The Pharma Innovation Journal 2021; 10(4): 1038-1044

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(4): 1038-1044

www.thepharmajournal.com
Received: 15-02-2021
Accepted: 17-03-2021

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Isolation of an endophytic fungus *Colletotrichum* sp. and study of its plant growth promoting traits

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DOI: https://doi.org/10.22271/tpi.2021.v10.i4o.6129

Abstract
Some fungi grow within plant parts and live as endosymbiont without causing apparent disease and enhance host growth, carrying defense response, nutrient acquisition, decrease biotic and abiotic stress, used as biofertilizer, also great role in medicine industry, known as endophytic fungi. Here five different fungi strains have been isolated from different plant parts of *Plumbago zeylanica* Linn. from the Departmental garden of Botany department of Burdwan university. These fungi strains show different plant growth traits like IAA production, Nitrogen fixation, phosphate solubilization, production of ammonia, polysaccharide, HCN, organic acid. These strains also can tolerate from different concentrations of salt. Among all fungal strains SL4 was identified as *Colletotrichum* sp. by 18S rRNA gene sequencing and gave the best result in most of the PGP traits of the fungal strains. Considering all these properties, SL4 strain can be used in agricultural field for cultivation of crops or in different nurseries as phytostimulator as well as biofertilizer.

Keywords: Endophytic fungi, plant growth promoting fungus, *Colletotrichum* sp. Biofertilizer

1. Introduction
Environmental pollution is the biggest problem of today's world which is one of the main cause of destruction of green world. Today's important pollutants are chemical fertilizer, pesticides, herbicides which is very harmful for all the living organisms including insects, plants, human-being etc. Use of chemical fertilizer gives a bad impact on soil quality by increasing the acid level and make toxic. These fertilizers leads to eutrophication, kill water animals. These types of fertilizers also help in increasing global warming, kill agricultural soil friend earthworms, decrease nutrition value of plants, bad for human health by consumption of chemicals through food. For all these reasons use of bio-products like bio-fertilizers have been increased. Fossil fuel, chemical fertilizers these compounds take a great role for making pollution. Fossil fuel such as coal, oil, natural gas release carbon dioxide, methane, nitrogen etc in the atmosphere by burning which cause disbalance of natural compounds. This leads to smog, acid rain, greenhouse effect. These all are the reason of global warming. If we use bio-fuel in the place of fossil-fuel we can reduce the rate of pollution. Another important area of today's world is the use of chemical medicines which sometimes damage the internal health of living organisms. By using bio-medicines we can recover that bad impact of our health. Endophytic fungi have a great role to solve the above mentioned problems. Endophytes help in host's growth by increasing the amount of plant growth promoting traits like IAA production, phosphate solubilization, nitrogen fixation, HCN production, organic acid production, ammonia production, EPS production, siderophore production etc [1]. Plant growth promoting fungi and bacteria (PGPF & PGPR) directly and indirectly help in plant growth promotion. PGPF have multipurpose beneficial role in plant growth promotion [2]. Endophytes now-a-days use in medicinal industries in huge amount. *Plumbago zeylanica* Linn. known as "Chitrak" have a number of medicinal properties having the metabolite "Plumbagin". *P. zeylanica* has anticancer, antibiotic, anti microbial [3], antineoplastic [4], antifertility, anti diabetic properties, sometimes used for abortion [5, 6]. Endophytes, PGPF from *P. zeylanica* used in medicine industry and also they have the the plant growth promotion ability so used in agriculture field for crop yielding.

2. Material and methods
2.1 Isolation of endophytic fungi from the plant *Plumbago zeylanica* Linn.
Fresh plants (*Plumbago zeylanica*) were collected from the departmental garden of Golapbag Campus of The University of Burdwan (Latitude- N 23˚14' 20.86", Longitude- E 87˚51'45.743").

1st time the plant was washed in running tap water and then repeatedly washed in distilled water. For isolation root (secondary and tertiary), stem samples were cut into 1cm length and 'Y' shapes were made and leaf samples were cut into 1cm^2 blocks. In front of laminar air flow plant parts were passed serially through sterilized distilled water, ethyle alcohol for 1 minute, 0.01% NaOCl solution for 1 minute and antibiotic (streptomycin) water for 1 min. Plant parts were transferred into Potato Dextrose Agar media [Potato infusion- 200 g/l, Dextrose (Glucose)- 20 g/l Agar- 20 g/l pH after sterilization (25℃) 5.6±0.2] and Nutrient Agar media [Peptone- 5g/l, NaCl- 5g/l, Yeast extract- 1.5g/l, Beef extract- 1.5g/l, Agar- 20 g/l pH after sterilization (25℃) 6.8±0.2] containing petriplates and incubated in BOD incubator at 30℃. After 72 hours 5 different fungal endophytes were isolated with distinct morphology. Pure culture was done and maintained in slants after each 15 days interval by subculturing the strains.

2.2 Morphological characterization of 5 Fungal Endophytes

Morphological characters like shape, colour, surface, margine of fungal colonies were observed from 5 day old colony. (Table 1)

2.3 Characterization of Plant Growth Promoting Traits (PGP) *in vitro* condition

2.3.1 Qualitative estimation of Nitrogen fixing ability

Fungal strains were inoculated in the petriplates containing Ashbay's Mannitol Agar media [Dipotassium phosphate- 0.2 g/l, Mannitol- 20 g/l, Sodium chloride- 0.2 g/l, Magnesium sulphate- 0.2 g/l, Calcium carbonate- 5 g/l, Agar- 20 g/l, pH (at 25℃) 7.4±0.2] without any nitrogen source for 4 days. O ccurrence of fungal colonies indicates its ability to fix atmospheric nitrogen. (Table 2).

2.3.2 Qualitative estimation of phosphate solubilizing ability

Fungal isolates were inoculated in PKV Agar media [7] [Ferrus sulphate- 0.0001 g/l, Manganese sulphate- 0.0001 g/l, Magnesium sulphate- 0.1 g/l, Potassium chloride- 0.2 g/l, Ammonium sulphate- 0.5 g/l, Calcium phosphate- 5 g/l, Dextrose- 10 g/l, Yeast extract- 0.5 g/l, Agar 20 g/l] incubated for 4 days at 30℃. Presence of clear halo zone surrounding the colony is the positive indication of phosphate solubilizing ability. Phosphate solubilisation index (PSI) was calculated by the following formula [8] (Table 3).

PSI = (Colony diameter + Halozone diameter) / Colony diameter

2.3.3 Quantitative estimation of phosphate solubilizing ability

Fungal strains were were inoculated in PKV broth in shaker incubator at 180 rpm for 5 days. After incubation period cultures were centrifused at 10000 rpm for 10 minutes. Then 1 ml of supernatant was added to 2.5 ml of Barton's reagent, volume made upto 5 ml with distilled water following the Ammonium- molybdate method [9]. OD was taken at 430 nm wave length and concentrations of solubilized phosphates were determined with the standard curve of KH_2PO_4. (Table 2, Fig 1)

2.3.4 Quantitative estimation of Indole Acetic Acid production ability

Fungal isolates were inoculated in Nutrient broth supplemented with 0.5% L-Tryptophan and incubated for 4 days at 30℃. After incubation period cultures were centrifuged at 7000 rpm for 10 minutes. 2 ml of supernatant was mixed with 2 ml of Salkowsky's reagent (35% perchloric acid mixed with 1 ml of 0.5 M $FeCl_3$) solution. The reaction mixtures were kept in dark condition for 15 minutes. Appearance of pink colour indicated the IAA production ability. OD value was measured at 530nm and estimated using a standard curve of Auxin [10]. (Table 2, Fig 2)

2.3.5 Production of HCN (Hydrogen cyanide)

Fungal isolates were inoculated in Nutrient Agar media supplemented with 4.4 g/l glycine and incubated for 6-7 days at 30℃. Whatman filter paper no. 1 was impregnated at the inner surface of the lid of petriplates and the filter papers were dipped in 2% sodium carbonate in 0.5% picric acid solution. Occurance of brownish orange colour in the filter papers indicated the HCN production ability of the fungal strains. Method of Lorck H (1948) [11] was followed. (Table 2)

2.3.6 Ammonia production ability

Fungal isolates were inoculated in Peptone broth following the method of Cappuccino *et al*, (1992) [12] and incubated for 4 days at 30℃. After incubation period 1 ml of each strain was addad to 0.5 ml of Nesseler reagent and development of yellowish orange colour indicated the capacity of ammonia production of fungal strains. (Table 2)

2.3.7 Estimation of Exo-polysaccharide production ability

Following the method of Dubois *et al* (1956) [13] fungal strains were grown in Nutrient broth in BOD incubator for 4 days at 30℃. After incubation cultures were centrifuged at 7000 rpm for 10 minutes. 1 ml of supernatant was added to 3 ml of Acetone and re-centrifused, this process was repeated for more 4-5 times. Then supernatant was suspended with distilled water. 1 ml of supernatant was mixed with 1 ml of 5% aquas phenol and then 5 ml of concentrated Sulphuric acid and kept for 20 minutes. Occurance of brownish red colour indicated the presence of exo-polysaccharide. OD value was measured at 490 nm and concentration was determined using the standard curve of glucose. (Table 2, Fig 3)

2.3.8 Production of Organic acids

Strains were grown in PKV broth media individually at 28℃ for 4 days to show the ability of organic acid production of the endophytic fungal strains. Before inoculation pH of the media was taken. After incubation period was over pH of was taken again. The decreament of the pH proved the presence of oraginc acids. (Table 2)

2.3.9 Production of Siderophore

Siderophore production was determined by following the method of Schwyn and Neilands (1987) [14]. Fungal strains were inoculated in the blue agar plates containing the dye chrome azurol S (CAS) and occurance of orange halo zone around the colonies of blue plates indicated the positive resul of siderophore production. (Table 2)

2.3.10 Salt stress tolerance ability test

The five fungal strains were assessed for resistance against different conc. of salts. PDA media was mixed with solution of different conc. of NaCl (2%, 4%, 6%, 8%, and 10%) and allowed to grow at 30ºc for 72 hours. Growth analysis was done and recorded. (Table 4)

2.4 Selection of best performing endophytic fungal strain
On the basis of PGPF traits SL4 was selected as best performing endophytic fungal strain. Molecular characterization of SL4 was carried out in National Centre for Microbial Resource (NCMR), Pune.

2.5 Cell density and amount of phosphate solubilization
Cell density of SL4 was measured by measuring the dry weight of the fungal culture and phosphate solubilization [9] was measured in every 24 hours interval for 6 days. (Fig 4)

2.6 Cell density and amount of IAA production
Cell density of SL4 was measured by measuring the dry weight of the fungal culture and IAA production [10] was measured in every 24 hours interval for 6 days. (Fig 5)

2.7 Cell density and amount of Exo-polysaccharide production
Cell density of SL4 was measured by measuring the dry weight of the fungal culture and EPS production [13] was measured in every 24 hours interval for 6 days. (Fig 6)

2.8 Identification of the selected endophytic fungal strain
Molecular identification was done using 18S rRNA gene sequencing method by NCMR-NCCS, Pune. Phylogenetic tree was prepared using MEGA 7.0 software. (Table 5, Fig 7)

3. Results

Table 1: Morphologiacal characterization of five fungal endophytes

Fungal strains	Shape	Surface	Margine	Colour
SRI	Irregular	Rough	Smooth	White
SS2	Irregular	Rough	Wavy	Yellowish white
SL2	Round	Smooth	Wavy	Off-white
SL4	Irregular	Rough	Wavy	Greenish
SL5	Round	Smooth	Smooth	Off-white

Table 2: Characterization of plant growth promoting traits of 5 fungal endophytes

Name of the isolates	Nitrogen fixation	Phosphate solubilization	IAA production	HCN Production	Ammonia Production	EPS Production	Organic acid Production	Siderophore Production
SR1	P	P	P	P	P	P	P	P
SS2	P	P	P	N	P	P	P	N
SL2	N	N	P	N	P	N	P	N
SL4	P	P	P	P	P	P	P	P
SL5	N	N	P	N	P	P	N	N

'P' and 'N' represent positive and negative results respectively. All data are mean value of three replicates.

Table 3: Phosphate solubilization index (PSI) of five fungal strains

PSI	SR1	SS2	SL2	SL4	SL5
	2.25	2.16	-	3.047	-

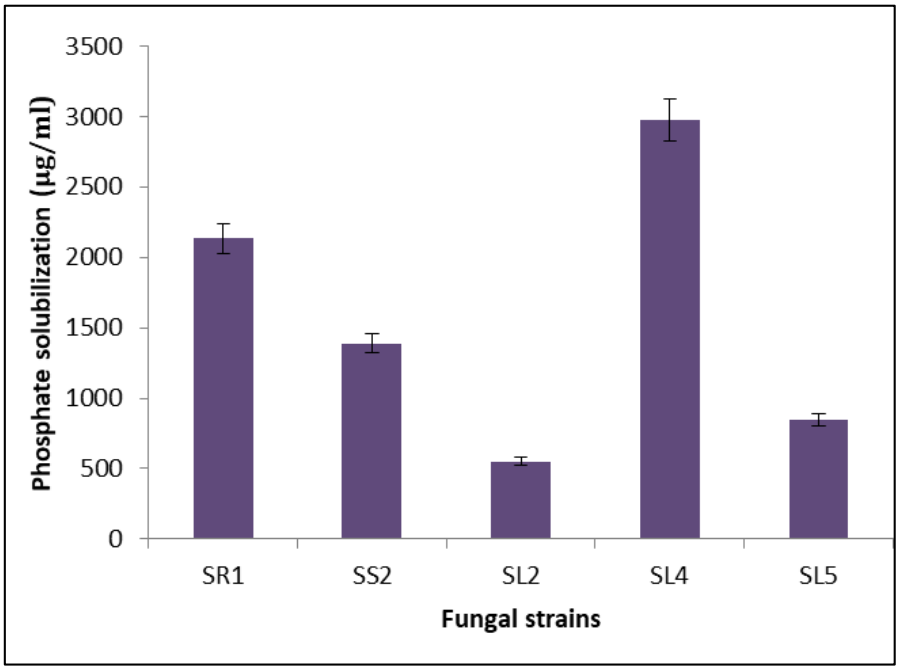

Fig 1: Quantitative estimation of phosphate

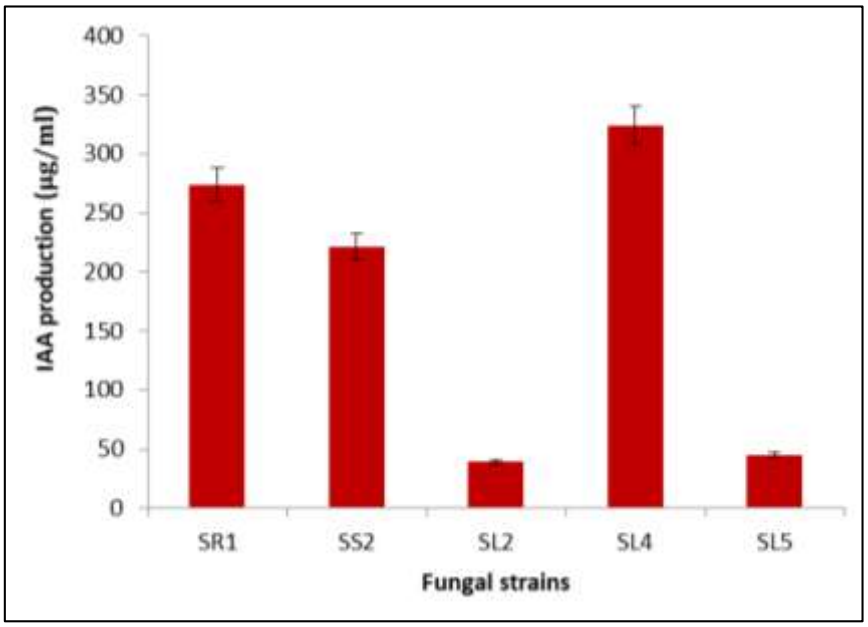

Fig 2: Quantitative estimation of IAA production

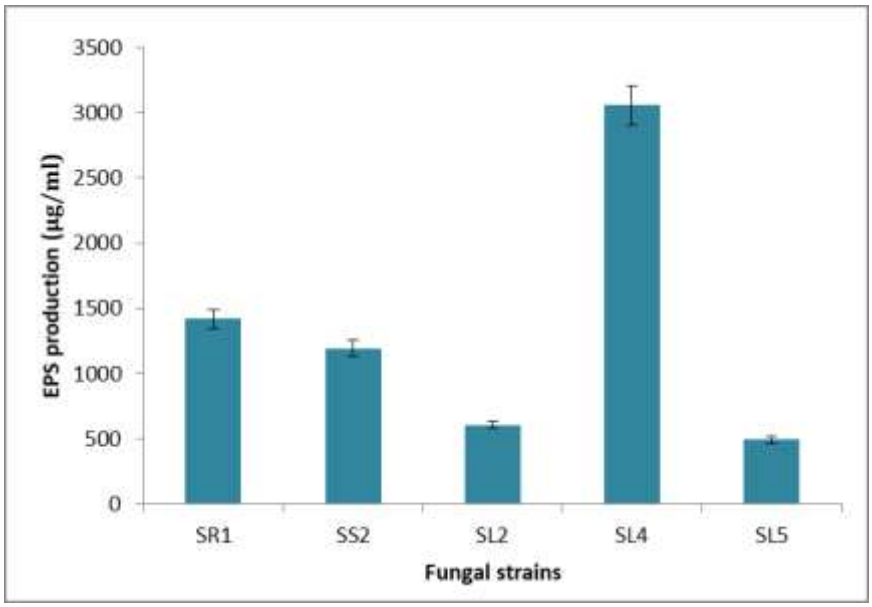

Fig 3: Quantitative estimation of EPS production

Table 4: Growth on different concentration of NaCl supplemented media

Fungal isolates	Growth on different concentration of NaCl supplemented media				
	2%	4%	6%	8%	10%
SR1	P	P	P	P	N
SS2	P	P	N	N	N
SL2	P	P	P	N	N
SL4	P	P	P	P	P
SL5	P	P	N	N	N

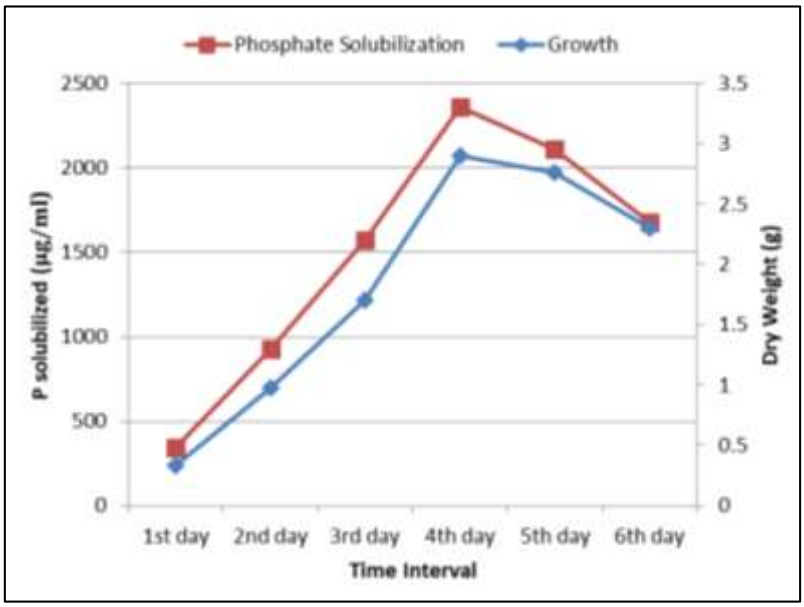

Fig 4: Growth curve and phosphate solubilization

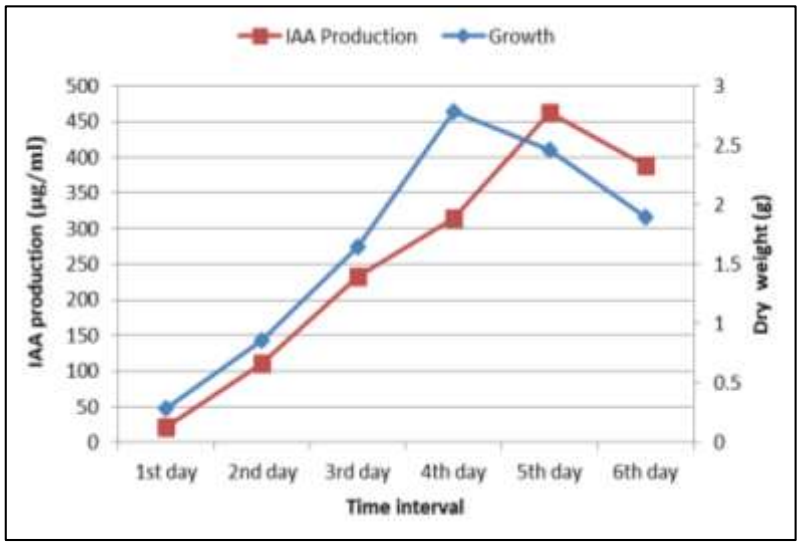

Fig 5: Growth curve and IAA production

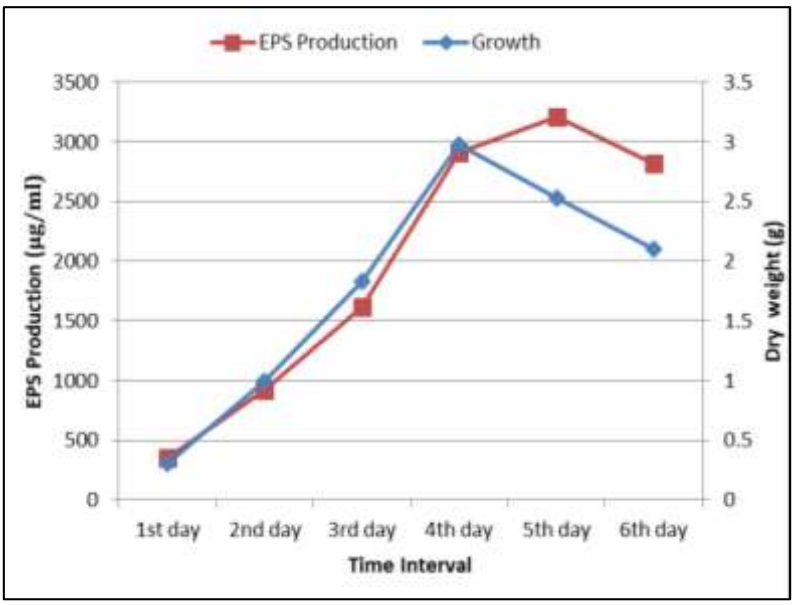

Fig 6: Growth curve and EPS production

Table 5: Molecular Identification of the fungal strain SL4

Strain No.	Closest Neighbour*		% Similarity
	Taxonomic Designation	Accession No.	
SL4	*Colletotrichum salicis* isolate RB157	MK541032.1	98.96
	Colletotrichum lupini isolate RB173	MK541036.1	98.57
	Colletotrichum simmondsii isolate RB179	MK541034.1	98.57

Sequence Text (in FASTA format) of SL4

TGGCTCATTATATAAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACA
TGCTAAAAATCCCGACTTACGAAGGGATGTATTTATTAGATTAAAAACCAATGCCCTTCGGGGCTCACTGGTGATTC
ATAATAACTTCTCGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGCTA
GAGTAGTGTTCTAGCATGGTTACAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAAACG
GCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACGATAAATAC
TGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGC
AAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTA
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TCATGCCCTTCACTGGGTGTGTGGGAAAACAGGACTTTTACTTTGAAAAAATTAGAGTGCTCCAGGCAGGCCTATG
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TAATAGGGACAGTCGGGGGCATCAGTATTCAATTGTCAGAGGTGAAAATTCTTGGATTTATTGAAGACTAACTACT
GCGAAAGCATTTGCCAAGGATGTTTTCATTTATCAGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCG
TAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTTTTTGACTCGTTCGGCACCTTACGAGAAAT
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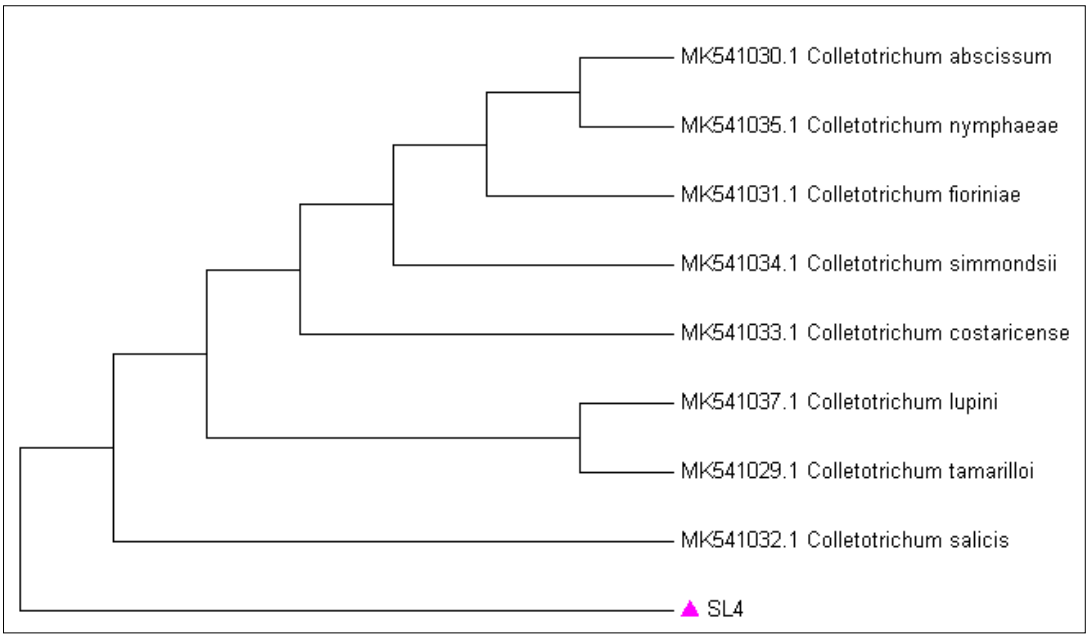

Fig 7: Phylogenetic tree of SL4

3.1 Microscopic Characteristics of SL4

Hyphae: hyaline to brown, branched, long hyphae. Appressoria: abundant, pale to medium brown with irregular in shape.Conidiogenous cells: cylindrical to clavate, hyaline, cylindrical. After 20 days conidia formed in a orange masses, hyaline, aseptate, straight, cylindrical, guttulate, rounded at both ends, measuring 9.52-13.78 (11.64) x 2.95-4.73 (3.91) µm. Ascoma: an ostiolateperithecium, dark brown, obpyriform to subglobose, 772-760 µm high, 610-730 µm wide. Ascospores: hyaline, fusoid to cylindrical, mostly straight to slightly curved, rounded at both ends, aseptate, 1-septate at maturity, septum median, 9.06-14.83 (12.29) x 3.02-4.16 (3.62) µm.

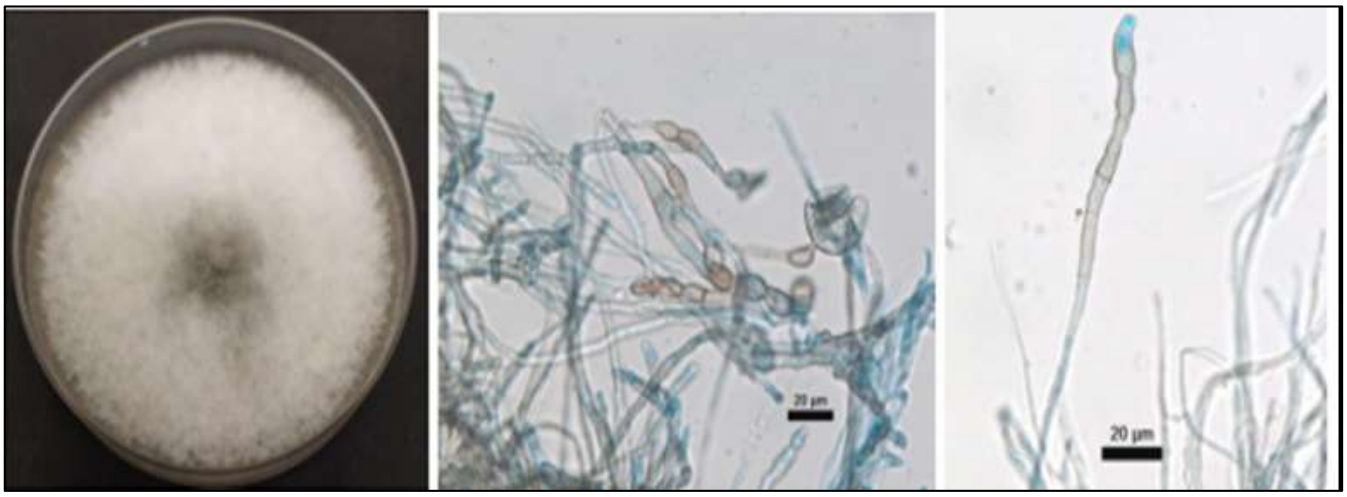

Fig 8: Photographs of the strain SL4

4. Discussion

In the isolation process five different fungal isolates were selected (from root 1, from leaf 3 and from shoot portion 1) with distinct morphology. After studying the colony morphologies, different plant growth promoting traits were studied like nitrogen fixation ability test, phosphate solubilization, HCN test, Ammonia test, Organic acid producing test, IAA production test, Exo-polysaccharide production test, Siderophore production. In most of the tests SL4 gave the best results among all the fungal isolates. Even in salt stress test, SL4 showed maximum capacity (10%) to tolerate. Regular estimation of IAA of SL4 was done with measuring the cell density for 6 days. On the 5th day of estimation IAA production was highest and then started to decline. Regular estimation of EPS production and phosphate solubilizition of SL4 was done along with measuring the cell density for 6 days in interval of 24 hours. EPS production showed best result on 5th day and phosphate solubilization showed best result on 4th day of the above mentioned test and then started to decline. SL4 showed best growth on 4th day of incubation period and the fungal mat looked like rough and pale white in colour, but from 6th day of incubation period the colour of the fungal mat turned into greenish. For optimum growth of this ascospore bearing fungus needs 30^{o}C temperature. According to NCMR-NCCS, Pune this particular fungal strain (SL4) showed the maximum similarity (98.57% - 98.96%) with *Colletotrichum* sp. (18S rRNA gene sequencing).

5. Conclusion

According to the above study it can be concluded that SL4 is itself very efficient plant growth promoting endophytic fungi which can be used as good bio-fertilizer mainly for the crop plants. It has very significant roles for producing IAA, HCN, siderophore etc. It will enhance the crop production and environment will remain harmless.

6. Acknowledgement

The authors wish to acknowledge Laboratory infrastructure and instruments provided by Mycology and Plant Pathology UGC-CAS department of Botany, the University of Burdwan and my supervisor Prof. Sikha Dutta for her continuous guidance and support.

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