Int.J.Curr.Microbiol.App.Sci (2017) *6*(12): 585-594

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 12 (2017) pp. 585-594**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.612.071

Molecular Characterization of *Erwinia chrysanthemi* Causing Soft Rot Disease in *Aloe vera*

M. Syamala* and C. Ciba

Department of Plant Pathology and Department of Horticulture, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore -641003, Tamil Nadu, India
**Corresponding author*

Keywords

Aloe vera, Soft rot disease.

Article Info

Accepted:
07 October 2017
Available Online:
10 December 2017

A B S T R A C T

Erwinia chrysanthemi is the causal agent of soft rot of Aloe vera. In the present study, 51 isolates of *Erwinia chrysanthemi* of Aloe vera from Tamilnadu were characterized. Molecular detection of *Erwinia chrysanthemi* was carried out using species specific primers and toxin analysis was carried out using PCR and HPTLC analysis. Morphological identifications were well correlated with the molecular detection method. All the 51 isolates expressed an amplified product size of approximately 560 bp and hence confirmed as *P. fluorescens*. One isolate which expressed an amplified produce approximately 546 bp was confirmed as *Bacillus subtilis*. The crude antibiotics, viz., phenazine and 2, 4 diacetylphloroglucinol (DAPG) extracted from Pf 32, Pf 45 and Bs5 respectively, were thermostable (100^0C) and highly anti-bacterial against *E. chrysanthemi*. The presence of Phenazine and DAPG was detected through thin layer chromatography (TLC) with an Rf value of 0.57 for the isolates Pf 32, Pf 45 and Pf 4 and for DAPG the Rf value 0.88 was detected for the isolates Pf 32 and Pf 45. Primers Phl2a and Phl2b amplified the predicted size of 745 bp fragment from the genomic DNA of Ph 1 producing *Pseudomonas* isolates Pf 32 and Pf 45. Primers PCA 2a and PCA 2b amplified the predicted 1,150 bp fragment from genomic DNA of *Pseudomonas sp* Pf 32 and Pf 45.

Introduction

Aloe vera (*Aloe barbadensis*) is an important medicinal crop worldwide. The leaves are used for cosmetic purposes. Diseases and pests cause considerable yield loss. Among these, bacterial soft rot of *Aloe vera* caused by E*rwinia chrysanthemi* is a major problem in most of the *Aloe vera* growing areas of India posing serious constraint in gel production by appreciably affecting the leaf yield. Management of these diseases by cultural methods and by using toxic chemicals as bactericides has reduced the biodiversity of soil microbes and lead to the development of resistant strains of the pathogen. Hence, the present investigation was taken under to develop eco-friendly management strategies like exploiting induced resistance by using native bacterial antagonistics and botanicals.

Molecular characterization methods include PCR-based typing methods and DNA sequence-based characterization, which is broadly applicable for typing bacterial species. Many applications of molecular characterization have focused on differentiation of important bacterial species

used in crop protection. Many of the fluorescent pseudomonads, predominantly *P. fluorescens*, were isolated from suppressive soil for the management of soil-borne and foliar diseases (Weller *et al.,* 2002). Fluorescent pseudomonads are saprophytic root-colonizing bacteria. Fluorescent pseudomonads associated with plants include *P. fluorescens*, *P. putida, P. aeruginosa* and *P. aureofaciens*, *P. aeruginosa* forms a tight cluster and grow above 41°C (Hildebrand *et al.,* 1992). Most of the plant associated *Pseudomonas* sp. belongs to *P. fluorescens* and *P. putida* complex. There was no clear distinction between *P. fluorescens* and *P. putida* (Sheath *et al.,* 1981). Polymerase chain reaction (PCR) based detection is a favoured approach as it is accurate, rapid and sensitive. The small-subunit 16S rDNA sequence has shown to be useful for the detection of bacteria (Seal, 1997; Stead *et al.,* 1997). Development of correlations between genetic types and biocontrol potentials of microorganisms will help for application of molecular characterization methods for rapid identification of effective bioagents and tracking of economically important biocontrol agents.

Materials and Methods

Molecular basis

Detection of fluorescent Pseudomonads among the rhizobacterial isolates

DNA extraction

T otal DNA was isolated from bacterial strains using standard protocols (Sambrook *et al.,* 1989). Bacterial cultures were grown on KMB for 48 h at 25^0C. Two bacterial colonies (2 mm diameter) were suspended in 100 µl of lysis solution [0.05 M NaOH, 0.25 per cent sodium dodecyl sulfate (SDS)] and incubated for 15 min at 100ºC. The suspension was centrifuged for one min at 12,000 rpm and diluted 50-fold with sterile distilled water. Five microliters of the diluted suspension was used for PCR.

PCR amplification

To confirm strains as *Pseudomonas fluorescens*, 16S-23S rRNA intervening sequence specific primers ITSIF (AAGTCGTAACAAGGTAG); ITS2R (GACCATATATAACCCCAAG) were used to get an amplicon with the size of 560 bp (Rameshkumar *et al.,* 2002). PCR reactions were carried out in 20 µl reaction mixture containing 10X buffer (with 2.5 mM $MgCl_2$), 2 µl; 2 mM dNTP mixture, 2 µl; 2 M primer, 5 µl; *Taq* DNA polymerase, 3 U; H_2O, 8 µl and 50 ng of template DNA samples were amplified in thermal cycler (Eppendorf Master Cycler Gradient, Westbury, New York) using the PCR conditions 92°C for 4 min, 28°C for 1 min and 72°C for 2 min. The total number of cycles was 40 with the final extension time of 10 min. The PCR products were resolved on two per cent agarose gel at 50 V, stained with ethidium bromide (0.5 µg ml^{-1}), photographed and analysed using gel documentation system (Alpha Innotech Corporation, San Leandro, California).

Detection of *Bacillus* species from among the rhizobacterial strains

Isolation of DNA

Bacillus spp. was grown in nutrient broth at 28^0C. Total DNA (including chromosomal and plasmid DNA) was extracted as described by Robertson *et al.,* (1990). Cultures grown for 16 h in nutrient broth were centrifuged into a pellet, washed in TE buffer (10 mM Tris pH 7.5/1 mM EDTA pH 8.0) and suspended in 10 per cent sucrose. Cells were incubated at 37°C in lysozyme solution (5 mg/ml lysozyme, 50 mM Tris pH 7.5, 10 mM

EDTA pH 8.0), followed by addition of 20 per cent SDS containing 0.3 per cent beta-mercaptoethanol. DNA was purified by organic extraction and ethanol precipitation. Purified DNA was quantified by UV spectrophotometry.

PCR amplification

To identify the isolates of *Bacillus* sp., 16S rRNA intervening sequence specific BCF1 (CGGGAGGCAGCAGTAGGGAAT); BCR2 (CTCCCCAGGCGGAGTGCTTAAT) primers were used to get an amplicon size of 546 bp (Cano *et al.,* 1994). PCR reactions were carried out in 20 µl reaction mixture containing 10X buffer (with 2.5 mM $MgCl_2$), 2 µl; 2 mM dNTP mixture, 2 µl; 2 M primer, 5 µl; *Taq* DNA polymerase, 3 U; H_2O, 8 µl and 50 ng of template DNA samples were amplified in thermal cycler (Eppendorf Master Cycler Gradient, Westbury, New York) using the PCR conditions with the initial denaturation of 94^0C for four min and followed by 94^0C for one min, 58^0C for one min and 72^0C for one min.

The total number of cycles was 40 with the final extension time of 10 min. The PCR products were resolved on two per cent agarose at 50 V, stained with ethidium bromide (0.5 µg ml^{-1}) and photographed and analysed using gel documentation system (Alpha Innotech Corporation, San Leandro, California).

Extraction of crude antibiotics produced by antagonistic bacteria

Strains of antagonistic bacteria were grown at 28±2˚C in Pigment production medium. (Peptone-20 g; Glycerol-20 g; NaCl-5 g; KNO_3-1 g and Distilled Water-1litre pH 7.2). The cultures were grown in Pigment production (PP) broth for five days and were centrifuged at 5000 rpm and the supernatants were adjusted to pH 2.0 with conc. HCl and it was extracted with an equal volume of benzene. The benzene layer was subjected to evaporation in water bath. After evaporation, the residues were resuspended in 0.1N NaOH.

Effect of bacterial crude antibiotics on the growth of *E. chrysanthemi*

The efficacy of crude antibiotic extracted from bacterial antagonists was tested against *E. chrysanthemi* by filter paper disc assay (Smale and Keil, 1966). *E. chrysanthemi* was cultured in a 100 ml flask containing 40 ml of Nutrient broth at 25°C for 2 days with shaking at 100 rpm. One ml of *E. chrysanthemi* inoculum from nutrient broth was suspended in 10 ml of sterile distilled water and mixed with 15 ml of molten agar at a temperature below 50°C; the mixture was then poured into Petri dishes (90 mm diameter) and cooled. The sterile filter paper discs (5 mm diameter) were placed at the centre on these agar plates and the discs were loaded with 150 µl of crude antibiotics. The plates were incubated at 25°C for 2 to 7 days and the diameter of the inhibition zones formed around the discs was measured. The experiment was replicated thrice for all the isolates and the results were analyzed statistically. Surface area of inhibition was measured by tracing the area of inhibition in a trace paper, plotting it on a graph sheet and comparing with that of the control.

Extraction and isolation of phenazine

The antagonistic bacterial strains were grown in nutrient broth at 30°C on a rotary shaker. The cells were collected by centrifugation at 3500 rpm for seven minutes. The pellets were suspended in pigment production broth and then incubated on a rotary shaker for four days at 30°C. The antibiotic phenazine-1-carboxylic acid (PCA) was isolated as per the procedure described by Rosales *et al.,* (1995).

The antibiotic was separated into respective fractions after acidifying the culture filtrate with 1N HCl to pH 2.0 and then extracting the culture filtrate with an equal volume of benzene. Then the benzene phase was extracted with five per cent $NaHCO_3$. Phenazine-1-carboxylic acid was recovered from the bicarbonate layer while oxychlororaphine remained in the benzene layer. The bicarbonate fraction was extracted once again with benzene to recover phenazine from bicarbonate fraction. The antibiotic was air dried and dissolved in methanol.

Effect of phenazine on the growth of *E. chrysanthemi*

The antibiotic phenazine was assayed for its antibacterial action as described in assay of crude antibiotic (3.11.1.1.1) by adding the antibiotic at the rate of 100 µl, 150 µl, and 200 µl /well. The surface area of inhibition was recorded.

Extraction of 2, 4 diacetylphloroglucinol (2, 4 DAPG)

Strains of antagonistic bacteria were grown in 100 ml of Pigment Production (PP) broth for four days on a rotary shaker at 30˚C. The fermentated broth was centrifuged at 3500 rpm for five minutes in a tabletop centrifuge and the supernatant was collected. It was acidified to pH 2.0 with 1N HCl and then extracted with an equal volume of ethyl acetate. The ethyl acetate extracts were reduced to dryness in vaccum. The residues were dissolved in methanol (Rosales *et al.*, 1995).

Effect of 2, 4 DAPG on the growth of *E. chrysanthemi*

2, 4 DAPG antibiotics were assayed for their microbial action at the rate of 100 µl, 150 µl, and 200 µl/well against *E. chrysanthemi* as described in assay of crude antibiotic (3.11.1.1.1). The surface area of inhibition was recorded and expressed as per cent of inhibition.

Detection of antibiotics by thin layer chromatography (TLC)

Bacterial cultures grown on pigment medium (peptone, 20 g; glycerol, 20 ml; NaCl, 5 g; KNO_3, 1 g; distilled water, 1 litre; pH 7.2) (for DAPG production) or KBB (for pyoluteorin) or minimal medium (peptone, 20 g; KH_2PO_4, 3 g; K_2HPO_4, 0.5 g; NaCl, 5 g; $MgSO_4.7H_2O$, 0.5 g; D-tryptophan, 0.61 g; $ZnSO_4$, 0.35 mM; $Mo_7(NH_4)_6O_{24}.4H_2O$, 0.5 mM and distilled water, 1 litre) (for pyrrolnitrin) at 27^oC for 3 days were centrifuged at 14,000 g for 20 minutes at 4^oC and 20 ml of the supernatants were extracted with an equal volume of ethyl acetate for two h a rotary shaker. The ethyl acetate extracts were dried in vaccum at 35^oC and were dissolved in 1.5ml of 65 per cent methanol. A volume of 5 µl was applied to aluminium coated sheets with silica gel (Merck, Silica gel 60 F_{254}). Separation was performed with acetonitrile/methanol/water (1:1:1) as a solvent system for phloroglucinol and chloroform-acetone (1:1 v/v) as the solvent solution system for pyoluteorin and pyrrolnitrin.

The plates were sprayed with dinitrosalicylicacid, Rf values of the spots was 0.41 as compared with migration of synthetic 2,4-diacetyl phloroglucinol and identical colour (Rosales *et al.,* 1995). The corresponding pyrrolnitrin spots were detected by spraying with 2 % DMAB (Sigma) dissolved in ethanol-sulphuric acid (1:1 v/v). Pyoluteorin spots were detected by spraying with an aqueous 0.5 % fast blue RR salt solution (Sigma). The Rf values were 0.80 for pyrrolnitrin and 0.50 for pyoluteorin as determined by migration of pure standard

and confirmed by identical color spot and R_f values reported (De Souza and Raaijmakers 2003).

Detection of phenazine carboxylic acid (PCA) and 2,4 diacetylpholoroglucinol (2,4 DAPG) genes in *P. fluorescens* by polymerase chain reaction (PCR)

Total DNA was isolated from bacterial strains by standard protocol (Sambrook *et al.,* 1989). PCR amplification was performed with 25 µl reaction mixture which contained either approximately 20 ng of total DNA or 5 ml of a diluted heat-lysed cell suspension, 200 mM each dATP, dTTP, dGTP, and dCTP (Genei), 20 pmol of each primer and 3 U of Ampli *Taq* DNA polymerase (Genei). The oligonucleotide primers Phl2a (GAGGAC GTCGAAGACCACCA) and Phl2b (ACCG CAGCATCGTGTATGAG) developed from sequences within the biosynthetic loci for DAPG of *P. fluorescens* Q2-87 were used for detection of DAPG and PCA2a (TTG CCAAGCCTCGCTCCAAC) and PCA3b (CCGCGTTGTTCCTCGTTCAT) developed from sequences within biosynthetic loci for PCA of *P. fluorescens* 2-79 were used to detect PCA (Raaijmakers *et al.,* 1997).

Amplifications were performed in a thermal cycler (Eppendorf Master Cycler Gradient, Westbury, New York). The PCR program consisted of an initial denaturation at 94ºC for 2 min followed by 30 cycles of 94ºC for 60 s, 67ºC for 45 s and 72ºC for 60 s. Samples (9 µl) of the PCR products were separated on a 1.2 per cent agarose gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.3]) containing 0.5 µg of ethidium bromide per ml at 75 V for three h. The amplified PCR products in agarose gel were visualized with a UV transilluminator and photographed using the gel documentation system (Alpha Innotech Corporation).

Results and Discussion

Detection of *P. fluorescens* and *B. subtilis* through PCR

In the present study, fifteen isolates were confirmed as *P. fluorescens* by PCR. Using ITS1F and ITS2R specific primers, an amplicon size of approximately 560 bp was obtained in these fifteen isolates Rameshkumar *et al.,* (2002). PCR amplification with specific primers BCF1 and BCR2 has confirmed that the antagonistic bacterial isolate BS32 isolated from different agroecosystems as *Bacillus* sp. with the amplicon size of approximately 546 bp. Cano *et al.,* (1994) confirmed that the bacteria isolated from four separate stingless bees specimens are *Bacillus* spp. based on the PCR techniques using same specific primers and got an amplicon size of approximately 546 bp. Zinniel *et al.,* (2002) identified six endophytic bacteria as *Bacillus* sp. with the most promising level of colonization in a range of host plants based on 16S rRNA gene sequences.

Efficacy of crude antibiotics eluted by antagonistic bacteria against *E.chrysanthemi in vitro*

The results on the efficacy of the crude antibiotics, of five isolates of *P. fluorescens* (Pf 4, Pf 16, Pf 26, Pf 32 and Pf 45) and one isolate of *B. subtilis* (Bs_5) tested for their antibacterial activity against *E. chrysanthemi*. The crude antibiotic isolated from Pf 32 recorded maximum (14.6 mm) inhibition zone followed by of Pf 45 and Pf 4 which recorded the inhibition zone of 11.5mm and 7.3mm respectively. The other isolates *viz*., Pf 26 (5.4mm), and Pf 16 (5.2mm) and Bs_5 (3.0mm) were less effective against pathogen (Table 1). The crude antibiotics produced by *P. fluorescens* (CHA0) suppressed damping off of cucumber (Maurhofer *et al.,* 1992). Leifert

et al., (1995) observed the antibiotic produced by of *B. subtilis* CL 27 and *Botrtis pumulis* CL 45 against *B. cinerea*. Kavitha (2004) reported that crude antibiotics of *Bacillus subtilis* and *P. fluorescens* inhibited the growth of *Pythium aphanidermatum* in turmeric.

Efficacy of crude antibiotics of effective bacterial isolates exposed at different temperatures against growth of *E.chrysanthemi*

The antibiotics produced by the antagonistic bacteria in the rhizosphere should remain stable without loosing its antifungal and antibacterial activity after getting exposed to different temperatures. The crude antibiotics of Pf 26, Pf 32 and Pf 45 were thermostable antibacterial to *E. chrysanthemi* (Table 2).

The crude antibiotics extracted from *Pseudomonas* isolates Pf 26, Pf 32 and Pf 45 were exposed to various temperatures were similar to the action of crude antibiotic at the room temperature. This indicated that the thermostable nature of antibiotics. Whereas, the crude antibiotics from the other isolates lost their antibacterial activity when these were subjected to 40°C, 60°C, 80°C and 100°C.

This indicates that the crude antibiotics from Pf 4, Pf 16,Pf 26, Pf 32 and Pf 45 Bs 5 isolates were thermostable and those from the other isolates were heat sensitive. Michereff *et al.,* (1994) reported that *P. fluorescens* and *P. marginalis* produced nonvolatile, thermostable antibiotics which inhibited the mycelial growth of *C. graminicolum.* Kavitha (2004) documented that the crude antibiotic from *P. chloraphis* isolate AA23 and *Bacillus* isolate CBE 4 were thermostable and tested against *P. aphanidermatum*. The antibiotic iturin produced by *Bacillus* sp. was thermostable (Bernal *et al.,* 2002).

Efficacy of phenazine produced by effective bacterial antagonists against *E. chrysanthemi*

The results on the efficacy of the phenazine eluted from the crude antibiotics produced by the isolates of *Pseudomonas sp* against *E. chrysanthemi* at different concentrations *viz*., 100 µl, 150 µl and 200 µl.

The phenazine eluted from *Pseudomonas aeruginosa* isolate Pf 32 recorded 24.24 mm growth inhibition of *E. chrysanthemi* at 200 µl concentration followed by Pf 45 and pf 4 which recorded the inhibition zone of 20.25mm (150µl) and 18.14 (200 µl) respectively. The other isolates *viz*., Pf 16, Pf 26, Pf 45 and Bs_5 were less effective when tested at different concentrations against pathogen. Lottmann *et al.,* (2000) reported that the growth of bacterial pathogen *E. carotovora var. atroseptica* was significantly inhibited due to the production and action of phenazine produced by the rhizobacterial isolates in potato. In the present study also the antibiotic phenazine isolated from the cell cultures of *P. fluorescens* isolates suppressed the growth of *E. chrysanthemi* (Table 3).

Efficacy of 2,4 diacetyl phloroglucinol produced by effective bacterial antagonists against *E. chrysanthemi in vitro*

The experimental results revealed that the antibiotic 2, 4 DAPG eluted from the cell cultures of P. fluorescens isolate Pf32 recorded the maximum growth inhibition (26.10mm) at 200 µl concentration which effectively suppressed the growth of *E. chrysanthemi*.

Gnanamanickam and Velusamy (2003) demonstrated that 2, 4 DAPG produced by *P. fluorescens* strain PTB 9. Suppressed the growth of the rice bacterial blight pathogen *Xanthomonas oryzae pv. Oryzae* (Table 4).

Table.1 Efficacy of crude antibiotics (150µl/disc) eluted by antagonistic bacteria found effective *in vitro*

Crude antibiotics	Inhibition Zone (mm) *
Pf_4	7.3^{c}
Pf_{16}	5.2^{d}
Pf_{26}	5.4^{c}
Pf_{32}	14.6^{a}
Pf_{45}	11.9^{b}
Bs_5	3.0^{e}
Control(Sterile distilled water)	0

* Mean of three replications
In a column, means followed by common letters are not significantly different at 5% level by DMRT

Table.2 Efficacy of crude antibiotics of effective bacterial isolates exposed at different temperatures on *E. chrysanthemi*

Crude antibiotics of	*Inhibition zone (mm)			
	40^0 C	**60^0 C**	**80^0 C**	**100^0 C**
Pf_4	5.1^{d}	5.1^{d}	5.1^{d}	0
Pf_{16}	4.4^{e}	4.3^{e}	4.3^{e}	0
Pf_{26}	11.0^{b}	10.1^{b}	10.1^{b}	10.1^{b}
Pf_{32}	12.2^{a}	12.1^{a}	12.2^{a}	12.0^{a}
Pf_{45}	7.4^{c}	7.4^{c}	7.4^{c}	0
Bs_5	5.1^{d}	5.0^{d}	5.1^{d}	5.1^{c}
Control (Sterile distilled water)	0	0	0	0

* Mean of three replications
In a column, means followed by common letters are not significantly different at 5% level by DMRT

Table.3 Efficacy of phenazine produced by effective bacterial antagonists against *E. chrysanthemi*

Phenazine produced by	Inhibition zone (mm)*		
	100 µl/well	**150 µl/well**	**200 µl/well**
Pf_4	10.12^{d}	14.26^{bc}	18.14^{b}
Pf_{16}	11.25^{bcd}	13.76^{cd}	12.27^{d}
Pf_{26}	12.07^{bc}	15.64^{b}	16.36^{bc}
Pf_{32}	14.40^{a}	20.25^{a}	24.24^{a}
Pf_{45}	12.59^{b}	15.88^{b}	16.14^{c}
Bs_5	10.74^{d}	11.71^{d}	12.91^{d}
Control	0.05	0.05	0.07

* Mean of three replications
In a column, means followed by common letters are not significantly different at 5% level by DMRT

Table.4 Efficacy of 2,4 diacetyl phloroglucinol produced by effective bacterial antagonists against *E. chrysanthemi in vitro*

Bacterial antagonists	Inhibition zone (mm)*		
	100 µl/well	150 µl/well	200 µl/well
Pf_4	10.12^{b}	11.87^{de}	14.50^{d}
Pf_{16}	10.41^{b}	10.07^{e}	13.04^{de}
Pf_{26}	14.30^{a}	14.61^{e}	16.41^{c}
Pf_{32}	15.20^{a}	20.60^{a}	26.10^{a}
Pf_{45}	14.00^{a}	19.16^{b}	24.10^{b}
Bs_5	11.37^{b}	12.11^{d}	12.60^{e}
Control	0	0	0

* Mean of three replications

In a column, means followed by common letters are not significantly different at 5% level by DMRT

Detection of antibiotics using Thin Layer Chromatography (TLC)

The secondary metabolites produced by the biocontrol agents are responsible for antimicrobial activity. Secondary metabolites of biocontrol agents were detected through TLC. In case of crude antibiotics, several spots were observed when visualized under UV light (254 nm). This showed presence of different compounds in the crude antibiotics. The secondary metabolite 2, 4 DAPG was detected in TLC plates by using 2, 4 DAPG standard. The 2, 4-DAPG isolated from Pf 4, Pf 16, Pf 26, Pf 32 and Pf 45, recorded the R_f value of 0.89. The results were in agreement with the findings of Rosales *et al.*, (1995). Since the gene is responsible for the production of DAPG the R_f value of DAPG from *P. putida* was similar to the R_f value of 2, 4 DAPG extracted from Pf 4, Pf 32 and Pf 45. Kavitha (2004) reported that R_f value was 0.88 for PA23 and CBE4.

In the present study, the R_f value of 0.57 was obtained for phenazine of Pf 32 and Pf 45 as compared to the standard. The presence of three different spots revealed the presence of three different phenazine compounds. Similar results were obtained by Kavitha (2004). Rosales *et al.*, (1995) reported the R_f value of phenazine as 0.50. This variation in the R_f value might be due to the environmental conditions and the solvent systems used.

Detection of phenazine and DAPG gene in *Pseudomonas sp* by using PCR

Detection of PCA by PCR in *Pseudomonas sp*

Primers PCA2a and PCA2b amplified and predicted 1,150 bp fragment from DNA of *Pseudomonas* isolate namely Pf 32 and Pf 45. But no products were amplified from DNA of the nonproducing isolates Pf 16 and Pf 26.

Detection of 2, 4 DAPG by PCR in *Pseudomonas sp*

The antibiotic phenazine and DAPG producing isolates of *P. fluorescens* were detected by using specific primers. The Phl2a and Phl2b primer specifically amplified a DNA fragment of approximately 745 bp in *P. fluorescens* isolates Pf 32 and Pf 45 But the desired fragment was not amplified in case of Pf isolate 35. It might be due to the sequence variation in the gene responsible for the antibiotic production in the bacteria. The observations of the present are similar to the findings of Raaijmakers *et*

al., (1997), Kavitha (2004) and Radja Commare (2004), in which Phl2a and Phl2b amplified 745 bp fragments in *Pseudomonas* isolates. The phl primers used in this study were directed against phlD, one of the six biosynthesis genes in the *phl* biosynthetic cluster of *Pseudomonas fluorescens* Q2-87 (Bangera and Thomashow, 1996).

Phenazine primers PCA2a and PCA3b amplified approximately 1150 bp fragment of DNA in isolate Pf 45. Similar results was reported by Raajjmakers *et al.*, (1997) that primers *PCA2a* and *PCA3b* amplified the predicted 1150 bp fragment from *Pseudomonas fluorescens* isolate 2-79. Similar results were reported by Kavitha (2004) and Radja Commare (2004). The *PCA* primers were directed against *phzC* and *phzD* two of the nine genes in PCA biosynthetic cluster of *P. fluorescens* 2-79 (Boronin *et al.*, 1995). Rajkumar (2006) reported that primers PCA2a and PCA3b amplified the predicted 1150bp fragment from DNA of *P. fluorescens* 1. *Pseudomonas fluorescens* namely Bpf_1, BPf_3, BPf_4 and BPf_8 produced pyrrolnitrin and to be an effective biocontrol agent for this pathogen.

References

Bangera, M.G., and Thomashow, L.S. 1996. Characterization of a genomic locus required for synthesis of the antibiotic 2, 4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. *Mol. Plant-Microbe Interact.*, 9 :83-90.

Bernal, G., Illanes, A. and Ciampi, L. 2002. Isolation and partial purification of a metabolite from a mutant strain of *Bacillus sp*. with antibiotic activity against plant pathogenic a/gents. *Electronic J. Biotechnol*., Pp. 1-4.

Cano, R.J., Boruck, M.K., Higby-Schweitzer, H., Poinar, H.N., Poinar, JR, G.O. and Pollard, K.J. 1994. *Bacillus* DNA in Fossil Bees: an Ancient symbiosis. *Appl. Environ. Microbiol.*, 60: 2164-2167.

Cano, R.J., Boruck, M.K., Higby-Schweitzer, H., Poinar, H.N., Poinar, JR, G.O. and Pollard, K.J. 1994. *Bacillus* DNA in Fossil Bees: an Ancient symbiosis. *Appl. Environ. Microbiol.*, 60: 2164-2167.

Hildebrand, D.C., Schroth, M.N. and Sands, D. C. 1992. *Pseudomonas*. In: *Laboratory guide for identification of plant pathogenic bacteria*. (Eds.) Schaad, N.W. 2nd edition. International Book Distributing Co. Lucknow. pp. 60-80.

Kavitha, K. 2004. Molecular and biochemical approaches for the selection of biocontrol agents for the management of turmeric rhizome rot. *Ph.D., (Ag) Thesis*, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore-3.

Leifert, C., Li, H., Chid Buree, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S. and Harbour, A. 1995. Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. *J. Appl. Bacteriol*., 78:97-110.

Lottmann, J., Heuer, H., de Vries, J., Mahn, A., During, K., Wackemagel, W., Smalla, K., Berg, G. 2000. Establishment of introduced antagonistic bacteria in the rhizosphere of transgenic potatoes and their effect on the bacterial community. *FEMS Microbiol. Ecol*., 33(1): 1574-6941.

Maurhofer, M., Keel, C., Schneider, U., Voisard, C., Haas, D. and Defago, G. 1992. Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. *Phytopathology*, 82: 190-195.

Michereff, S.J., Silveira, N.S.S. and Mariano, R.L.R. 1994. Antagonism of bacteria to *Colletotrichum graminicola* and potential for biocontrol of sorghum anthracnose, *Fitopatologia Brasileira*, 19:541-545.

Raajimakers, J.M., Weller, D.M. and Thomashow, L.S. 1997. Frequency of antibiotic producing *Pseudomonas* spp. in natural environments. *Appl. Environ. Microbiol.*, 63: 881-887.

Radja Commare, R. 2004. Molecular and biochemical markers aided selection of effective Bio-control microbial strains for the eco-friendly management of major diseases in

Rice and Vanilla. *Ph.D.,(Ag) Thesis*. Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore – 3. p. 226.

Rajkumar, 2006. Molecular and biochemical approaches for the selection of biocontrol agents for the eco-friendly management of rhizome rot of banana caused by *Erwinia carotovora* sub sp. *Carotovora*. Ph.D. Thesis. Tamil Nadu Agricultural University, Coimbatore. pp. 107-119.

Rameshkumar, N., Thirumalai Arasan, V. and Gunasekaran, P. 2002. Genotyping of antifungal compounds producing plant growth – promoting Rhizobacteria, *Pseudomonas fluorescens*. *Curr. Sci.*, 82: 1436- 1466.

Robertson, D.L., Bragg, T.S., Simpson, S., Kaspar, R., Xie, W. and Tippets, M.T. 1990. Mapping and characterization of the *Bacillus anthracis* plasmids pX01 and pX02. *Salisbury Med Bull.*, 68:55-58.

Rosales, A.M., Thomashow, L., Cook, R.J. and Mew, T.W. 1995. Isolation and identification of antifungal metabolites produced by rice associated antagonistic *Pseudomonas* sp. *Phytopathology*, 85: 1028-1032.

Sambrook, J., Fritsch. E.F. and Maniatis, T. 1989. *Molecular cloning: a laboratory manual* 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Seal, S. 1997. Cost analysis of detection of bacteria and phytoplasmas in plant tissue cultures by PCR. In: *Pathogen and microbial management in micropropagation* (Eds.) Cassells, A.C. Kluwer, Dordrecht, pp123–130

Sheath, P.H.A., Stevens, M. and Sackin, M.J. 1981. Numerical taxonomy of *Pseudomonas* based on published records of substrate utilization. *Antonie Van Leeuwenhoek*, 47: 423-448.

Smale, B.C. and Keil, H.C. 1966. A biological study of the intervarietal resistance of *Pyrus communis* to fire blight. *Phytopathology*, 5: 1113-1120.

Stead, D.E., Hennessy, J. and Wilson, J. 1997. Modern methods for identifying bacteria. In: *Pathogen and microbial management in micropropagation* (Eds.) Cassells, A.C. Kluwer, Dordrecht, pp61–74.

Weller, D.M., Raaijmakers, J.M., McSpadden Gardener, B.B. and Thomashow, L.S. 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol*., 40: 309–348.

Zinniel, D.K., Lambrecht, P., Beth Harris, N., Feng, Z., Kuczmarski, D., Higley, P., Ishimaru, C.A., Arunakumari, A., Barletta, R.G., and Vidaver, A.K. 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl. Environ. Microbiol*, 68: 2198-2208.

How to cite this article:

Syamala, M. and Ciba, C. 2017. Molecular Characterization of *Erwinia chrysanthemi* Causing Soft Rot Disease in *Aloe vera*. *Int.J.Curr.Microbiol.App.Sci.* 6(12): 585-594.
doi: https://doi.org/10.20546/ijcmas.2017.612.071