

RESEARCH ARTICLE

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Isolation and Proto-cooperation of *Pseudomonas putida* TS 18 from Water Calyx Fluid of *Spathodea campanulata* P. Beauv.

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Abstract

The study was to isolate, characterize and identify bacteria present in the water calyx fluid of the African tulip, *Spathodea campanulata* P. Beauv. Nutrient agar and nitrogen free malate (Nfb) semi-solid media were used to isolate the bacteria. Bacteria was identified using 16S rDNA sequencing. *Pseudomonas putida* TS 18 was found to be a new strain based on highest homology during the BLAST. Morphological and biochemical characterization of the identified bacteria was done by routine methods. This is the first study to report bacteria from the water calyx fluid of *S. campanulata*. Identified bacteria along with fluid content might be playing a unique role in the flower development by showing nitrogen fixing potential under *in vitro* conditions.

Keywords: Water calyces; *P. putida*; 16S rDNA sequencing.

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INTRODUCTION

Water calyces are the peculiar structures produced by the *Spathodea campanulata* P. Beauv., belonging to family Bignoniaceae from which the entire flower develops. The water calyces are ought to serve as a shield against floral herbivores; in protecting the sex organs from unfavourable consequences like high transpiration there by providing a cooling effect and also with antimicrobial activity (Jane et al. 2007; Burck, 1910; Killedar et al. 2011; Pat, 2011). Apart from its protective role, water calyces are ascribed to be beneficial to plant due to presence of endophytic microflora and biomolecules (Burck, 1910). Endophytic communities are vast and form microbial niches with in the host plant having direct influence on their growth, yield, by suppressing pathogenicity, solubilizing phosphate, synthesizing phytohormones and assimilating nitrogen to plants and (Monica et al. 2006). Not a single plant species is known to exist without endophytic colonization. Survival of the endophytic organisms inside the host plant involves complex physiological manipulations including majorly the bypass of plant defensive mechanism which is seen as a highest accomplishment by plant-colonizing bacteria (Gail MP, 2004). Most of the reports on endophytic communities in the last two decades are plant growth promoting microorganisms and their applications in the field of agriculture as modern organic biofertilizers (Vessey, 2003; Silva et al. 2006). Among which *Bacillus* and *Pseudomonas* forms are predominate (Podile, 2006) and one such dynamic plant growth promoting bacteria belongs to genus *Pseudomonas* which is heterogeneous and ecologically significant group among bacteria (Tasic et al. 2014). Various species of *Pseudomonas* are widely used as bioinoculant, biofertilization and phyto-stimulation (Wafa et al 2018). They are known to inhabit foliar surfaces, root surfaces, intercellular spaces in leaves and roots. Many of the aforementioned functionalities are known to be possessed by many species of *Pseudomonas* such as *Pseudomonas aureofaciens*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, *Pseudomonas solanacearum*, *Pseudomonas stutzeri* and *Pseudomonas syringae*. (Bernard, 2012; Islam et al. 2016). Similar kind of association is also assumed to be involved in the flower development of *Spathodea campanulata*.

The literature available on the water calyx fluid is largely confined to its mortality, mineral and nitrogen content, antimicrobial activity and biomolecules (Charyulu et al. 1990; Trigo et al. 2000; Martin et al. 2019, Jayanthi et al. 2019) But, the report and the role of bacteria within the water calyces still remains unexplored except for the findings of Treub (Burck, 1910). Hence, the present study was conducted to isolate, identify and indicate its role in the water calyx fluid.

MATERIAL AND METHODS

Sampling

Freshly collected flower buds of *S. campanulata* of different flowering seasons were collected from different locations in the city of Bengaluru.

Microscopic examination

Preliminary microscopic examinations on the calyx fluid were carried out using Magnus MLX plus light microscope and Magnus phase contrast microscope. Gram staining, capsule staining and hanging drop technique for motility were conducted following routine microbial methods (James et al. 1999).

Isolation

For extracting calyx fluid, unopened floral buds were washed in distilled water to remove surface contaminants. Under *in vitro* condition, the floral buds were washed in 0.1% mercuric chloride for 10 minutes followed by three washes in autoclaved distilled water for 5 minutes. The flower buds were again surface sterilized with absolute alcohol. Using sterile syringe, calyx fluid from the calyces was extracted. Isolation of bacteria was carried out by inoculating loopful of calyx fluid on nutrient agar by spread plate technique. The plates were incubated at room temperature $28 \pm 2^\circ\text{C}$ and observed for colony characters.

Evaluation of isolate for PGPR potential

The isolated bacterium from the nutrient agar plates was screened *in vitro* for various plant growth promoting properties such as nitrogen fixing ability on nitrogen free malate-semisolid media (Nfb). Loopful of calyx fluid was also inoculated directly in Nfb tubes. Pellicles obtained from Nfb tubes were sub-cultured further to obtain pure bacterial culture.

Table 1. Biochemical characterization of the *Pseudomonas putida* TS 18

Biochemical Tests	Results
Gram Staining	Negative rods
Motility	+
Capsule	+
Catalase	+
Oxidase	+
Starch hydrolysis	-
Lysine	-
Ornithine	-
Urease	-
TDA	-
Nitrate reduction	-
H ₂ S production	-
Glucose	+
Adonitol	-
Lactose	-
Arabinose	+
Sorbitol	-
Mannitol	+
Rhamnose	-
Sucrose	-
Indole	-
Methyl Red	-
Voges-Proskauer	-
Citrate Utilization	+

Biochemical characterization

The isolate from Nfb tubes were screened for gram staining, capsule staining, catalase, oxidase, starch hydrolysis and IMViC test (James GC, et al. 1999). Test for Lysine, ornithine, urease, TDA, nitrate reduction, H₂S production, carbohydrate utilization through glucose, adonitol, lactose, arabinose, sorbitol, mannitol, rhamnose, sucrose using HiAssorted Biochemical test kit KB001 and KB002.

DNA Extraction

Extraction of Genomic DNA of the isolate was done using Chromous Genomic DNA isolation kit (RKT09). Suspension buffer of 750 µl of 1X was mixed with 100mg of bacterial pellet followed by addition of 5µl of RNaseA with intermittent mixing for 5-6 times followed by incubation at 65°C for 10 minutes. Later, lysis buffer of 1 ml was added with intermittent mixing for 5-6 times and incubated at 65°C for 15minutes. The mixture was centrifuged at 13000g and the supernatant was collected in a 2ml vial. Supernatant was loaded onto the spin

column and centrifuged at 13000g for 1 minute at room temperature. The content of the collection tube was discarded. Then, 500µl of 1X wash buffer was added to the column and centrifuged at 13000g for 3 minutes at room temperature. Later, the spin column was placed in a fresh 1.5ml vial followed by the addition of 50 µl of warm elution buffer (kept at 65°C) and centrifuged at 13000g for 1 minute at room temperature. The eluted DNA was collected and electrophoresed on 1% agarose gel to estimate the quality and quantity of the extracted DNA with template used subsequently for PCR amplification.

Amplification of 16S rDNA

Amplification was done using 16s rRNA degenerative primers (16s Forward primer 5' - CMGSCVTDACACAWGCHAGYC - 3' and Reverse primer 5' - GGCGSMTGWGTNCAAGSV - 3'). Polymerase chain reaction was carried out using 1µl extracted DNA, 4µl dNTPs Mix, 10µl of 10X Taq DNA polymerase assay buffer, 1µl of Taq Polymerase enzyme (3U/µl) and with water to make up total reaction volume of 100µl. PCR was programed for 35 cycles with initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 90 seconds and final extension at 72°C for 7 minutes. The PCR product of about ~1.4 kb size band was determined on 1% agarose gel electrophoresis (Fig. 1). All reagents used in the experiment were of Chromous make.

DNA sequencing and identification

The amplified PCR product was subjected to Sanger's Dideoxy method of sequencing. This was carried out using ABI 3500 Genetic



Fig. 1. Visualization of PCR amplified 16srDNA fragment (~1.4kb) on 1% agarose gel. TS: amplified bacterial genomic DNA; L: 500bp DNA ladder.

Analyzer. Sequencing reaction composed of BigDye terminator cycle sequencing kit-ready reaction mix of 4 μ l, template (100ng/ μ l) of 1 μ l, primer of 2 μ l, milli Q water of 3 μ l. PCR was programmed for 25 cycles of initial denaturation at 96°C for 5 minutes, denaturation at 96°C for 30 seconds, hybridization at 50°C for 30 seconds and elongation at 60°C for 90 seconds. The sequence obtained was later compared against available database of 16S rDNA using the Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) for its identity. The data was then submitted to NCBI to obtain a GenBank accession number MG768972.

Molecular phylogenetic analysis

The 16S rDNA gene sequence obtained from the isolated bacteria was subjected to phylogenetic analysis using MEGA X. The evolutionary history was inferred using the Neighbor-Joining method (Li et al. 2017).

RESULTS AND DISCUSSION

Morphological and Biochemical characteristics of *Pseudomonas putida* TS 18

Identical colonies of gram –ve, rod shaped, motile and capsulated bacterium was obtained on nutrient agar after incubating the plates for 24-36 hours at 28 \pm 2°C. The colonies were creamish yellow in color, entire form, elevation raised, margin smooth, opaque and motile. Biochemical characterization showed presence for catalase, oxidase and positive reactions for glucose, mannitol and citrate utilization and negative for other tests (Table 1). Thus indicating that the isolate plays a significant role in colonizing the host plant competitively by its flagellar activity and thereby utilizing different kinds of carbon source (Turnbull et al. 2001; Weisskopf et al. 2011). White pellicles of microaerophilic, gram -ve, and rod shaped, motile bacteria was obtained on Nfb semisolid media. Pellicles were formed after incubating the tubes at 28 \pm 2°C for 7-10 days. Thus indicating the isolate is capable of fixing atmospheric nitrogen micro aerobically under *in vitro* conditions.

This is the first study aimed at understanding the role of associative symbiosis of alkalophilic nitrogen fixing bacteria present in water calyx fluid of *S. campanulata*. Flowering phenomenon of *S. campanulata* is interesting and

unique. The uniqueness of the plant is production of water calyces which often contains watery fluid. The presence of reported bacteria adds to its peculiarity. The development of the floral whorls under bathing alkaline fluid of pH 8.5 is unique among all flowering plants. The alkaline condition of the fluid would have further increased by the identified bacteria and it could be one of the reasons for the ammonical nature of calyx fluid which was reported earlier (Martin PA, et al. 2019). The amino acid pool in the calyx fluid would have also been enhanced by the identified bacteria as nitrogen fixers are known to contribute to amino acid production, other nitrogenous compounds required for chlorophyll containing organisms (Gonzalez-Lopez et al. 1995; Takuji et al. 2017; Ronald, 2001; Jayanthi et al. 2019).

Nitrogen fixing is considered to be second most vital process occurring in plants after photosynthesis influencing primary productivity (Rao, 2014). Nitrogen fixing is an important plant growth promoting character exhibited by the plant growth promoting rhizobacteria. It is well known fact that even Proteobacteria members like *Pseudomonas* can fix atmospheric nitrogen (Yongliang et al. 2008). Many authors have also proven that *P. putida* strains can fix nitrogen under natural and induced conditions (Yongliang et al. 2008; Ran et al. 1987; Chan et al. 1944; Setten et al. 2013; Li et al. 2017). In the present study the isolate showed 99% homology to *P. putida* H8234. The complete genome sequence of *P. putida* H8234 reveals presence of nitrogen metabolism, nitrogen fixation proteins coding for *NifU* and other similar related proteins (KEGG). Since, the isolate shows very close similarity to *P. putida* H8234, probably identified bacterial strain may be involved in fixing molecular nitrogen to ammonia and thereby helping in inner floral whorl development. The anaerobic environment created by the gamosepalous condition must be an ideal advantage for oxygen liable nitrogenase enzyme. In the present study, the microaerophilic culture conditions exhibited by the isolated bacteria are correlating with other studies as nitrogenase is reported to be functioning even at microaerobic conditions. (Mus et al. 2006). The isolate has also other plant growth enhancing traits which are not reported here.

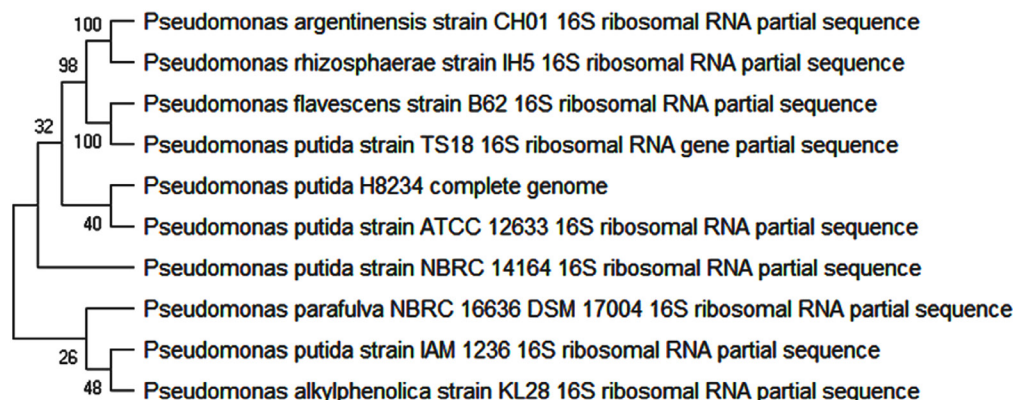


Fig. 2. Phylogenetic tree of *Pseudomonas putida* TS 18.

For any given plant, flowering is a complex and energy driven event and in cases like *S. campanulata* where it is exuberant and rapid, the nitrogen assistance provided by the bacteria will definitely be an additional source of nitrogen for the host plant (Salvagiotti F, et al. 2008). There are evidences showing nitrogen fixation is at peak during flowering (Lahiri K, et al. 2004). Additionally it has been shown that nitrogen fixation occurs till most of the seed fill period and the nitrogen is primarily transported into developing seed as glutamine and asparagine leading to the synthesis of seed proteins which is undoubtedly the reason for *S. campanulata* to produce hundreds of seeds in one single capsule (Rainbird RM, et al. 1984).

CONCLUSION

This is the first ever report on the presence of bacteria in the water calyx fluid of *S. campanulata*. The plant and the bacteria seem to be evolving as proto co-operative or a primitive associative organisms. The uniqueness of *Spathodea campanulata* flower development itself is its water calyx structure. The occurrence of the bacteria inside these structure perhaps should be influencing the flowering peculiarity with the developing floral buds pumped with dual sources of endogenous nitrogen.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

MP and JD conducted laboratory experiments. All authors have borne the expenses incurred during the study. All authors have contributed in preparation of the manuscript intellectually.

FUNDING

None.

DATA AVAILABILITY

Bacterial 16s rDNA gene sequence available under the mentioned Genbank accession number.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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