DAPG through PCR based approach, seven isolates were found to be positive for DAPG. Further, the antibacterial activities of these *Pseudomonas* isolates were evaluated *in vitro* against *Xanthomonas axonopodis pv punicae* and it was found that the isolate AFPF19 exhibited highest antagonistic activity against phytopathogen. AFPF19 isolate was used to clone DAPG synthesizing gene cluster phlACBD into cloning vector pTZ257R/T and confirmed by sequencing.

Introduction

Pseudomonas fluorescens is a common, non pathogenic, gram-negative and rod-shaped bacterium that colonizes primarily in soil, plant and water. *Pseudomonas fluorescens* belongs to group of well characterized plant growth promoting rhizobacteria (PGPR) which protect plant from various pathogens and plays a major role in the plant growth promotion, induced systemic resistance and also biological control of phytopathogen. *Pseudomonades* are known to produce wide spectrum of metabolites including antibiotics, siderophores and other volatiles. Antibiosis is one of the key bio control mechanism which is used by *Pseudomonades*. The antagonistic fluorescent *Pseudomonas* is well characterized for their ability to produce different anti microbial metabolites of interest for controlling plant pathogens. Phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn), pyoluteorin (Plt) (Weller,

2007), hydrogen cyanide and surfactants (Couillerot *et al.,* 2009) are most common antibiotics produced by *Pseudomonas* bio control agents.

Among the different antimicrobial metabolites produced by *Pseudomonas fluorescens*, DAPG is a form of phloroglucinol derivative with a broad spectrum of antiviral, antibacterial, antifungal and antihelminthic properties (Bangera and Thomashow, 1999) and it acts as a major determinant in the bio control of phytopathogens. Strains of *P. fluorescens* that produce DAPG also play a key role in natural suppression of take all disease of wheat (Velusamy *et al.,* 2006). The gene cluster responsible for the biosynthesis of DAPG is organized on 6.5 Kb DNA fragment in *P. fluorescence* Q2-87 (Yang and Cao, 2011). Later, with the advancement in nucleotide sequencing techniques genes involved in biosynthesis, regulation, export and degradation of DAPG were identified and it has been predicted that *phI* gene cluster contains eight ORFs within it (Moynihan *et al.,* 2009). The biosynthetic locus of DAPG includes phIACBD and from the different expression studies, it has been identified that product of all the four genes *phIA*, *phIB*, *phIC* and *phID* are necessary for the production of DAPG precursor monoacetylphloroglucinol (MAPG) and for conversion of MAPG into 2, 4-DAPG (Gupta *et al.*, 2015).

Pomegranate is one of the economically important fruit crop of India belongs to the family Punicaceae. Since from ancient time, this fruit is well known for its high nutritional with therapeutic value and believed to have originated from Iran but extensively cultivated in Mediterranean region especially in Spain, Morocco, Egypt and Afghanistan. It is also grown in drier parts of Southeast Asia, Burma, China, Japan, USA, West Indies (Priya *et al.*, 2016). India is one of the leading countries in pomegranate production and presently more than 1.32 lakh ha area is under cultivation. Out of this, nearly 94,000 ha area is covered in Maharashtra, which produces fruits of over one lakh mt worth about 400 cores. Karnataka is the second largest pomegranate producing state accounting for 19.2 per cent of total production of pomegranate in the country. The state is producing about 1, 98,600 mt of pomegranate from an area of 18400 ha. The productivity of pomegranate in the state is 10.75 t/ha. The major producing belts are Chitradurga, Vijayapur, Tumkur, Dharwad and Bagalkot (Sahana, R. T, 2016).

The pomegranate crop is prone to various fungal and bacterial diseases and among which the bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae*. is one of the major constraint in pomegranate growing areas which adversely affects both yield and quality of fruit. This disease was first reported in Karnataka from Bangalore during 1959 with minor economic importance and later this disease turned into epidemic form which brings down the production of pomegranate up to 60-80% in India (Mondal and Mani, 2009). The Phytopathogen can infect and damage plant with irrespective of its growth stage. Many attempts have been made to control this disease either by mechanical or chemical methods, but complete control has not been achieved yet. So, one of the alternative approaches to control this disease is usage of bio control agents and also there are reports on effect of bio control agents *viz*., *Bacillus subtillis* and *Pseudomonas fluorescens* against *Xanthomonas axonopodis* pv. *punicae* (Poovarasan *et al.*, 2013).

In view of 2, 4-DAPG role in biological control, the present study was conducted to isolate and identify efficient 2, 4-DAPG producing *Pseudomonas fluorescens* from

rhizosphere soil collected from different locations of Karnataka. PCR based approach was used to identify *Pseudomonas fluorescens* isolates and to explore efficient isolate, antagonistic activity of these isolates were screened against pomegranate bacterial blight causing pathogen *X. axonopodis* pv. *punicae*. The genes encode for production of 2, 4-DAPG was cloned and Sequenced.

Materials and Methods

The present study was carried out to isolate efficient isolate of *Pseudomonas fluorescens*, cloning and sequencing of 2,4-DAPG synthesis genes(phlACBD). The materials used and methods employed are as follows.

Sample collection and isolation of *Pseudomonas fluorescens*

Rhizosphere soil samples of crops including rice, coffee, finger millet, red gram, green gram, potato and also soil samples from forest herbs from different locations of Karnataka were collected. Serial dilution and spread plate methods were used for isolation of rhizosphere bacteria. The serially diluted suspensions of rhizosphere soil samples were plated onto King's B agar medium (King *et al.,* 1954) and plates were incubated at 28 °C for 48 hours. Single colonies exhibiting Greenish yellow fluorescens under UV-light (365nm) were picked and further purified on fresh King's B agar medium. The purified fluorescent *Pseudomonas* isolates were stored in 50% glycerol at -80 °C.

Genomic DNA isolation and molecular identification of *Pseudomonas fluorescence* isolates through PCR

The fluorescent *Pseudomonas* isolates were inoculated into king's B broth and kept in shaking incubator for overnight at 120 rpm. The genomic DNA was isolated from overnight grown cultures by using HiMedia Kit following instructions provided in user guide. The DNA samples were quantified by using nano drop spectrophotometer. PCR amplification of *cumin deoxygenase* (*cds*) gene was performed using forward cds F:TTGAGCCCCGTTACATCTTC and reverse cds R:GGGGAACCCACCTAGGA TAA, which were developed from the *cumene dioxygenase* gene sequence in the GenBank accession no. D37828.1 (Mohammed. A.H. 2015). The PCR amplification was carried out in 20 µl reaction mixtures that consisting 50 ng of DNA, 1X PCR buffer, 10 mM of dNTPs mixture, 10 pmol of each primer and 1 unit of Taq DNA polymerase.

The PCR program included initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 45s, 56°C for 45s, 72°C for 60s, and then a final extension at 72 °C for 10 min. the PCR amplified products were separated on 1% agarose gel stained with EtBr and bands were visualized using gel documentation system.

Screening for 2, 4-DAPG production by PCR amplification of *phID* gene

The PCR amplification of *phID* gene was performed using forward primer phlD (5'-GAG GAC GTC GAA GAC CAC CA-3') and reverse primer phlD (5'-ACC GCA GCA TCG TGTATG AG-3'), which were developed from the *phlD* sequence of *Pseudomonas fluorescens* Q2-87 (Raaijmakers *et al.,* 1997). PCR amplification was carried out in 20 µl reaction mixtures that containing 50 ng of DNA, 1X PCR buffer, 10 mM of dNTPs mixture, 10 pmol of each primer and 1 unit of *Taq* DNA polymerase. PCR cycling program was used as described by Wang *et al.,* (2001), with minor modification in annealing temperature. Amplification was performed using the following PCR conditions: initial denaturation

at 94°C for 5 min followed by 35 cycles of 94°C for 60s, 60°C for 60s, 72°C for 90s, and then a final extension at 72 °C for 10 min.

Collection and isolation of pomegranate bacterial blight pathogen *Xanthomonas axonopodis* pv. *punicae*

The diseased pomegranate leaves and fruits were collected from pomegranate cultivating areas of Karnataka. The infected parts of leaves and fruits were separated and further sterilized by treating with 70% ethanol for 10 minutes followed by 3-4 times sterile water wash in a laminar hood. The sterilized infected leaf and fruits were squeezed to release pathogenic extracts. The extracts were cultured on nutrient agar plates containing glucose and incubated at 28 °C for 72 hours. After incubation single colonies having circular, convex, mucoid, shiny and pale yellow colour morphological characteristics of *X. axonopodis pv. punicae* were picked by sterilized loop and purified cultures were obtained by streaking on fresh NA medium (Poovarasn *et al.,* 2013). Further colonies were inoculated in nutrient broth for genomic DNA isolation.

Genomic DNA isolation and molecular identification of *Xanthomonas axonopodis* pv. *punicae* through PCR

The isolated and cultured Xanthomonas axonopodis inoculated into nutrient agar media and kept in shaking incubator for overnight at 120 rpm. The genomic DNA was isolated from overnight grown cultures by using HiMedia Kit following instructions provided in user guide. The DNA samples were quantified by using nano drop spectrophotometer. PCR amplification of nucleotides of C-terminus region in the *gyrB* gene using *gyrB* specific primer. A primer set, gyrB forward GTTGATGCTGTTCAC CAGCG' and Reverse CATTCATTT CGCCCAAGCCC were used and which were taken from the 530 nt C terminus region of gyrB gene with amplican size of 491 bp is specific to only *Xanthomonas axonopodis* pv.*Punicae*. The PCR condition followed consisted of initial denaturation at 94°C for 5', then 30 cycles comprising denaturation at 94°C for 30 Sec, annealing at 60°C for 60sec, extension at 72°C for 90sec, followed by an final extension cycle at 72°C for 3 minutes and final shock at 4°C. A 20 µl PCR reaction mixtures that consisting 50 ng of DNA, 1X PCR buffer, 10 mM of dNTPs mixture, 10 pmol of each primer and 1 unit of Taq DNA polymerase.

Screening for antibacterial activities

Antibacterial activity of isolates of *P. fluorescens* were screened by using agar well diffusion method (Balouiri *et al.,* 2016) against *Xanthomonas axonopodis* pv. *punicae*. The agar plates were inoculated by spreading with 10^6 CFU/ml of *X. axonopodis* pv. *punicae* suspension over the entire agar surface. Then, a hole of 8 mm diameter was made aseptically by using sterile cork borer and 100 µl antagonists solution was introduced into the well. The plates were incubated at 28 °C for 78 hours and the zone of inhibition was measured after 2-4 days after incubation.

Those isolates can able to produce higher inhibition zone was considered as efficient isolate that was used for isolating and cloning of DAPG coding genes.

Cloning and sequencing of DAPG (phlACBD) from AFPF19 isolate

The total DNA was isolated from efficient isolate of *P. fluorescens* isolate (AFPF19) by following the protocol of Sambrook and Russell (2001) with some modification. PCR Cloning of the DAPG gene was performed by the T/A cloning method following user's

manual (MBI Fermentas) using DAPG gene specific primers. DAPG gene specific primers were designed for reported gene sequence (Accession No: AB636682.1) by using Gene Tool Lite Software and synthesized at SUMANA ENTERPRISESP (SIGMA) Bangalore, India. DAPG-F (5' GCGCGCATGAACGTGAAAAAGATAGG TATTGTCAGCT 3') and DAPG-R (5' TCAGGCGGTCCACTCGCCCACCG 3').

PCR was carried out in a Eppendorf Master Cycler gradient in 25 µl reaction volume containing 100 ng DNA template, 3.0 mM $MgCl_2$, 5 pmole of each primer, 2.5 mM dNTPs, 1X *Taq Pol* buffer and 1U *pfu* DNA polymerase (#EP0501) and with 95°C for 4 min. Initial denaturation followed by 40 cycles of 95°C denaturation for 1 min, 55°C annealing for 1 min and 72°C extension for 1 min with a 72°C final extension for 20 min.

Cloning of PCR product

The purified PCR amplicon of DAPG coding gene cluster (phlACBD) was ligated to pTZ257R/T cloning vector (2868 bp), as described in InsT/A cloneTM PCR product cloning kit (K1214) of MBI, Fermentas, USA. For ligation, an optimal molar ratio of 1:2 vector: insert was calculated. The ligation mixture along with linerised vector and amplicon DNA were mixed in 0.5 mL micro-centrifuge tubes and incubated at 16^{o}C for 16 h. for ligation.

Preparation of competent cells and Transformation of *E. coli* DH5α

The competent cells of *E. coli* DH5α were prepared by following the protocol mentioned by Sambrook and Russell (2001) with minor modifications. About 100ul of freshly prepared competent cells were taken in a chilled centrifuge tube and 10:l of ligation mixture was added and mixed gently. The mixture was chilled in ice for 45 min and heat shock was given by shifting the chilled mixture to preheated 42°C water bath for exactly 2min.It was immediately transferred to ice to chill for 5 min. The culture was pre incubated and spread on the plates having Luria agar with Amp50, X-gal, IPTG and incubated overnight at 37°C. The recombinant clones were identified by blue/white assay.

Confirmation of clones by PCR and by sequencing

The Confirmation of the presence of cloned insert was done by PCR amplification of recombinant vectors with respective primers and by restriction analysis by using restriction enzymes. The total DNA and cloning vector were used as positive and negative controls in the process. The full length 4 kb of DAPG gene amplicon cloned in pTZ257R/T was sequenced using M13 primers walking technique at Chromous Private Ltd., Bangalore. The sequences were subjected to analysis using BLAST algorithm available at http://www.ncbi.nim.nih.gov.

Results and Discussion

Isolation and identification of *Pseudomonas fluorescens*

In the present study, a total of 158 *Pseudomonas fluorescens* isolates were isolated from different place of Karnataka (Table 1) and observed under UV light at 365 nm for few seconds to confirm their fluorescing property (Fig. 1).

The isolates which exhibited fluorescens under UV light were further identified by PCR amplification of *Cumene deoxygenase* (Cds) gene. It was found that all the fluorescent isolates showed amplification of unique sequence of Cds region with ~498 bp (Plate.1).

PCR based screening for DAPG producing isolates

Pseudomonas fluorescens isolates which produces 2, 4 DAPG were identified by using phlD primers. The presence of amplicon of around 745bp confirms the presence of DAPG (Plate 2) in seven isolates AFPF19, DWDPF2, CoSRPF2, SHIMPF, SKPPF1, UBPF3and MBPF3 using DAPG markers. the DAPG positive isolates were further used for screening against bacterial pathogen *Xanthomonas axonopodis* pv. punicae.

Isolation and identification of *Xanthomonas axonopodis* pv *punicae*

The bacterial pathogen was isolated from infected parts of leaves and fruits of pomegranate trees. Isolates showing pale yellow colour mucoid shining properties on nutrient glucose agar media were identified as *Xanthomonas axonopodis pv punicae* (Fig. 2). The isolates were further confirmed by PCR amplification with *Gyrase* B specific primers with a amplification product of ~495 bp (Plate.3).

In vitro efficacy of DAPG producing *Pseudomonas fluorescens* isolates against *Xanthomonas axonopodis* pv. *punicae*

The seven *Pseudomonas* isolates confirmed for DAPG production were evaluated *in vitro* against *Xanthomonas axonopodis pv punicae*. Among seven 2,4 DAPG producing *Pseudomonas fluorescens* isolates screened, five isolates AFPF19, DWDPF2, CoSRPF2, MBPF3 and SHIMPF were proved effective against *X*. *axonopodis* pv. *punicae*.

The isolate AFPF19 was found to be exhibit efficient antibacterial activity against *X*. *axonopodis* pv. *punicae* by forming maximum inhibition zone of 20 mm (Fig. 3). *Pseudomonas fluorescens* isolates SKPPF1 and UBPF3 did not show any antibacterial activity against *X*. *axonopodis* pv. *punicae*.

Cloning and Sequencing of DAPG from AFPF19 isolate

DAPG synthesizing gene cluster phlACBD was amplified from AFPF19 isolate using phlACBD specific primers designed using reported phlACBD nucleotide sequence from the database. The amplicons so obtained were separated on 0.8 per cent agarose gel is presented in Plate 4. From the gel, it is clear that an amplicon of 4Kb was obtained from amplification of phlACBD gene. This amplicon was cloned into cloning vector pTZ57R/T. The recombinant molecules was transferred into *E. coli* DH5α using 5 µl of ligation mixture.

The transformed cells were picked up and streaked on Luria agar containing amplicillin (100mg/mL), X-gal and isopropyl -D-thiogalactosidase (IPTG). The clones containing recombinant molecules were selected based on blue-white colonies. Plasmids were isolated from white colonies contained phlACBD gene and the clones were confirmed through PCR amplification by using specific primers (Plate. 4) and by restriction analysis. The confirmed recombinant vectors with DAPG was named pNCDCV1607 and Figure 4 represent the map of pNCADCV1607.

The construct pNCDCV1607 was sequenced completely using M13 primers and by employing primer walking technique. The complete nucleotide sequence of DAPG gene is presented in Figure 5. Sequenced DAPG gene was analyzed for the presence frequently used restriction sites (Fig. 6). The blast analysis of DAPG (phlACBD) sequence were analysed by aligning with reported DNA sequence of *Pseudomonas fluorescens* and it was having 99 per cent homology (Fig. 7).

Table 1: *Pseudomonas fluorescens* isolates collected from different locations of Karnataka

Place of collection	Code	Host	No. of *P. fluorescens* isolates obtained
Bangalore, GKVK	MBPF	Mungbean	08
Chinthamani	CoSPF	Ragi	12
Tumkur	TUMPF	Maize	06
Sakaleshapur	SKPPF	Coffee	20
Chikmagalur	CKMPF	Forest herb	16
Shimoga	SHIMPF	Forest herb	12
Agumbe	AFPF	Forest herb	28
Thirthahalli	THPF	Rice	23
Puttur	DKPF	Cashew	13
Brahmavar	UBPF	Forest herb	03
Dharwad	DWDPF	Cotton	17

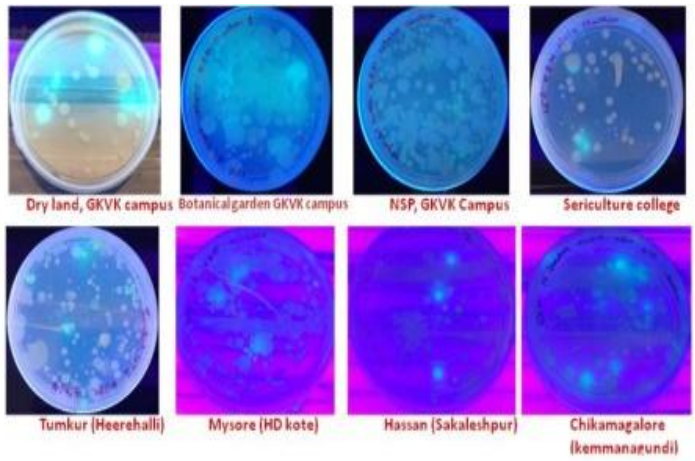


Fig. 1 Identification and confirmation of *Pseudomonas fluorescens* grown on King's B Base agar media Under UV light

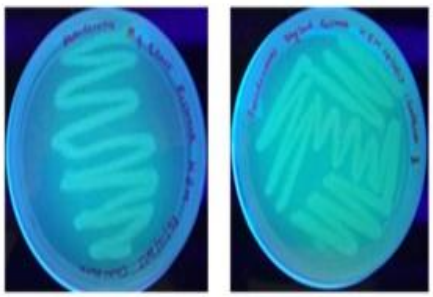


Fig.1a Purification of isolated *Pseudomonas fluorescens* on king's B Base media

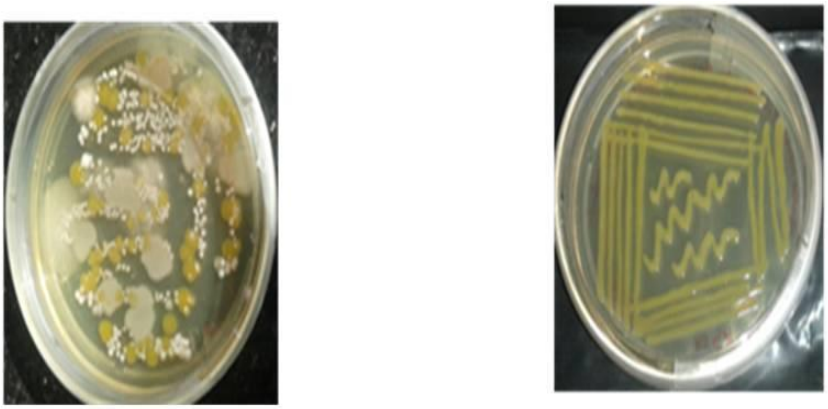


Fig.2 Isolation and purification of *Xanthomonas axonopodis pv punicae* on NGA media

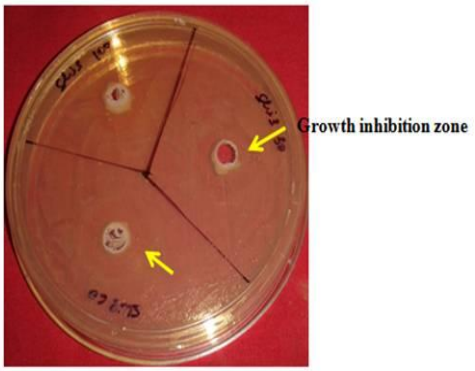


Fig. 3 *Pseudomonas fluorescens* isolate AFPF19 showing highest inhibition zone

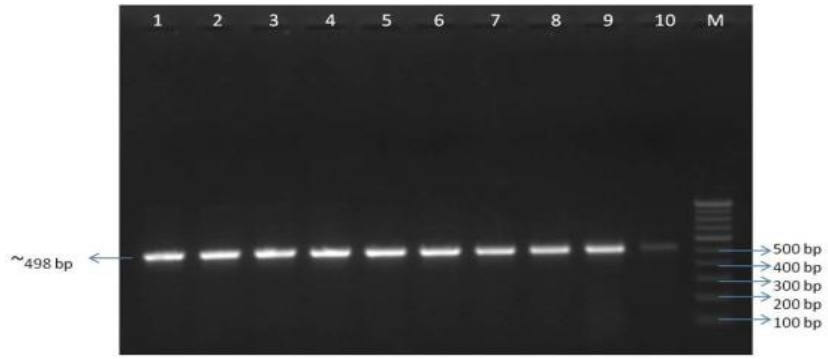


Plate.1 Molecular confirmation of Pseudomonas isolates by PCR amplification of *Cumene deoxygenase* (Cds) gene.
Lane 1-10: *P. fluorescens* isolates,
M: 100 bp ladder.

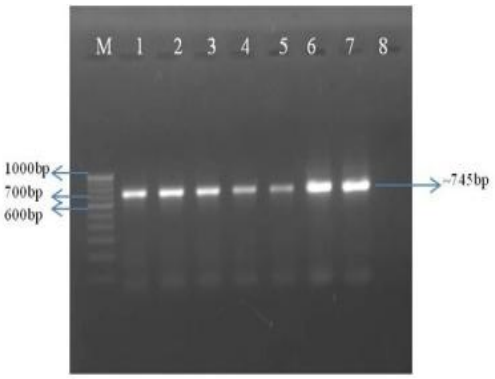
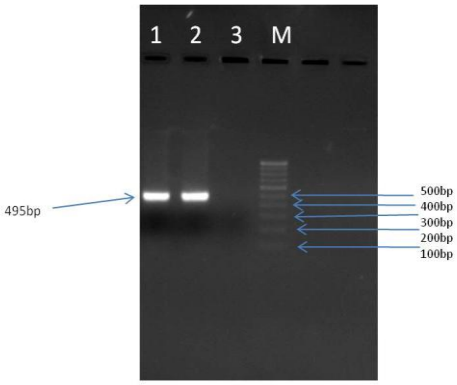
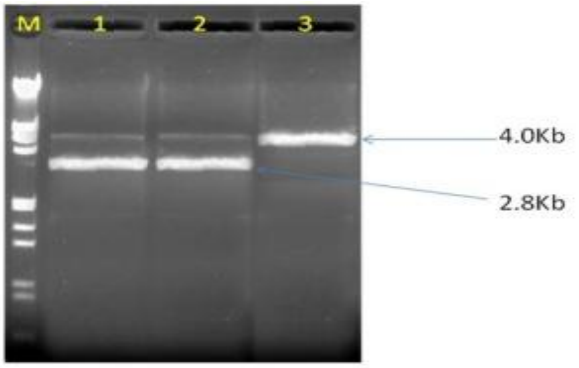


Plate. 2 PCR amplification of *PhlD* gene in *Pseudomonas fluorescens* isolates.
Lane: M: 100 bp Ladder,
Lane.1-7: AFPF19, DWDPF2, CoSRPF2, SHIMPF, SKPPF1 UBPF3 and MBPF3 isolstes



Plat. 3: *Confirmation of Xanthomonas axonopodis* pv *punicae by* PCR amplification of *Gyrase* B by specific primers
Lane.1&2: *Xanthomonas axonopodis* isolate
Lane.3: Negative control
M: 100bp marker



Plat.4 PCR and Restriction digestion confirmation of construct pNCDCV1607
M: Marker(lambda DNA/EcoRI-HindIII)
Lane 1&2: Restriction digestion of clone1 &2
Lane 3: PCR amplification of construct pNCDCV1607

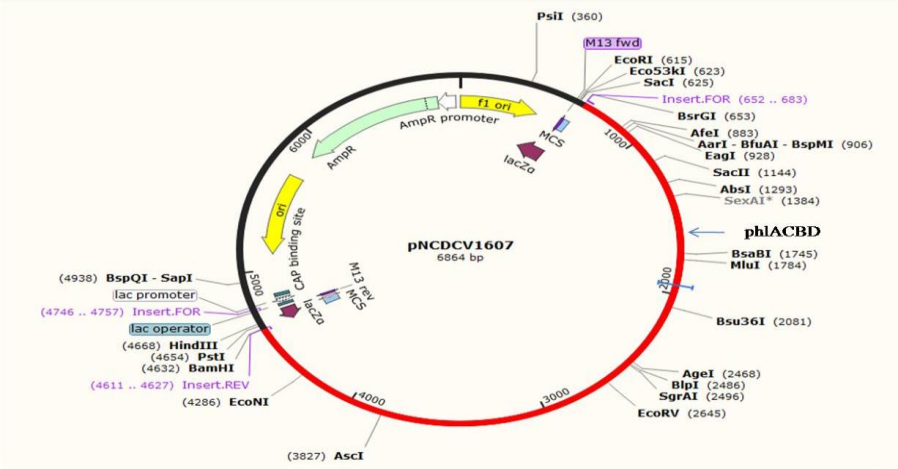


Fig. 4 Map of construct pNCADCV1607 containing phlACBD gene cluster

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caagttttggcctccagcccgttgcttgattctgtctttcagagtccagcttttgcttgttattgcctaaacatgtttctaacttttattggctttt
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ttcgacagcggacctgtcaatggcgcgacgggcatgttggcggcctcggcccgggttttcaccgctgaaatggcggtcggcaagtgggtcgcctga
```

Fig.5 represents the complete sequence of phlACBD (DAPG)

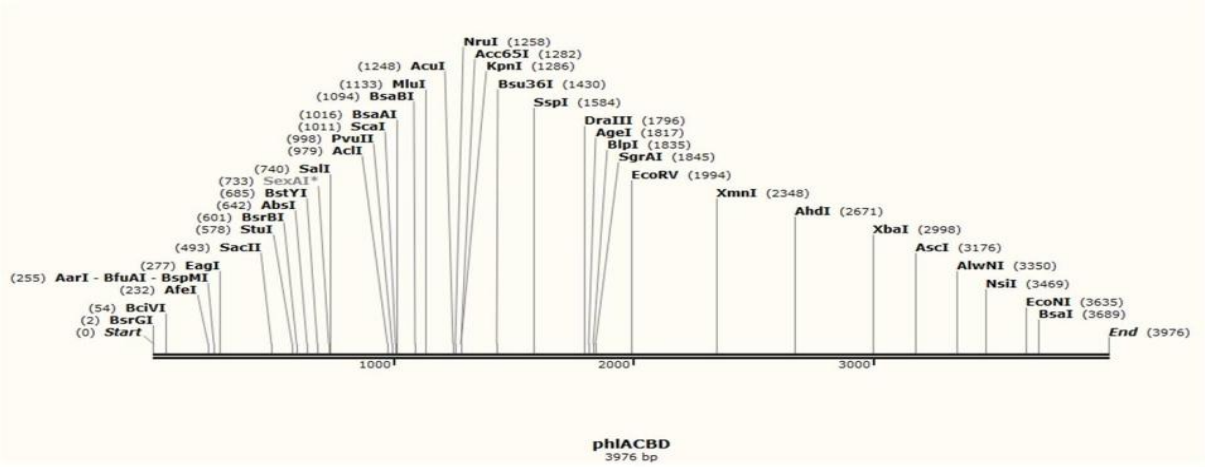

Fig.6 Restriction sites in phlACBD DNA sequence

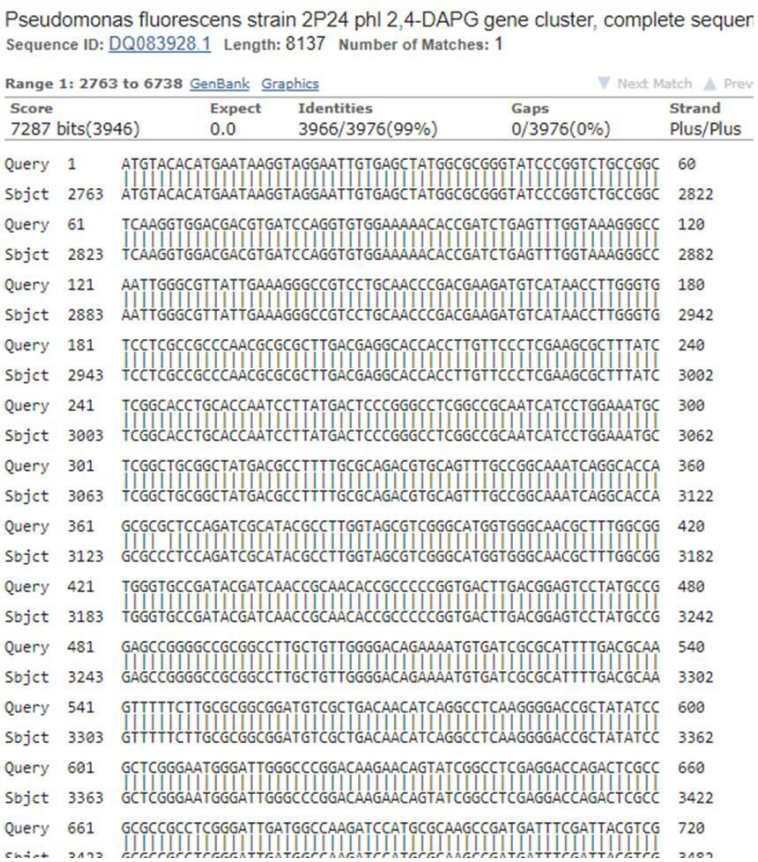

Fig. 7 Represents the blast analysis of Sequenced phlACBD gene cluster

In our studies, 158 isolates of *Pseudomonas fluorescens* were confirmed by using cds marker, that were isolated from rhizosphere of different crops collected from different districts of Karnataka, India. *Cumene deoxygenase* (*Cds*) gene primers were used for PCR amplification which is particular for *Pseudomonas fluorescens* and not for other *Pseudomonas* spp. (Mohammed, 2015). of these, seven isolates were positive for 2,4-DAPG production based on PCR studies using phlD specific primers. For detection of 2,4-DAPG-producing strains of *P. fluorescens* from rhizosphere soils, the *phlD* gene is used as a genetic marker (Raaijmakers *et al.*, 1997). The antimicrobial metabolite DAPG produced by *Pseudomonas fluorescens* is a principal factor enabling this bacteria to control plant diseases caused by soil-borne pathogens. The species capable of DAPG biosynthesis have been documented in numerous experimental studies and review articles (Bossis *et al.*, 2000; Haas and Keel2003; De La Fuente *et al.*, 2006a; Weller *et al.*, 2007; Sonnleitner and Haas 2011).

The phenolic metabolite 2,4-DAPG is an important component of the natural suppressiveness of certain agricultural soil to take all disease of wheat and black root of tobacco, and the active ingredient of many of the key biocontrol strains of *Pseudomonas fluorescens* (Picard and Bosco 2006). The existence of *phID* gene was evidenced through detection of a ~500bp DNA fragment in seven isolates AFPF19, DWDPF2, CoSRPF2, SHIMPF, SKPPF1, UBPF3and MBPF3 using primers phlD specific primers. Similar observations has also made by Sherathia *et al.*, (2015) in *P. putida* DAPG4 and *P. fluorescens* FP46. The amplification of 745 bp internal DNA fragment of *phID* gene

has been extensively utilized to identify 2, 4 DAPG producing *Pseudomonas fluorescens* from the rhizosphere of maize (Bangera and Thomashow. 1999) and groundnut (Sherathia *et al.,* 2016). phlD positive *P. fluorescens* were subjected for In vitro antagonistic studies indicated that two isolates of *P. fluorescens* were highly effective against pathogen *Xanthomonas axonopodis pv punicae* which was isolated from infected pomegranate plants. Among two isolates AFPF19, was found to be exhibit efficient antibacterial activity against *X. axonopodis* pv. *punicae* by forming maximum inhibition zone of 20 mm. these results supported the antibacterial activity of 2,4 DAPG.

Bangera and Thomashow (1996) were the first to isolate gene cluster responsible for the biosynthesis of DAPG from *P. fluorescens* Q2-87 and reported that seven genes (*phlFACBDE*) were involved in biosynthesis of 2, 4 DAPG. Studies have revealed that product of four genes *viz*., *phlA, phlB, phlC* and phlD are necessary for the production of MAPG and 2, 4 DAPG. ~4 kb DNA fragment of *phIACBD* was amplified from *Pseudomonas* AFPF19 isolate using specific primers and cloned into pTZ257R/T cloning vector. The cloned phlACBD gene cluster was confirmed by PCR and the confirmed recombinant vectors with DAPG were named pNCDCV1607. The sequencing of pNCDCV1607 construct was done by using M13 primers walking technique at Chromous Private Ltd., Bangalore. The sequenced DNA data were subjected to BLAST analysis and it was 99% similarity with reported phlACBD sequence. Zhou *et al.*, (2012) isolated 4 kb *phIACBD* gene fragment from *P. brassicacearum* J12 strain and expressed in *E. coli* DH5α. The main antimicrobial compound of J12 was identified as 2,4-diacetylphloroglucinol (2,4-DAPG) by HPLC–ESI-MS analysis. The gene cluster phlACBD, which is responsible for 2,4-DAPG production, was identified and expressed in the bacterial strain Escherichia coli DH5α.

From the current study, it can be concluded that *Pseudomonas fluorescens* isolate AFPF19 exhibited more antagonistic activity as compared to DWDPF2, CoSRPF2 and SHIMPF isolates under *in vitro* assay, but SKPPF1 and UBPF3 isolates did not show any inhibitory activity against *X. axonopodis* pv. *punicae*. The difference in their bio control activity may be attributed from their genotype since different genotypes may produce varied quantities of 2,4-DAPG. Our results provide a scope for over expression of 2,4- DAPG antibiotic in recombinant *E. coli* BL21 by sub-cloned to pET28a (+) expression vector and utilize this antimicrobial compound for further studies.

Acknowledgment

We gratefully acknowledge the Science & Engineering Research Board (A statutory body of Department of Science and Technology, Govt. of India) for financial support for the conducting research and University of Agricultural Sciences, Bangalore for providing facilities to carry out the Research.

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How to cite this article:

Ningaraju, T.M., H.V. Chaithra, and Anitha Peter. 2019. Isolation, Cloning and Sequencing of phlACBD Gene Cluster Encoding Antibiotic and Phloroglucinol Derivative 2, 4-diacetylphloroglucinol (2, 4-DAPG) from *Pseudomonas fluorescens*. *Int.J.Curr.Microbiol.App.Sci.* 8(03): 822-835. doi: https://doi.org/10.20546/ijcmas.2019.803.099