The Pharma Innovation Journal 2018; 7(6): 290-296

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(6): 290-296

www.thepharmajournal.com
Received: 10-04-2018
Accepted: 14-05-2018

Rathi Devi P
Department of Microbiology& CLT, Dr. Umayal Ramanathan College for Women, Karaikudi, Tamil Nadu, India

Packialakshmi N
PG & Research Department of Microbiology, Jamal Mohammed College (Autonomous) Tiruchirappalli, Tamil Nadu, India

Correspondence
Rathi Devi P
Department of Microbiology& CLT, Dr. Umayal Ramanathan College for Women, Karaikudi, Tamil Nadu, India

Screening of phosphate solubilizing fungi from *Cardiospermum halicacabum* roots and its bioactive compounds

Rathi Devi P and Packialakshmi N

Abstract
Endophytic fungi are promising tools for plant growth promotion. The present study is aimed to identify the potential phosphate solubilizing endophytic fungi from the roots of *Cardiospermum halicacabum*. Initially 25 fungal isolates were isolated from the roots of *C. halicacabum* and screened for phosphate solubilization using Pikovskaya's medium. Two of the fungal species PSF11 and PSF 9 were efficient phosphate solubilizers producing significant halo zones ranging from 2.4cm-3.1cm. Further studied for organic acid production in NBRIY medium with bromocresol purple as indicator. Fungal isolates were identified as *Aspergillus* species by shape, size colony morphology, and spore structures and based on molecular characterization, the sample has been identified as *Aspergillus oryzae* with Genbank accession number MG770589. FTIR analysis of *Cardiospermum halicacabum* ethanolic leaf extracts showed the presence of functional group for flavonoids and phenols that prevents phytopathogens, may be inherited from the endophytic fungal metabolites.

Keywords: Endophytic fungi, *Cardiospermum halicacabum*, phosphate solubilization, *Aspergillus oryzae*, ftir analysis, molecular characterization

1. Introduction
Cardiospermum halicacabum (Sapindaceae) is an herbaceous climber of about 2–4 m long. It is an evergreen, branched having inflated fruits containing white heart-shaped pattern on the seed. It is widely distributed in tropical and subtropical regions of Africa and Asia, and it is often consumed as green leafy vegetable in India. *C. halicacabum* is commonly known as Balloon vine, heart pea (England), Parol-paralon (Philippines), Jia hu gua (China) and Mudakathan Keerai (Tamil Nadu, India). *C. helicacabum* used in Indian traditional folk medicine system for the treatment of rheumatism, lumbago, cough, hyperthermia nervous diseases, stiffness of limbs and snake bite(R. Jeya devi *et al*., 2013) [16]. But its medicinal values is still unexplored and considered as a dangerous weeds in some countries. *Cardiospermum halicacabum* is eliminated intentionally and such novel medicinal plants are now extinct. All plants are inhabited internally by diverse microbial communities comprising bacteria, archaea, fungi, and Protista (Hardoim *et al.* 2015; Verma *et al*., 2008) [9, 21] The association between fungal endophytes and their host plant is due to the result of unique adaptations which enable the endophytes to harmonize their growth with that of their host (Verma *et al*. 2012) [22]. These endophytic communities are accountable to either partial or complete biosynthesis of host plant secondary metabolites Rajagopal *et al*., 2012; Ludwig-Muller. J.2015) [17, 11] Endophytic biotechnology can be used for the efficient production of agriculturally, industrially and economically important plants and plant products. The rational application of endophytes to manipulate the microbiota, intimately associated with plants, can help in enhancement of production of the agricultural product, increased production of key metabolites in medicinal and aromatic plants, aswell as adaption to new bio-geographic regions through tolerance to various biotic and abiotic conditions (Waru *et al*., 2015).
Phosphorus is the most important key element in the nutrition of plants. It plays an prime role in all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration and nitrogen fixation in legumes() Among the fungal members Aspergillus sp is a promising organism that solubilize the available rock phosphate and make it accessible for plant nutrition.
Phosphorus is abundant in soils, available both inorganic and organic forms, in an unavailable form for root uptake. Inorganic P occurs in soil, are insoluble, precipitated forms which

cannot be absorbed by plants (Rengel and Marschner 2005). Injudicious applications of chemical P fertilizers, leads to the loss of soil fertility by altering microbial diversity, and diminishing yield of crops.

Finally, as the host plants has never previously been utilized for isolating fungal endophytes, it is expected that the present study on this plant would be a novel pioneering attempt, and, a real advantage. Geographical locations of this plant may be needed to protect as an intellectual property. To protect the medicinal plants and determine the diversity of endophytic fungi and its inherent phosphate solubilization ability the present study is an ecofriendly approach to induce crop productivity and sustainability in agriculture.

Materials and Methods

Plant material

Fresh roots of *Cardiospermum halicacabum* was collected from the bushes near Karaikudi, Sivagangai, Tamil Nadu, India and identified taxonomically at The Rapinat Herbarium and Centre for Molecular systematic, Botanic gardens Conservation International, St. Joseph's college, Tiruchirappalli, Tamil Nadu, India.

Isolation of Endophytic fungi

Cardiosperm halicacabum plants were collected and for sampling the plants were uprooted carefully and bound soil were removed by gentle shaking. Using a sterilized blade, the rhizoids were excised and brought to the laboratory in a sterile glass vials, for isolation of endophytic fungi. Then the rhizoids were aseptically submerged in 50mL of 0.1M sodium phosphate buffer (pH7.0) and crushed into small fragments in a mortar and pestle. Sterile potato dextrose agar (PDA) (HIMEDIA Laboratories, India)was prepared and serially diluted root suspension was then poured onto sterile petriplate. Pour plate technique was performed and incubated at 25 °C for 24-48hours (Snega *et al*, 2018)

Screening for phosphate solubilization by the isolates.

Isolated endophytic fungi was cultured on nutrient broth for overnight and culture inoculums having 0.5 OD at 600nm was streaked o to Pikosvikaya's agar medium(PKA) (HIMEDIA Laboratories, India) (Pikosvikayas, RI,1948 and Rodriguez *et al*, 1948,1999) and after incubation at 28 °C for 48 hours, single colonies showing maximum halozones were picked and subcultured on PVK slants and maintained at 4 °C for further experiment (Mehta and Nautiyal, 2001) [13].

Measurement of phosphate Solubilization potential and organic acid production

Phosphate solubulizing fungal broth culture was carefully spotted on to National Botanical Research Institute's Phosphate growth medium devoid of Yeast extract(NBRIY) agar plates (pH7) (Nautiyal, 1999) supplemented with insoluble Tricalcium phosphate(5g/L) (HIMEDIA Laboratories, India) for the measurement of Solubilization Index(SI).Each fungal strain was inoculated in triplicates and incubated at 25° C for 3 days. Solubilization Index was measured using the formulae SI=Solubilization halo diameter/Colony diameter. Decrease of pH due to organic acids in the medium was observed on NBRIY broth incorporated with 0.01% bromocresol purple(HIMEDIA Laboratories, India) as an indicator for 5 weeks and phosphate solubilization was measured by standard method(Mehta and Nautiyal, 2001) [13]

Morphological Identification of Fungal Isolates

Fungal isolates PSF (1-25) were placed on glass slide and pressed with a coverslip and covered with Lactophenol cotton blue (HIMEDIA Laboratories, India).Colony Morphology, shape and spore structures are identified by microscopic observation.

Molecular Characterization of phosphate solubilizing endophytic fungi

DNA Extraction

Cells grown in monolayer should be lysed by suspending1-3 colonies aseptically and mixed with 450 µl of "B Cube" lysis buffer in a 2 mlmicro centrifuge tube and lyse the cells by repeated pipetting. Then 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer were mixed. The content were vortexed and incubated the tubes for 30 minutes at 65˚C in water bath. To minimize shearing the DNA molecules,DNA solutions were mixed by inversion.

Then centrifuged the tubes for 20 minutes at 14,000 rpm at 10 ˚C. Following centrifugation, the resulting viscous supernatant was transferred into a fresh 2 mlmicro centrifuge tube without disturbing the pellet. Added 600 µl of "B Cube" binding buffer to the content and mixed thoroughly by pipetting and incubated at room temperature for 5 minutes. Next 600 µl of the contents wre transferred to a spin column placed in 2 ml collection tube, and centrifuged for 2 minutes at 14,000 rpm and discarded flow-through. Reassembled the spin column and the collection tube then transferred the remaining 600 µl of the lysate. Again centrifuged for 2 minutes at 14,000 rpm and discarded flow-through.

Added 500 µL "B Cube" washing buffer I to the spin column and centrifuged at 14,000 rpm for 2 mins and discard flow-through. Reassembled the spin column and add 500 µl "B Cube" washing buffer II and centrifuged at 14,000 rpm for 2 mins and discard flow-through.

The contents were taken in afresh microcentrifuge tube and added 100 µl of "B Cube" Elution buffer at the middle of spin column. Care should be taken to avoid touch with the filter.

The tubes were incubated for 5 minutes at room temperature and centrifuged at 6000 rpm for 1min. Repeated the above mentioned step 14 and 15 for complete elution. The buffer in the microcentrifuge tube contains the DNA.DNA concentrations were measured by running aliquots on 1% agarose gel. The DNA samples were stored at -20 °C until further use.

PCR Protocol

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Primer Details Primer Name	Sequence Details	Number Of Base
Lr7	5' Tac Tac Cac Caa Gat Ct 3'	17
Lror	5' Acc Cgc Tga Act Taa Gc 3'	17

About 5 µL of isolated DNA was added in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). PCR was performed using the following thermal cycling conditions

Purification of PCR Production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM® Big Dye TM Terminator Cycle Sequencing Kits with Ampli Taq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Bioinformatics protocol

The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004).The resulting aligned sequences were cured using the program Gblocks 0.91b.ThisGblocks eliminates poorly aligned positions and divergent regions (removes alignmentnoise) (Talavera and Castresan a2007) [19]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be atleast as accurate as other existing phylogeny programs using simulated data, while beingone order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al*.,2008) [5]

FTIR analysis of Cardiospermum halicacabum leaf ethanolic extract

FT-IR is an advanced tool for determining types of chemical bonds (functional groups). FT-IR spectra were recorded on a BRUKER Optik GmbH, MODEL No - TENSOR 27 SOFTWARE - OPUS version 6.5 FT-IR spectrometer. Ethanolic Extract sample was dried and ground with potassium bromide powder and pressed into pellet for spectrometric measurement in the frequency range of 4,000–400 cm-1.

Results

Plant material was submitted for taxonomic identification and under processing. The excised roots suspension were serially diluted and used for fungl isolation. About 25 isolates were obtained and designated as PSF1-PSF25.Based on colony morphology the isolates were grouped and identified the results were shown in Fig1 and Table1.Endophytic fungi colonise in intact roots, similar results were reported by Verma *et al*., 2012 [22], totally 167 endophytic fungi were isolated

Table 1: Isoation of endophytic fungi from *C. halicacabum* root suspension

Isolate	Phosphate solubilization	Colony morphology	Size(cm)
PSF1,PSF8,PSF9PSF11,PSF12,PSF 22,PF23,PSF25	++	White cottony appearance with green spores, filamentous mold	0.8-0.9cm
PSF2,PSF4, PSF7,PSF19,PSF20,PSF21, PSF24	+	Black filamentous growth	1.8-2.2cm
PSF3	+	Pale green cottony mass	1.6cm
PSF4	+	Green colored colonies	1.3cm
PSF5	-	Yellow round colonies	1.2cm
PSF6	-	Brown filamentous growth	1.6cm

Isolated strains when screened for phosphate solubilization Pikosvikaya's agar, significant halozones were formed. Halozones ranged from 2.0-3.1cm which is shown in Fig 2 and Table.2.PSF 11, PSF12 and PSF 9 showed halozones of 2.325cm, 2.125 cmand 2.0 cm respectively, in which PSF11 showed maximum phosphate solubilization index.

Phosphate solubilization potential was further analysed by using NBRIY broth with bromocresol purple as an indicator. Colour change from purple to yellow colour was an evidence for phosphate solubilization due to organic acid production. Change in pH from 6.8 to 4.3 also infers that acid production induced phosphate solubilization. Concentration of tricalcium phosphate was estimated and amount of soluble phosphate was maximum for PSF11 which ranged from 300-450 microgram/mL in2-4 weeks of incubation, the results have been shown in Graph 1, Table. 1 and Table 2

Table 2: Phosphate solubilization efficiency of the isolated fungal strains

Isolate	Colony diameter(cm)	Halozone diameter(cm)	Solubilization index(cm)
PSF1	0.8	2.5	3.125
PSF2	0.5	2.3	1.533
PSF3	1	2.8	2.8
PSF4	1.8	2.4	1.33
PSF5	1.2	2.9	2.41
PSF6	1.6	2.4	1.5
PSF7	1.5	2.5	1.6
PSF8	1.8	3.5	1.9
PSF9	1.8	3.6	2
PSF10	1.9	3.6	1.8
PSF11	1.6	3.7	2.3125
PSF12	1.6	3.4	2.125
PSF13	1.1	2.7	2.454
PSF14	1.21	2.8	2.31
PSF15	1.9	2.5	1.35
PSF16	1.4	2.5	1.7857
PSF17	1.6	2.9	1.8125
PSF18	1.5	3	2
PSF19	1.4	2.3	1.642
PSF20	1.3	2.2	1.692
PSF21	1.32	2.4	1.818
PSF22	1.4	3	2.142
PSF23	1.9	3.1	1.631
PSF24	1.9	2.8	1.4736
PSF25	1.95	2.9	1.487
Mean value	1.4792	2.828	1.638

Fig 1: Morphological characteristics of *Cardiosperm halicacabum* plants

Fig 2: Isolation of Endophytic fungi

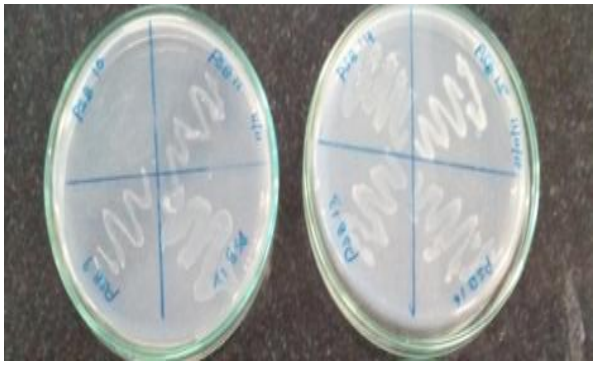

Fig 3: Screening for phosphate solubilization by the isolates

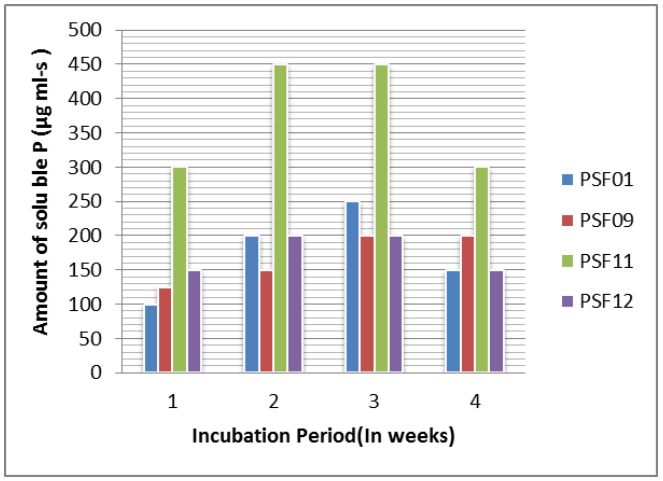

Fig 4: Phosphate solubilization potential of efficient isolates

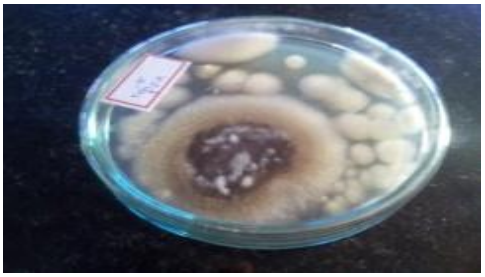

Fig 5: Aspergillus species

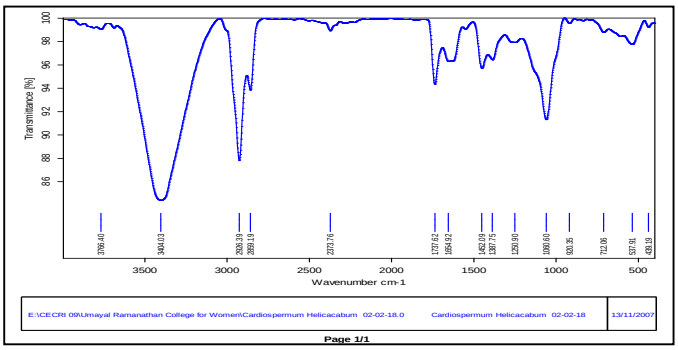

Fig 6: FTIR analysis of *Cardiospermum helicacabum* extracts

Table 3: FTIR analysis of Ethanolic leaf extract

S. No	Peak Value	Functional group	Interpretation of compound
1.	3404.03	N-H	Secondary amine
2.	2926.39	O-H	Carboxylic acid
3.	2859.19	O-H	Carboxylic acid
4.	1737.62	C=O	(for δ-Lactone) Esters
5.	1654.92	C=C	Conjugated alkane
6.	1452.09	C-H	Alkane
7.	1387.75	CH=O	Aldehyde
8.	1250.90	C=O	Aromatic Esters
9.	1080.60	C-O	Primary alcohol
10.	920.35	C-H	Alkene

>Contig

PSF_IITAACTGGCGAGTAGAAGCGGCAAGAGCTCAAATTTGAAAGCTGGCTCCTTCG

GGGTCCGCATTGTAATTTGCAGAGGATGCTTCGGGTGCGGCCCCTGTCTAAGTGCCC

TGGAACGGGCCGTCAGAGAGGGTGAGAATCCCGTCTGGGATGGGGTGTCCGCGCCC

GTGTGAAGCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGT

AAATTTCATCTAAAGCTAAATACTGGCCGGAGACCGATAGCGCACAAGTAGAGTGA

TCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAA

AGGGAAGCGCTTGCGACCAGACTCGCCTCCAGGGTTCAGCCGGCATTCGTGCCGGT

GTACTTCCCTGGGGGCGGGCCAGCGTCGGTTTGGGCGGCCGGTCAAAGGCTCCCGG

AATGTAGTGCCCTCCGGGGCACCTTATAGCCGGGAGTGCAATGCGGCCAGCCTGGA

CCGAGGAACGCGCTTCGGCACGGACGCTGGCATAATGGTCGTAAACGACCCGTCTT

GAAACACGGACCAAGGAGTCTAACATCTACGCGAGTGTTCGGGTGTCAAACCCGTA

CGCGCAGTGAAAAGCGAACGGAGGTGGGAGCCCCCTCGTGGGGCGCACCATCGACC

GATCCTGATGTCTTCGGATGGATTTGAGTAAGAGCGTAAATGTGGGGACCCGAAAG

ATGGTGACTATGCCTGAATAGGGCGAAGCCAGAGGAAACTCTGGTGGAGGCTCGCA

GCGGTTCTGACGTGAAATCGATCGTCAAATTTGGGTATA

Fig 7: BLAST sequences of PSFII

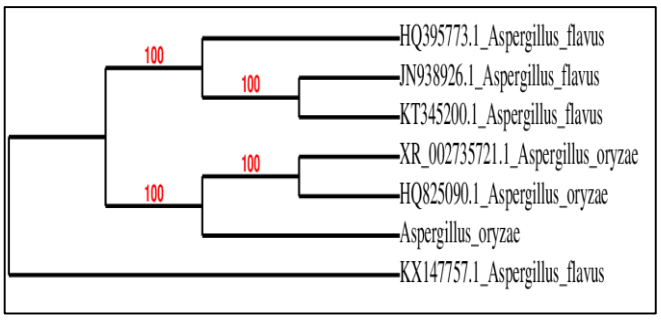

Fig 8: Phylogenetic tree for the Isolate PSF II

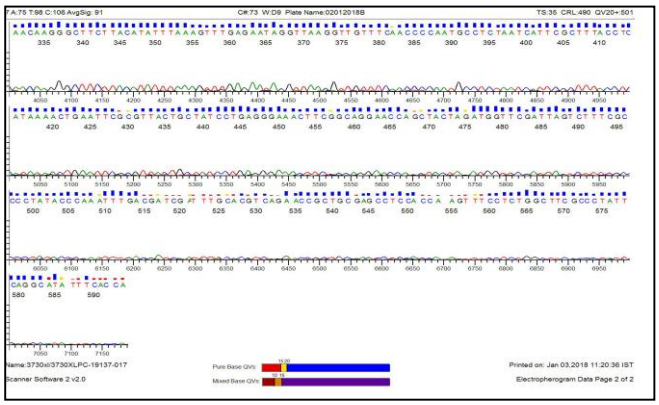

Fig 9: Electropherogram of the Sequences

Molecular Characterization

DNA sequencing studies was carried out for PSF 11 by isolating DNA and amplified by PCR and sequenced by 18srRNA sequencing protocol. DNA contig sequences thus obtained was BLAST searched and converted into FASTA file shown in Fig 6,and submitted for accessioning Gen Bank in online. The isolate PSF 11 was identified as *Aspergillus oryzae* and accession number was assigned by Genbank as MG770589 and phylogenetic tree represented in Fig 7reveals that there is no 100% matching with any strain. Electropherogram has been shown in Fig 8 confirmed that 594bp genomic sequence has been analysed and identified as *Aspergillus oryzae* isolate 1217_542_017_PCR_PF_II_LR7_D09 large subunit ribosomal RNA gene, partial sequence.

FTIR analysis

Cardiospermum halicacabum is used as green leafy vegetable in India, but less documented in medicinal uses. FT-IR spectra of ethanolic leaf extract are shown in Table.3 &Fig.5. Strong and broad absorption peak at 3404.03cm-1 for N-H stretching and 2926.39cm- for OH stretching vibrations, and also peaks at 2859.19cm-1and 2373.76cm-1 were obtained.1452.09 cm-1 for CH stretching vibrations, and a strong extensive absorption in the region of 1250.90 for aromatic ester(C-H)stretching and 1080.60 cm-1 for primary alcohol vibrations were observed in the spectra. Peaks at 1737.62cm-1 showing C=O stretching for δ-Lactone were also observed.

In addition there are characteristic peaks at 1654.92cm-1 (for CH stretching), 1,387.75cm-1 (for C-H stretching) and 920.35cm-1(alkene) were also observed in Fig. 5. TheFT-IR spectrum of ethanolic leaf extract confirms the presence of functional groups for phenolics and flavonoids, which are essential for their antioxidant potential. Several reports have confirmed that Flavonoids and phenolic acids have antibacterial, antifungal, antiviral, hepatoprotective, immunomodulating, and anti-inflammatory properties was preported by Jeya devi *et al*, 2013 [16] and Havsteen B *et al,1983* [10]

Discussion

In the present study fungal endophytes were isolated from laterite soil of Sivagangai district in Tamil Nadu, India, which is very poor in available phosphorus. *Cardiospermum halicacabum* can grow profusely in such phosphate deficient soil even under extreme conditions. To determine any possible interaction of phosphate solubilizing plant growth promoting fungal strains with C. halicacabum. Roots excised

from C. halicacabum were used to isolate the desired fungal strains that may contribute phosphate solubilization in such deficient soil.

25 fungal colonies have been isolated from the root suspension of C. halicacabum on PDA plates. Further the isolates were screened for phosphate solubilization on Pikosvikaya's agar plates. Prominent halozones were obtained for PSF11, PSF 1,PSF 9 andPSF12 respectively Table 1,Fig 1,2,&3.Based on Phosphate solubilization indices after 21 days and TCP solubilization potential on NBRIY broth was analysed and PSF11 was the strain identified as potential phosphate solubilizers. Concentration of tricalcium phosphate was estimated and amount of soluble phosphate was maximum for PSF11 which ranged from 300-450 microgram/mL in2-4 weeks of incubation, the results have been shown in Fig.4

Morphological identification of the isolates were based on lactophenol cotton blue mounting, revealed PSF11 found to be a filamentous fungi of the *Aspergillus* family, showing a hyphae that is hyaline and septate, and conidiophore, which ends at a round-shaped vesicle with long filaments giving rise to a conodial chain, appearing as long fluffy strands. The spores, are produced within the ascocarp, or the fruiting body was reported by Machhida *et al*, 2008

Based on 18srRNA sequencing and phylogenetic tree analysis, the isolate PSF11 was identified as *Aspergillus oryzae*.18srRNA sequence are 100% matching with *Aspergillus oryzae*. Nikkuni *et al*, 1998 reported that partial sequencing of *Aspergillus oryzae* have identity with other members in the genus. Fig 6,7 & 8

Machhida *et al*, 2008 reported that *Aspergillus oryzae* have the greatest potential in efficient production of enzymes within the *Aspergillus* genus, and is therefore employed in the fields of genetic engineering and biotechnology to create industrial enzymes. Chaturvedi and S. Uma Gowrie 2016 have reported that fungal colonized in *Cardiospermum halicacabum* and shown prolific secondary metabolite production. In the present study FTIR analysis of ethanolic leaf extract of *cardiospermum halicacabum* demonstrated the presence of functional groups for flavonoids and esters which are secondary metabolites of fungal endophytes (Table 3 & Fig 5).

Conclusion

The present study is a pioneering attempt to isolate endophytic fungi from *Cardiospermum halicacabum* roots and screening for phosphate solubilization. Endophytic fungi are capable of promoting plant growth and also their secondary metabolites are found to elicit antibacterial and antiviral role. Novel plants species are under the stage of extinction and also less awareness among people. When such beneficial symbiotic endophytic fungus are employed as plant growth promoting bioinoculants it may confer its medicinal values on their host.

Funding

This research received no external funding.

Acknowledgments

Very thankful to Jamal Mohammed college for permitting do this research work

References

1. Andreea Cosoveanu, Samuel Rodriguez Sabina, Raimundo Cabrera. Fungi as Endophytes in Artemisia thuscula: Juxtaposed Elements of Diversity and Phylogeny J Fungi. 2017, 2018; 4:17
2. *Aspergillus oryzae* Final Risk Assessment." Biotechnology Program under the Toxic Substances Control Act (TSCA). United States Environmental Control Agency, 1997.
3. Castresana J. Selection of conserved blocks from multiple alignments for the iruse in phylogenetic analysis. Molecular Biology and Evolution. 2000; 17:540-552.
4. Chathurdevi G, Uma Gowrie S. Endophytic Fungi Isolated From Medicinal Plant-A Promising Source of Potential Bioactive Metabolites. International Journal of Current Pharmaceutical Research. 2016; 8(1)
5. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F *et al*. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 2008; 1:36.
6. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32(5):1792-1797.
7. Edwards U, Rogall T, Blocker H, Emde M, Bottger EC. Isolation and directcomplete nucleotide determination of entire genes: characterization of a gene coding for16S ribosomal RNA. Nucleic Acids Res. 1989; 17:7843-7853.
8. Goldstein AH, Krishnaraj PU. Phosphate solubilizing microorganisms vs. phosphate mobilizing microorganisms: What separates a phenotype from a trait? In: Velázquez E, Rodríguez-Barrueco C (eds). First international meeting on microbial phosphate solubilization. Development in Plant and Soil Sciences. Springer, Dordrecht. 2007; 102:203-213.
9. Hardoim PR, van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A *et al*. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiol. Mol. Biol. Rev. 2015; 79:293-320. [CrossRef] [PubMed]
10. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol. 1983; 7:1141-1148
11. Ludwig-Müller J. Plants and endophytes: Equal partners in secondary metabolite production? Biotechnol. Lett. 2015; 37:1325-1334. [CrossRef] [PubMed]
12. Machida M, Yamada O, Gomi K. "Genomics of *Aspergillus oryzae*: Learning from the History of Koji Mold and Exploration of Its Future." Oxford Journals: DNA Research. 2008; 15(4):173-18321.
13. Mehta S, Nautiyal CS. An efficient method for qualitative screening of phosphate-solubilizing bacteria. Current Microbiology. 2001; 43(1):51-56.
14. Nikkuni S. Evolutionary relationships among *Aspergillus oryzae* and related species based on the sequences of 18S rRNA genes and internal transcribed spacers. J Gen Appl Microbiol. 1998; 44(3):225-230.
15. Pikovskaya RI. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Microbiologia. 1948; 17:362-370.
16. Jeyadevi R, Sivasudha T, Ilavarasi A, Thajuddin N. Chemical Constituents and Antimicrobial Activity of Indian Green Leafy Vegetable Cardiospermum halicacabum. Indian J Microbiol. 2013; 53(2):208-213.
17. Rajagopal K, Maheswari S, Kathiravan G. Diversity of

endophytic fungi in some tropical medicinal plants-A report. Afr. J Microbiol. Res. 2012; 6:2822-2827
18. Sneha Ogale, Karan Singh Yadav, Shrutika Navale. Screening of endophytic bacteria from the pharmacologically important medicinal plant *gloriosa superba* for their multiple plant growth promoting properties. The Pharma Innovation Journal. 2018; 7(1):208-214
19. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Systematic Biology. 2007; 56:564-577.
20. Verma VC, Gond SK, Kumar A, Kharwar RN, Boulanger LA, Strobel GA. Endophytic Fungal Flora from Roots and Fruits of an Indian Neem Plant Azadirachta indica A. Juss., and Impact of Culture Media on their Isolation. Indian J Microbiol. 2011; 51:469-476. [CrossRef] [PubMed].
21. Verma VC, Gond SK, Kumar A, Mishra A, Kharwar RN, Gange AC. Endophytic Actinomycetes from Azadirachta indica A. Juss.: Isolation, Diversity, and Anti-microbial Activity. Microb. Ecol. 2008; 57:749-756.[CrossRef] [PubMed]
22. Verma VC, Singh SK, Kharwar RN. Histological Investigation of Fungal Endophytes in Healthy Tissues of Azadirachta indica A. Juss. Kasetsart J Nat. Sci. 2012; 46:229-237.
23. Wani ZA, Ashraf N, Mohiuddin T, Riyaz-Ul-Hassan S. Plant-endophyte symbiosis, an ecological perspective. Appl. Microbiol. Biotechnol. 2015; 99:2955-2965. [Cross Ref] [PubMed]