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Antifungal and Plant Growth Promoting Properties of Endophytic *Pseudomonas aeruginosa* from *Zingiber officinale*

B. Jasim, Sabu Rohini, C. Anisha, C. Jimtha John, Mathew Jyothis and E.K. Radhakrishnan*

School of Biosciences, Mahatma Gandhi University, Priyadharshini Hills PO, Kottayam Dist, Kerala - 686 560, India.

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***Zingiber officinale* (ginger) is commercially cultivated in many parts of the world especially in India because of its use in culinary and medicinal purposes. One of the major disease that limit the yield of ginger is the rhizome rot disease caused by *Pythium myriotylum*. A feasible ecofriendly method is yet to be devised to prevent the plant from this threatening disease. Since there are reports on endophytic bacteria capable of enhancing plant growth both by growth promoting effects and disease resistance, such studies from ginger is very important. In the current study, an endophytically associated *Pseudomonas aeruginosa* with plant growth promoting properties and activity against *Pythium myriotylum* was isolated from ginger.**

Key words: Phenazine, endophytic *Pseudomonas aeruginosa*, *Pythium myriotylum*, Plant growth promoting bacteria, Siderophore.

Zingiber officinale (ginger) is well known for its use in culinary and medicinal purposes because of the presence of various bioactive metabolites. India is one of the major producers of ginger and the serious biotic stress limiting the yield of ginger is the rhizome rot disease caused by microorganisms including *Pythium myriotylum*. There are many methods that have been used to protect the ginger from disease[1]. However these methods are not fully successful in preventing the disease. The usage of chemicals can adversely affect the quality of ginger as well as environment and is expensive also[2]. Thus alternate ways of disease control methods which are cost effective, easily available and ecofriendly are much attractive. A promising approach based on these will be exploration of antifungal properties of bacteria associated endophytically with the plant to manage the disease. As endophytes are indigenous microbes, identifying those with antifungal properties can provide better applications due to the effective colonisation in the host plant[3]. Also such reports will be a strong support to the investigations on unravelling the possible microbial basis of disease resistance exhibited by some resistant varieties.

Among the various bacterial strains, fluorescent *Pseudomonas* sp. including *Pseudomonas aeruginosa* are found to be inhabited in the rhizosphere soil and also with in plants as endophytes. They can affect plant growth through the production of growth promoting factors like Indole 3 Acetic Acid (IAA), ACC deaminase, phosphate solublisation, siderophore etc. Also these are known to impart beneficial effect

* To whom all correspondence should be addressed.
E-mail: radhakrishnanek@mgu.ac.in

to the host plants by suppression of plant pathogens[4]. Many previous studies suggest the phenazine derivatives as one of the important antifungal products of *Pseudomonas* sp. as proved by their presence in the culture media[5]. Very interestingly, *Pseudomonas* sp. with phenazine mediated activity against *Pythium myriotylum* causing root rot of *Xanthosoma sagittifolium* (cocoyam) was previously reported[6]. But presence of endophytic *Pseudomonas* sp. in *Z. officinale* itself is least investigated. So identification of *Pseudomonas* sp. associated endophytically with rhizome of ginger can provide opportunities to explore its antifungal potential against *Pythium myriotylum* and also its plant growth promoting effects. In our ongoing research on endophytic bacteria from ginger, we identified a fluorescent *Pseudomonas aeruginosa* and the isolate was found to have excellent activity against *Pythium myriotylum* and also plant growth promoting properties.

MATERIALS AND METHODS

Isolation of endophytic bacteria

Rhizome of ginger (*Zingiber officinale)* was collected from Kerala Agricultural University, Thrissur and was used as the source material for the isolation of endophytic bacteria. The rhizome pieces were washed with tap water to remove soil and were made to 1 to 2 cm long pieces. This was further treated with Tween 80 for 10 minutes with vigorous shaking. This was followed by wash with distilled water for several times to remove Tween 80. After the treatment, the samples were dipped in 70% ethanol for 1 min and then with 1% sodium hypochlorite for 10 min. The samples were then washed several times with sterilized distilled water and the final wash was spread plated onto nutrient agar plate (g/l; peptone 5, beef extract 2, yeast extract 3, sodium chloride 5 and agar 18, pH 7.0) as control. For the isolation of endophytic bacteria, the outer surface of the sterilized plant material was trimmed. After trimming the pieces were placed onto nutrient agar plates. All plates including the control were incubated at room temperature for 5 days and observed periodically for bacterial growth. Colonies with green pigment was selected, subcultured and was used for further study.

Identification of the isolates by 16S rDNA sequencing

For this, genomic DNA was isolated from selected bacterial strain as per the method described by Ausubel *et al.*,[7]. Here the isolate was cultured overnight in Luria Bertani broth and the cells were collected by centrifugation. The cells were then resuspended in 567µL of TE buffer followed by lysis using 30µL of 10% SDS and 3µl of 20mg/mL protienase K. The mixture was then incubated for 1 hour at 37°C. After which the lysate was mixed thoroughly with 100µL of 5 M sodium chloride and 700µL chloroform : isoamyl alcohol (24:1) and was centrifuged at 8000 rpm for 10 min. The aqueous layer was then transferred to a fresh tube and equal volume of isopropanol was added. This was then inverted for several times and centrifuged at 8000 rpm for 10 minutes. The pellet was finally washed in 70% ethanol (v/v) and air dried at room temperature. The dried DNA pellet was resuspended in 100µl TE buffer and visualized in 0.8% agarose gel (w/v). Genomic DNA isolated was used as template for PCR using primers 16SF (5'-AgA gTT TgA TCM Tgg CTC-3') and 16SR (5'-AAg gAg gTg WTC CAR CC-3') specific to 16S rDNA[8]. PCR was carried out in a 50 µL reaction volume containing 50 ng of genomic DNA, 20 pmoles of each primer, 1.25 units of Taq DNA polymerase (Bangalore Genei), 200 µM of each dNTPs and 1X PCR buffer. PCR was carried out for 35 cycles in a Mycycler™ (Bio-Rad, USA) with the initial denaturation at 94^0C for 3 min, cyclic denaturation at 94^0C for 30 sec, annealing at 58^0C for 30 sec and extension at 72^0C for 2 min with a final extension of 7 min at 72^0C. The PCR product was checked by agarose gel electrophoresis. The product was further purified and subjected to sequencing, the sequence data was analyzed by BLAST[9]. The sequence was also used for phylogenetic analysis with MEGA 5 using neighbor-joining method with 1,000 bootstrap replicates[10].

Screening of the endophytic isolate for antifungal activity

Screening for antifungal activity of the isolate was done by streak-plating technique using the method described by Shomura *et al.*,[11] with suitable modifications. The isolate was streaked on one side of the nutrient agar plates in a single line and uninoculated nutrient broth was streaked on the other side of the plate as negative control.

Fresh mycelia of *Pythium myriotylum* was spot inoculated on the centre of the streak line of the isolate and control medium. The plates were then incubated at 28°C for 3 to 5 days and were observed for the antifungal activity. The inhibition of the growth *Pythium myriotylum* was observed as the positive result.

Molecular screening of the isolate for phenazine gene

The genomic DNA of the isolate was also used for the PCR amplification of *Phz*E and *Phz*F of the phenazine gene cluster. The primers for *Phz*E and *Phz*F were designed as per the previous reports of Schneemann *et al*., [12]. The primer sequence of *phz*E used was *phz*Ef (5'-gAA ggC gCC AAC TTC gTY ATC AA-3') and *phz*Er (5'-gCC YTC gAT gAA gTA CTC ggT gTg-3') and that of *phz*F were *phz*Ff (5'-ATC TTC ACC CCg gTC AA Cg- 3') and *phz*Fr (5'-CCR TAg gCC ggT gAg AAC-3'). PCR was carried out using 50 µL reaction volume containing 50 ng of genomic DNA, 20 pmoles of each primer, 1.25 units of Taq DNA polymerase (Bangalore Genei), 200 µM of each dNTPs and 1X PCR buffer. PCR reaction was carried out in for 35 cycles in a Mycycler™ (Bio-Rad, USA) with initial denaturation of 94 °C for 2 min followed by cyclic denaturation at 94 °C for 60 s, primer annealing at 54.7 °C (for *phz*E) and 57 °C (for *phz*F) respectively for 60 s, primer extension at 72 °C for 120 s and final extension of 72 °C for 7 min. The PCR product was analysed using a 1.5% agarose gel (w/v). The amplified product was gel eluted and was sequenced using Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystem). The sequence data obtained was analyzed further by BLAST for similarity[9].

Screening of plant growth promoting activities

IAA Production

The bacterial isolate obtained was screened for IAA production in the presence of tryptophan. For this, Erlenmeyer flask (250 mL) containing 50 mL of Nutrient broth supplemented with L-tryptophan (0.2%) was inoculated with 1 mL of bacterial suspension and incubated in the dark at 30°C for10 days on shaker at 200 rpm. The production of IAA in broth culture was analysed by using Salkowski's method[13].

ACC deaminase activity

Dworkin-Foster (DF) salts minimal medium (14) supplemented with ammonium sulfate (DF-Ammonium Sulfate) was used for ACC deaminase assay. The composition of DF salts minimal medium were: 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4$.$7H_2O$, 1 mg $FeSO_4.7H_2O$, 10 µg H_3BO_3, 10 µg $MnSO_4$, 70 µg $ZnSO_4$, 50 µg $CuSO_4$, 10 µg MoO_3, 2 g glucose, 2 g gluconic acid, 2 g citric acid, 12 g agar (for solid media), and 1000 mL distilled water. The amount of ammonium sulfate added to DF salts minimal medium was 2 gm/L.

Phosphate solubilization Activity

Qualitative analysis of the phosphate solubilizing activity of the isolate was carried out on Pikovskaya agar[15]. Briefly, isolates were plated onto agar medium containing inorganic phosphate (constituents -g/L) agar, 15; glucose, 10; $(NH_2)_2SO_4$, 5; NaCl, 0.2; $MgSO_4 7H_2O$, 0.1; $Ca_3(HPO_4)_2$, 5; $FeSO_4.7H_2O$, 0.002; Yeast extract, 2; Bromophenol Blue, 2.4mg/mL; pH 7.2) and incubated at 28°C for up to 48 h.

Siderophore Production

The isolate was checked for the production of siderophores on Blue agar CAS medium containing chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators[16]. The CAS agar medium inoculated with the bacterial isolate ZoB 5 obtained and was incubated at 28°C for 24 hrs.

RESULTS AND DISCUSSION

Isolation and identification endophytic bacteria

Inoculation of the surface sterilized rhizome on nutrient agar medium resulted in the isolation of a fluorescent bacterial isolate and was designated as ZoB 5. The absence of any bacterial colony on control plate which contained the final wash of the surface sterilized material confirmed the obtained bacterial isolate as endophytes.

The PCR amplification of the 16S rDNA of the endophytic bacteria resulted in the formation of 1500bp product as identified by agarose gel electrophoresis. The 16S rDNA gene sequence based identification is a generally used method for rapid and efficient identification of bacterial species[17]. The BLAST analysis of the 16S rDNA sequence of ZoB 5 showed its 100% identity to *Pseudomonas aeruginosa* (JQ900522) and this was further confirmed by phylogenetic analysis where the isolate formed cluster along with *Pseudomonas aeruginosa* (Fig. 1). Various species of

Pseudomonas are known to have endophytic association with plants like pepper, sugarcane, black gram etc[18]. Even some of the endophytic *Pseudomonas* are known as PGPR like bacteria because of their potential impact on the plant growth.

Screening of the endophytic isolate for antimicrobial activity

For screening the antifungal properties of *Pseudomonas aeruginosa* (ZoB5) identified in the study, streak plate method was used. The incubated plates were observed for inhibition of

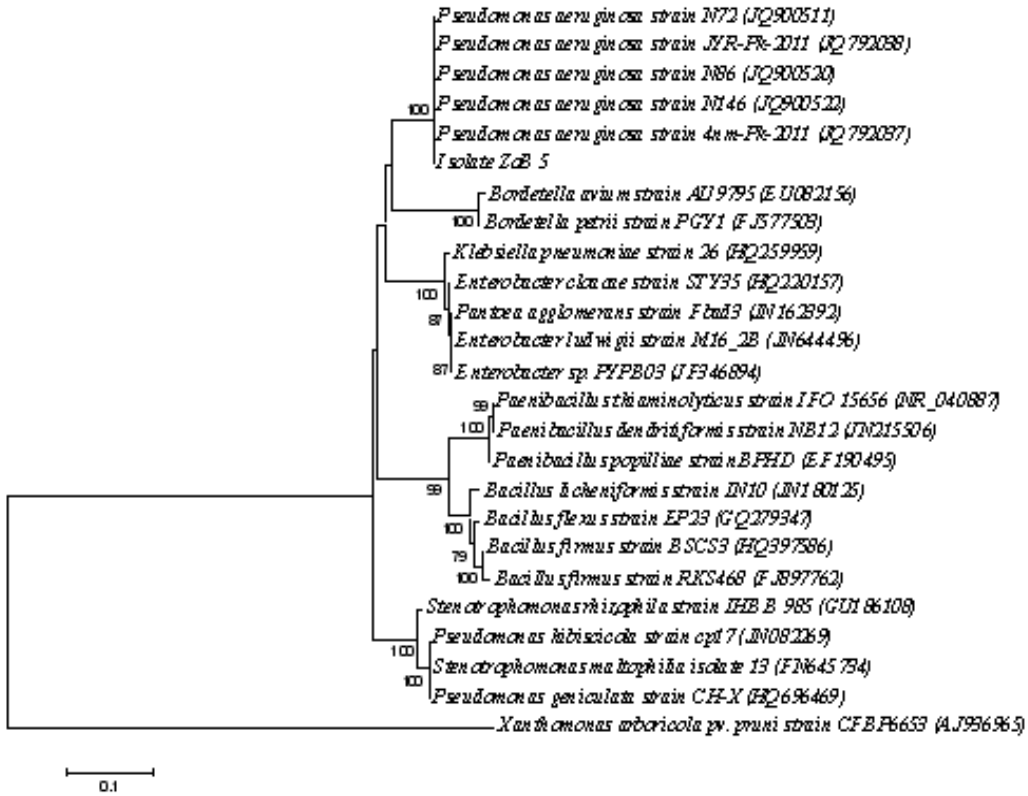

Fig. 1. The phylogenetic analysis of partial 16S rDNA sequence of the isolates obtained in the study along with sequences from NCBI using MEGA 5 with neighbor joining method using 1000 bootstrap replicates

Plate A – Nutrient agar plate inoculated with ZoB 5 and uninoculated nutrient broth as control on either sides and *Pythium myriotylum* on the centre of the two streak lines after 3 days of incubation.
Plate B – Nutrient agar plate inoculated with *Pythium myriotylum* alone after 3 days of incubation.
Plate C – Nutrient agar plate inoculated with ZoB 5 and uninoculated nutrient broth as control on either sides and *Pythium myriotylum* on the centre of the two streak lines after 6 days of incubation.
Plate D – Nutrient agar plate inoculated with Pythium myriotylum alone after 6 days of incubation.

Fig. 2. Screening of antimicrobial activity of isolate ZoB5

Pythium myriotylum by the isolate ZoB 5. From second day the plate showed inhibition of *Pythium myriotylum* by the isolate (Fig. 2a and b). Result observed on sixth day clearly reveals the inhibition of the pathogen (Fig. 2c and d). This confirmed the antifungal nature of endophytic *Pseudomonas aeruginosa* identified in the study against the *Pythium myriotylum* and this can have much application to develop strategies to provide disease resistance to ginger.

Pseudomonas sp. isolated from the rhizosphere soil is widely studied for the production of antimicrobial compounds. Also studies suggest that production of an array of compounds including phenazines by these bacteria can provide resistance towards a range of plant pathogens. The studies of Aravind *et al.*,[19] suggests that *Pseudomonas aeruginosa* endophytically associated with *Piper nigrum* has a promising role in the suppression *Phytopthora capsici,* the causative agent of foot rot disease in the plant. Several reports are there which supports the possibility of phenazines based biological activity rendered by the root-colonizing *Pseudomonas* sp against fungal plant pathogens[20].

The ability of *Pseudomonas aeruginosa* to produce phenazine is already known and due to this it is even used as reference organism for the comparison of phenazine biosynthesis by other bacteria[21]. There are many reports that states the biocontrol ability of the fluorescent *Pseudomonas* sp. against a wide range of phytopathogenic fungus in different plants like chickpea and pigeon pea[22,23], bean[22] and cocoyam[6]. However information on presence of endophytic *Pseudomonas aeruginosa* with antifungal property from ginger is very limited. In the current study the antimicrobial compound produced by the endophytic *Pseudomonas aeruginosa* was shown to inhibit the growth of *Pythium myriotylum* to a significant level.

As *Pythium myriotylum* is an important threat to ginger due to its role in rhizome rot, identification of antifungal endophytic bacteria from the rhizome proves its possible biocontrol applications. As the antifungal activity of *Pseudomonas aeruginosa* in plants like chickpea and pigeon pea[22, 23], bean[22] and cocoyam[6] was known to be mediated due to phenazines, the presence of gene clusters for phenazine biosynthesis was also investigated for the isolate used in the study.

Molecular screening of the isolate for phenazine gene

The presence of phenazine biosynthetic gene cluster in the isolated *Pseudomonas aeruginosa* strain was identified by PCR based screening for the *Phz* E and *Phz* F genes. Use of such an approach is an effective and rapid method for identifying organisms with the potential to form phenazine derivatives. The agarose gel electrophoresis analysis of the PCR clearly revealed the formation of expected 450 bp product for both cases. This was further confirmed by the DNA sequence analysis where both *Phz* E and *Phz* F sequences of the isolated *Pseudomonas aeruginosa* showed 100% identity to *Phz*E (EKA53849) and *Phz*F (ACT64244) genes of *Pseudomonas aeruginosa* respectively. The result of this clearly confirms the presence of phenazine biosynthetic gene clusters in the in the isolated *Pseudomonas aeruginosa* and supports greatly its activity against *Pythium myriotylum* as possibly due to phenazine compounds. This is also supported by the previous reports on phenazine mediated activity of *Pseudomonas* sp. against *Pythium myriotylum* causing root rot of *Xanthosoma sagittifolium* (cocoyam) [6].

Plant Growth Promoting Properties

The endophytic *Pseudomonas aeruginosa* was screened for IAA production and was found to have the ability to produce IAA. The IAA has the capability to increase the root size and distribution which can result in greater nutrient uptake from the soil[24, 25]. There is ample number of reports that suggests the ability of endophytes to promote plant growth by producing the phyto-hormone like IAA[26, 27]. However the ability of endophytic microbe to promote growth by producing both phytohormones and antimicrobial compounds is very limited. Ability of the isolated endophytic *Pseudomonas aeruginosa* to grow on DF salts minimal medium supplemented with ammonium sulfate as source of nitrogen indicated its positive ACC deaminase activity. ACC deaminase cleaves ACC (the immediate ethylene precursor) into alfa- ketobutyrate and ammonia, and as a result, the ethylene level is decreased. Ethylene is a potent plant growth regulator that affects diverse developmental processes, including

fruit ripening, senescence, and stress responses[28, 29]. But the isolate obtained was found to be negative for phosphate solubilization property.

Screening of endophytic *Pseudomonas aeruginosa* for siderophore production using the chrome azurol S (CAS) agar gave positive result as indicated by the formation of an orange halo around the colonies due to the chelation of iron. Siderophore produced could remove the iron from the dye complex which inturn changed the colour of the medium from blue to orange[16]. Siderophores producing bacteria promote plant growth by sequestering the limited iron which reduces its availability for growth of phytopathogens[30]. Pseudomonas species are shown to have the ability to form various siderophores. Structural studies of Abdallah[31] and Budzikiewicz[32] suggest that there are more than 50 structurally related siderophores like pyoverdins, which are produced by different species of *Pseudomonas*.

As endophytic bacteria are known to have promising plant growth promoting and antiphytopathogenic properties, their role in plant health is very significant. The microbial basis of plant disease resistance is just begun to study, it can have a wide promising applications to develop strategies to effectively control plant diseases. In the present study, endophytic *Pseudomonas aeruginosa* isolated from ginger is shown to have excellent activity against the *Pythium myriotylum*, which is one of the major pathogens of the plant. Very interestingly, the isolate obtained was also found to have multiple plant growth promoting properties. This clearly confirms its possible application as biocontrol and growth enhancer agent.

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