IJBPAS, October, 2021, 10(10): 3479-3491

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

EX-SITU EVALUATION OF ENDOPHYTE *COLLETOTRICHUM SIAMENSE* ISOLATED FROM *CAPSICUM ANNUUM* L. OF ASSAM AGAINST CHILI PATHOGEN *FUSARIUM LICHENICOLA*

BARMAN I[1*], GOGOI J[1] AND DUTTA AM[2]

1: Programme of Biochemistry, Faculty of Science, Assam Down Town University

2: Programme of Chemistry, Faculty of Science, Assam Down Town University

***Corresponding Author: Ms. Indrani Barman: E Mail: rcheindrani@gmail.com; Contact Number: (91)8638783226**

Received 24th Nov. 2020; Revised 30th Dec. 2020; Accepted 9th Jan. 2021; Available online 1st Oct. 2021

https://doi.org/10.31032/IJBPAS/2021/10.10.5655

ABSTRACT

An important cash crop of India is chili (*Capsicum* spp.). This spice crop suffers huge losses in storage and transportation due to microbial infection. *Fusarium lichenicola* is well known as a causal agent of fruit rot in chili fruits. The aim of the study was to find out the antifungal activity of the isolated endophyte against the isolated pathogen from spoiled chili. The present study was conducted by isolating fungal endophyte from fresh leaves of *Capsicum annuum* and its metabolites were extracted. In ex-situ study, these metabolites were treated against chili (*Capsicum frutescens* and *Capsicum assamicum*) brought from the market and stored in normal room temperature. Molecular analysis revealed the presence of the endophyte *Colletotrichum siamense*. Metabolite detection showed the secretion of alkaloid and reducing sugar in ethyl acetate fraction. Treatment against spoilage of *C.frutescens* showed best results when treated with ethyl acetate fraction of *C.siamense*. But in case of *C.assamicum*, mixture of ethyl acetate fraction of *C.siamense* and chloroform fraction of *Phyllantus emblica* showed better efficacy followed by only ethyl acetate fraction of the endophyte against spoilage. So, it could be concluded that ethyl acetate fraction of *C.siamense* has a potent metabolite against chili

pathogen, *F.lichenicola*. Also, the present study is the first report of the endophyte *C.siamense* found in *C.annuum* grown in North-east Guwahati region of Assam.

Keywords: ex-situ study, *C.siamense*, *F.lichenicola*, *C.frutescens*, *C.annuum*

INTRODUCTION

Capsicum annuum L. is widely recorded to be an economically important and major spice crop for different countries in the world **[1]**. Our subcontinent India holds the place of largest chili producer in the world scenario with million tons as its annual production **[2]**. Chili benefits were already in knowledge of pre-Hispanic people and evidences from modern science largely confirmed both its medicinal and nutritional value **[3]**. Stored and transported chili (*Capsicum annuum and Capsicum assamicum*) fruits are prone to varying degrees of decay in terms of discoloration as well as breaking of pericarp, pedicel detachment and formation of spore dust inside the fruit. Numerous management practices have been employed but these agricultural chemicals are a major threat to environmental pollution as well as healthcare system. Therefore, the major search for environment friendly, toxicologically safe and cheap biocontrol agent remains the need of the research **[4]**.

Pharmaceutical as well as agricultural industries are in continual hunt to screen out contemporary products. In this regard, natural selection stands superior to chemical synthetic chemistry for discovering unique compounds that could be formulated into industrial products **[5]**. Thus, endophytic fungi represent a biotope for the exploration of novel secondary metabolites. They grow internally in host plant tissue without causing any overt negative effect and present an unexplored trove of metabolic relationship between fungus and host **[6, 7]**. They are chemical synthesizers inside host plant that synthesize biologically active compounds which are utilized by plants for their protection against pathogens **[8]**. Fungal endophytic strains are known to be potentially important in pigment production, immunosuppressant, biocontrol agents and anticancer substances **[9, 10, 11]**.

Many fungal endophytes can produce potent antimicrobial agents which could lead to a promising new pavement to combat drug-resistant strains of human and plant pathogens. The production of phytoalexins by endophytic fungus and their ecological occupation pops up to be the prime reason towards plant protection by these endophytes. They can synthesize both single and multiple array of antibiotics starting from

alkaloids, terpenoids, polypeptides to aromatic compounds **[12]**. Amongst these, alkaloids are known to be strong suppressors of microbes. Altersetin is an example of an alkaloid isolated from *Alternaria* spp. Possess antibacterial properties against pathogenic bacteria **[13]**. In Assam, however, studies on fungal endophytes from chili plant as biocontrol agents have not been recorded. The main aim of the study was to isolate endophytic fungi from leaves of chili plant species *Capsicum annuum L.* and extract bioactive secondary metabolites and further *ex-situ* evaluate the efficacy of these fungal metabolites as antimicrobial agents in treating chili fruit (*C.frutescens* and *C.assamicum*) pathogens during storage.

MATERIALS AND METHODS

Collection of sample:

Leaf sheath of chili variety (*Capsicum annum* L.) was collected from Kundil nagar area of Narengi, Guwahati, Assam. Fresh and uninfected leaf samples were used in this study.

Isolation of fungal endophytes and pathogen:

Fresh leaves were surface sterilized by immersing in 70% ethanol, 0.5% NaClO and distilled water using standard procedure. The leaves were cut aseptically into smaller segments and were carefully placed on potato dextrose agar (PDA) petri plates and incubated at 28 ºC for 72 hrs **[14]**. The cultures were routinely maintained on slants for further analysis.

Pathogen was isolated from the spoiled chili fruits **(*Capsicum frutescens and C.assamicum*)** by allowing it to grow on to a Potato Dextrose Agar (PDA) media and incubated at 28 ºC for 24-96 hrs **[15]**. The cultures were routinely maintained on slants for further studies.

Morphological and molecular identification of the fungal isolates (both endophyte and pathogen):

The isolated endophyte and pathogen were morphologically identified using lactophenol cotton blue as per standard protocol **[16]**.

Further, for molecular analysis, genomic DNA was isolated from the mycelial mat as per an established protocol **[17]**. Endophytic and pathogenic fungal species was identified by sequencing (ABI 3500 Genetic Analyzer**)** the ITS forward primer (5' GRAAGNAHADGTVGKAAYAWSG-3') and ITS reverse primer (5'-TCCTNCGYTKATKGVTADGH-3'). Sequence homology of nucleotide was compared using BLAST search program. The alignment of sequences and phylogenetic tree were constructed using weighbor software.

The bootstrap replications were used as a statistical support for the nodes in the phylogenetic tree.

Metabolite extraction:

The endophytic isolates were further processed using solvent-solvent extraction, where the endophytes were grown in PDA broth and biomass was separated by filtration with slight modification **[18, 19]**. The broth was incubated at room temperature for 21 days. It was filtered using cheesecloth to separate the supernatant and mycelia. The supernatant was used for secondary metabolites extraction by addition of organic solvents (hexane, chloroform, ethyl acetate and butanol) treated thrice in the same ratio as 1:1 v/v. The different extracts were separated from broth medium and crude extract was concentrated by using a rotary evaporator under reduced pressure **[20]**.

Phytochemical screening of the extracts:

The portions of the dry extract were subjected to the phytochemical screening. Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars and cardiac glycosides using standard protocol **[21-24]**.

After the performance of phytochemical analysis through various test mentioned above, different separation techniques was performed for further confirmation about the presence of secondary metabolites such as- TLC (thin layer chromatography) and paper chromatography. Different solvent systems was used for both the techniques and results were being analysed **[25]**.

Ex-situ evaluation studies to treat fungal infection in *C.frutescens* and *C.assamicum* fruits [26]:

The metabolites detected were used in treatment of fungal infection in the chili fruits during storage. A set of five treatment combinations (T1-T5) were prepared for the ex-situ evaluation studies: T1 (control), T2 (streptomycin), T3 (alkaloid from endophyte), T4 (endophyte alkaloid and alkaloid from *Phyllantus emblica)* and T5 (alkaloid from *Phyllantus emblica*). The control set was the normal untreated chili samples studied for a period of 7 days for *C.assamicum* and 5 days for *C.frutescens*. The metabolites were prepared using 2% DMSO prepared in methanol. The chili fruits were topically applied with the treatment combination solutions at 10^{-3} dilution using atomizer and colony forming unit (cfu) count was monitored for an interval of 1,3,5,7 days for *C.assamicum* and 1 to 5 days for *C.frutescens*. All the combinations were compared with the control set and standard and each treatment was replicated three times.

RESULTS AND DISCUSSION

In the present study, fungal pathogen was identified as *Fusarium lichenicola* which belongs to the phylum Ascomycota. The infection was observed in two days in the spoiled chili samples. Morphology of the pathogen showed mat colony with distinct cottony white aerial mycelium. Under microscope, production of elongate, filiform, conidiophores were observed **(Figure 1)**. This is in accordance with another study done by **[27]** which showed similar features.

Its phylogenetic analysis was confirmed by using ITS1 and ITS2 using 1000 bootstrap replication using weighbor software **(Figure 2)**. Similar study was performed for *F.lichenicola* in Pomelo (*Citrus maxima*) which was reported to be the first report causing fruit spoilage **[28]**.

Endophyte was isolated from young healthy leaves of *C.annuum*. Morphological characters showed white mat colony on PDA plate. The organism was slow growing as compared to the pathogen and took approximately one week to cover the entire PDA plate. A unique grey to pale brown colour was observed on the backside of the mat making it distinct from the pathogen. Microscopic features showed the presence of internal, septate, aerial and branched mycelium **(Figure 3)**. Molecular characterisation of this endophyte identified it as *Colletotrichum siamense* belonging to the phylum Ascomycota. ITS1 and ITS2 sequence with 1000 bootstrap replication using Weighbor software was used for molecular study **(Figure 4)**. Earlier reports suggests that *Colletotrichum* species was a dominant endophyte isolated from *C.annuum* at fruiting stage in Korea **[29]**. Also a report from Assam highlighted the presence of *C. gloeosporioides* from tea plant **[30]**. But in north-east Guwahati region of Assam, this is the first report of the presence of *C.siamense* endophyte in leaves of *C.annuum*.

Ex-situ evaluation of the metabolite was extracted from *C.siamense*. The extract was prepared using solvent extraction method after incubation of the culture for 21 days from PDB culture. The extract showed the presence of alkaloid and reducing sugar in ethyl acetate fraction **(Table 1)**. The prevalence of phytochemicals in endophytes is a clear indication towards development of synthetic drugs and explored into medicinal and industrial applications **[31]**.

The dominant extracted metabolite was further chromatographed using different combinations of solvent system. Our study revealed that using relatively high polar solvents like chloroform: methanol (8:2) was best in detection of alkaloid from *C.siamense*

and spot detection was done using iodine fumes. Brown spot development on the TLC plate confirmed the presence of alkaloid which is in accordance with the result of qualitative detection of alkaloid. Other reports also demonstrate the presence of orange /brown spot on TLC plate that correlates with our present study **[25]**.

For ex-situ evaluation, chili varieties (*C.frutescens* and *C.assamicum*) were purchased from the market. The study was carried out at an interval of odd days for *C.assamicum* and consecutive 5 days for *C.frutescens*. Various combinations were used where T3, T4 and T5 against T1 (control) and standard T2 (streptomycin) was studied. The effectiveness of plant extracts on microorganism has been studied worldwide **[29]**. Over the last few years, reports were found on endophytic fungi exhibiting substantial and sustainable antimicrobial activity **[32]**, where inhibitory activity against *Pestalotiopsis* spp. was reported **[33]**. A study in Assam demonstrated saprophytic antagonists and its bio-formulations being evaluated in effective management of bacterial wilt disease in Naga chili. Different bioformulations including talc based formulation, vermicompost (VC) and mustard oil cake (MOC) were applied to seed treatment, seedling treatment, soil application **[34]**.

While treating *C.frutescens* with all the ex-situ treatment groups, the microbial count was found to be increased in combination treatment (T4) as compared to control on day 1 and 2. Since the extract showed the presence of reducing sugar, so it might increase the microbial load in initial days but later the secondary metabolite showed its better result in reducing the microbial load in comparison to control from day 3 to day 5. Best result was seen with T3 in all days compared to standard T2 **(Figure 5a)**.

Ex-situ treatment study on *C.assamicum* also showed better treatment result with T3 as compared to standard T2.Efficiency of T4 was increased as compared to T3 on day 5. So, combinatorial treatment (T4) showed best activity in *C.assamicum* as compared to best result T3 in *C.frutescens* **(Figure 5b)**.

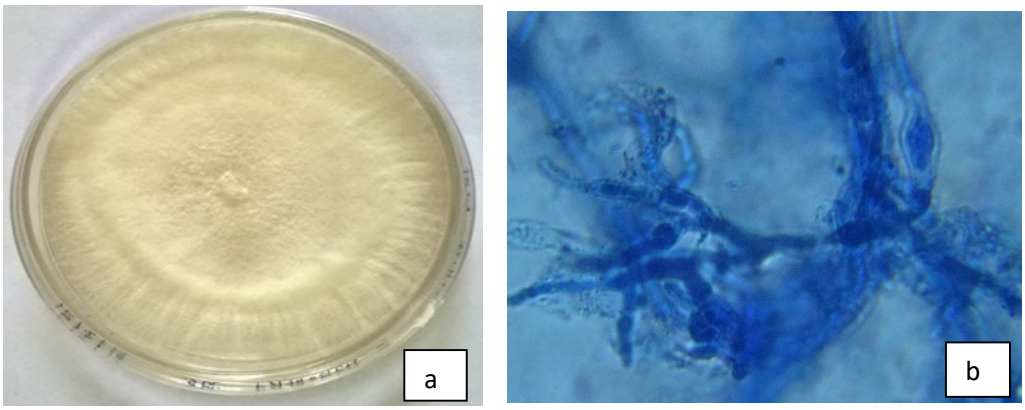

Figure 1: a) Fungal pathogen, *F.lichenicola* on PDA plate b) Morphology based on lactophenol cotton blue staining

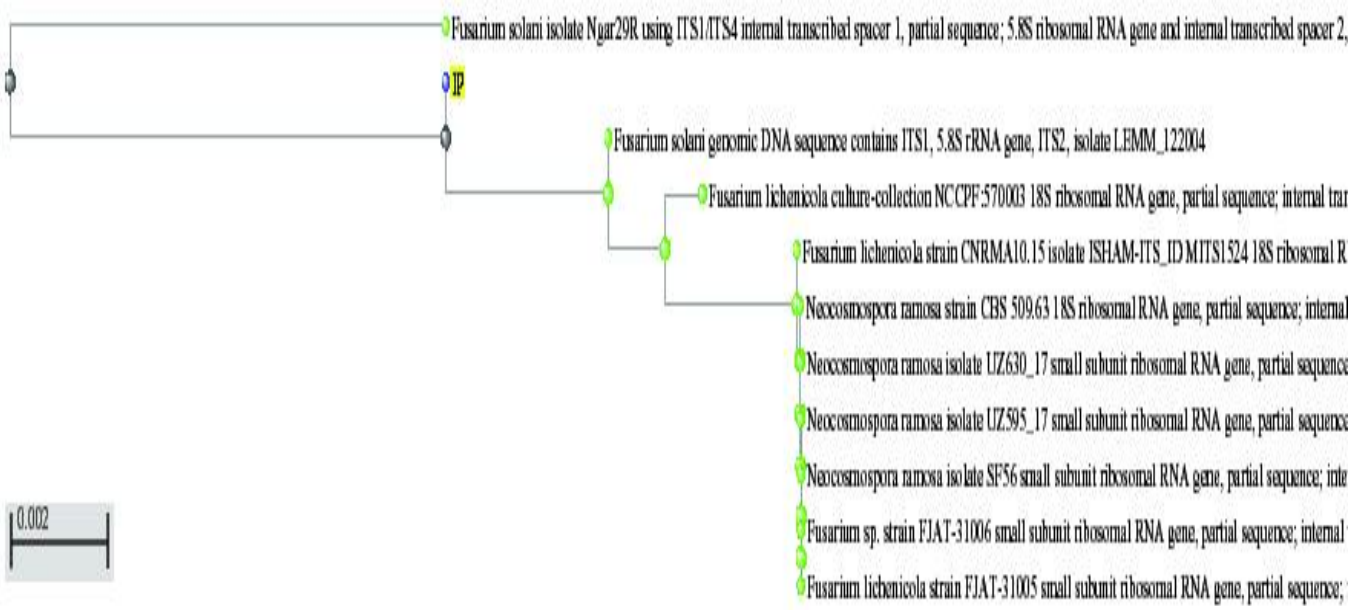

Figure 2: Phylogenetic tree formed by Weighbor joining method using ITS sequence of fungal pathogen *Fusarium lichenicola* and related fungi

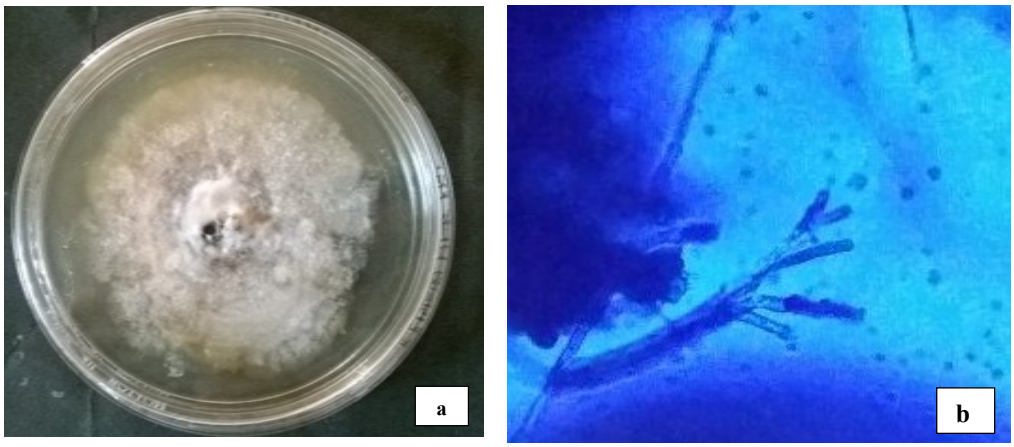

Figure 3: a) White cottony mat colony of *C.siamense* on PDA plate. b) Lactophenol cotton blue staining showing morphology of the fungus

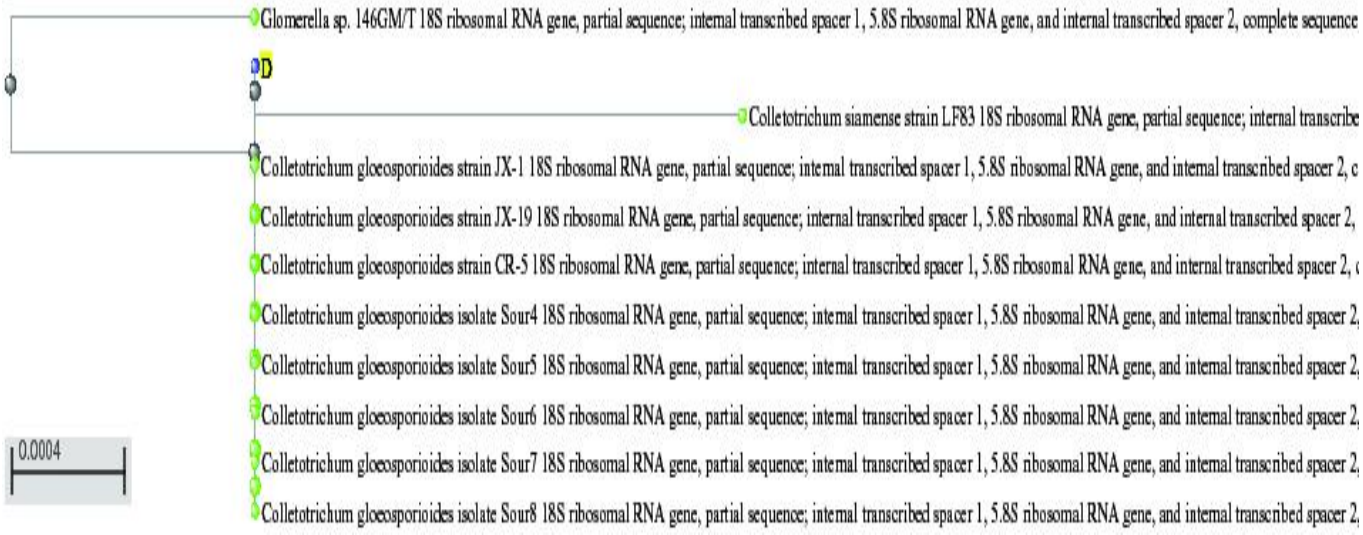

Figure 4: Phylogenetic tree formed by Weighbor joining method using ITS sequence of fungal endophyte *C.siamense* and related fungi

Table 1: Metabolite detection in crude extract of *C.siamense*

Metabolites	Hexane	Chloroform	Ethyl acetate	Butanol
Alkaloid	-	-	+++	-
Phenol	-	-	-	-
Flavonoid	-	-	-	-
Reducing sugar	-	-	+	-
Tannin	-	-	-	-
Steroid	-	-	-	-

**presence of metabolite: sparingly (+), mildly (++), dominant (+++) and absence (-)

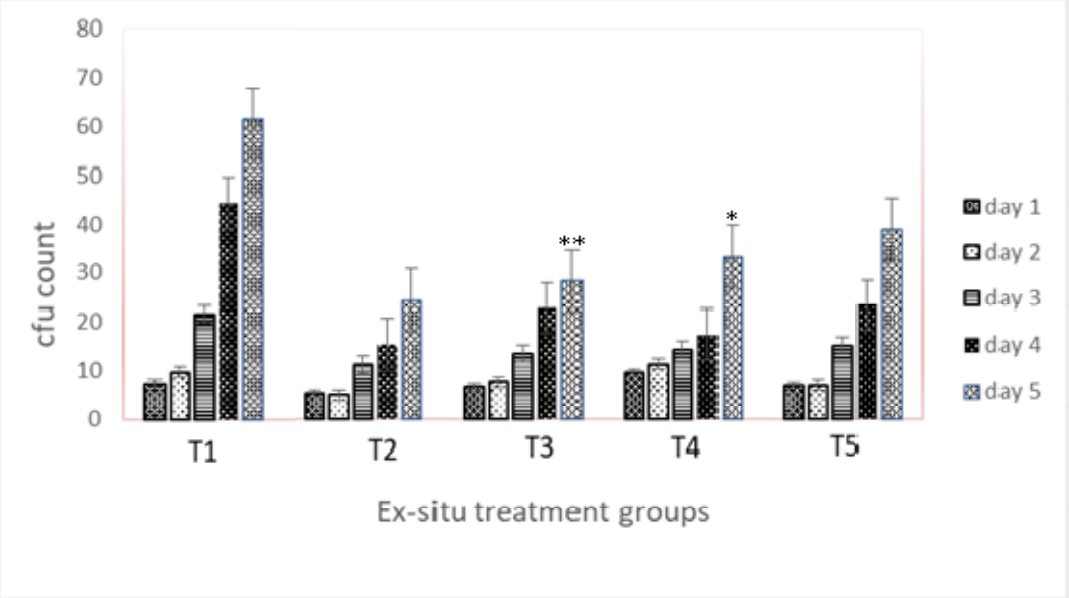

Figure 5a: Graph showing ex-situ data for *C.frutescens*

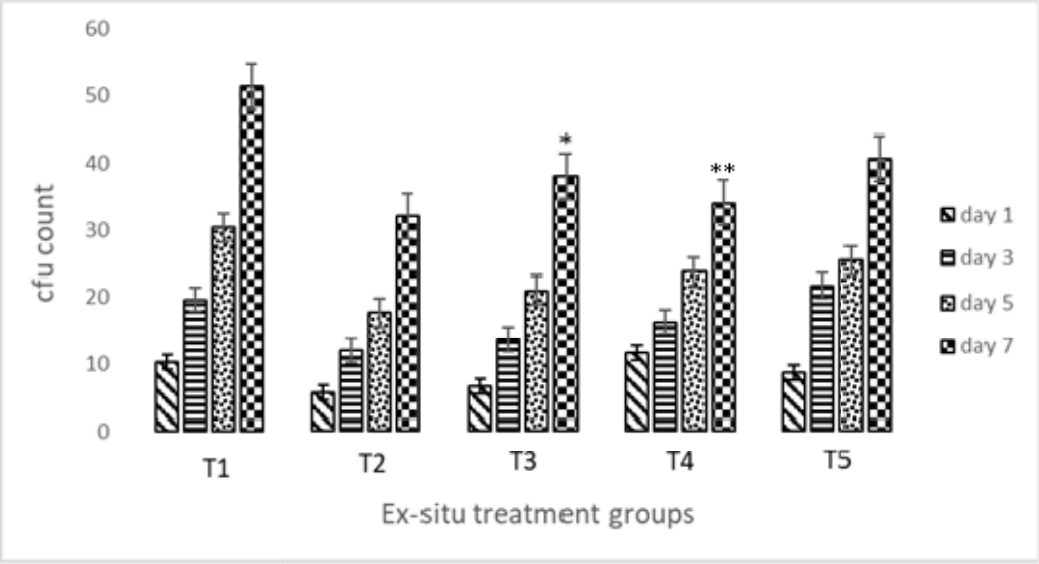

Figure 5b: Graph showing ex-situ data for *C.assamicum*

CONCLUSION

From the present study, it can be concluded that ex-situ evaluation of *C.siamense* secondary metabolite has efficient antimicrobial activity in the formulation of 2% DMSO in methanol. *C.siamense* has been first reported from *C.annuum* from north-east Guwahati region of Assam which has strong antimicrobial activity. Further, chemical study of this metabolite and enrichment of endophyte would help in generating a potent antimicrobial agent for chili infection during storage and transportation.

ACKNOWLEDGEMENT

The authors would like to thank Assam down town University for providing the necessary facilities in conducting the experimental work. Also, we are thankful to Chromous Biotech, Bangalore for conducting the sequencing studies of our samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCE

[1] Allu S, Kumar NP and Audipudi AV. Isolation, Biochemical and PGP characterization of endophytic Pseudomonas aeruginosa isolated from chilli red fruit antagonistic against chilli anthracnose disease. Int. J. Curr. Microbiol. App. Sci. 3, 2014, 318-329.

[2] Khalid S, Iftikhar S, Munir A, Ahmad I. Potato diseases in Pakistan. PARC Islamabad. 2000:165.

[3] Serra I, Yamamoto M, Calvo A. Association of chili pepper consumption, low socioeconomic status and longstanding gallstones with gallbladder cancer in a Chilean population. Int J Cancer.8, 2002, 407–11.

[4] B.K. Prasad, DR Sahoo, M Kumar and N Narayan. Decay of chilli fruits in India during storage. Indian Phytopathology Souzailp. 2012.

[5] Schulz B, Boyle C, Draeger S, Rommert AK and Krohn K. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol. Res. 106 (9), 2002, 996-1004.

[6] Petrini, O. Fungal endophytes of tree leaves. In Microbial Ecology of Leaves (J. Andrews & S. Hirano, eds), 1991, 179-197.

[7] Wilson, D. Endophyte -the evolution of a term, and clarification of its use and definition. Oikos 73, 1995, 274-276.

[8] Owen N.L., Hundley N. Biodiversity of Marine derived fungi and

identification of their metabolites. Sci. Prog. 87, 2004, 79–99.

[9] Gangadevi V.Rani. Screening Endophytic Fungi Isolated from a Medicinal Plant, *Acalypha Indica* L. for Antibacterial Activity. Indian Journal of Science and Technology 2008, 5.

[10] Wang, J. A cytotoxin produced by *Paecilomyces* sp. And *Aspergillus clavatus* isolated from *Taxus mairei* and *Torreya grandis*. FEMS Immunol. Medical Microbiol 2002, 34: 51-57.

[11] Stinson M. An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds, Plant Sci. 165, 2003, 913-922.

[12] Gao F, Dai C and Liu X. Mechanisms of fungal endophytes in plant protection. African Journal of Microbiology Research. 4(13), 2010, 1346-1351.

[13] Hellwig V, Grothe T, Mayer-Bartschmid A, Endermann R, Geschke FU, Henkel T, Stadler MA. Altersetin, a new antibiotic from cultures of endophytic Alternaria spp. taxonomy, fermentation, isolation, structure elucidation and biological activities. J. Antibiot. 55, 2002, 881-892.

[14] Tayung K, Jena SK. Endophytic fungal communities associated with two ethno-medicinal plants of Similipal Biosphere Reserve, India and their antimicrobial prospective. J Appl Pharm Sci, 3(4S-1), 2013, S7–17.

[15] Srividya, Thapa, Bhat, Golmei and Dey. Streptomyces sp. 9p as effective bio control against chilli soil borne fungal phytopathogens. Ind.J.Micro, 2(1), 2012, 163-173.

[16] Astrid. L. Preparation of Lactophenol Cotton Blue Slide Mounts. Community Eye Health 12(30), 1999, 24.

[17] Khan, S.A., M., Hamayun, Z.K.,Shinwari, A.L., Khan, I.J., Lee and J.G. Kim. Isolation of plant growth promoting endophytic fungi from dicots inhabiting coastal sand dunes of Korea. Pak J. Bot., 44(4), 2012, 1453-1460.

[18] Kwon H.R., Son S.W., Han H.R., Choi G.J., Jang K.S., Choi Y.H., Lee S., Sunog N.D., Kim J.C. 2007. Nematicidal activity of bikaverin and fusaric acid isolated from *Fusarium oxysporum* against pine wood

nematode *Bursaphelenchus xylophilus*. Plant Pathol. J. 23 (4): 318–321.

[19] Zin N.M., Sarmin N.I., Ghadin N., Basri D.F., Sidik N.M., Hess W.M., Strobel G.A. Bioactive endophytic streptomycetes from the Malay Peninsula. FEMS Microbiol. Lett. 274 (1), 2013, 83–88.

[20] G. Naga Rathna Supriya and Amrutha V. Audipudi. Screening for antimicrobial activities of endophytic fungi isolated from ripened fruit of *Capsicum Frutescence* L. World J Pharm Sci. 3(2), 2015, 258-262.

[21] Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. BMC Complement Altern Med. 12, 2012, 221.

[22] Al-Rimawi F, Rishmawi S, Ariqat SH, Khalid MF, Warad I, Salah Z. Anticancer Activity, Antioxidant Activity, and Phenolic and Flavonoids Content of Wild *Tragopogon porrifolius* Plant Extracts. Evidence-Based Complementary and Alternative Medicine. 2016:1-7.

[23] Dogra NK. Phytochemical Analysis and In vitro Antioxidant Studies of Plumeria obtusa L. Leave. Indian Journal of Pharmaceutical Sciences. 78(1), 2016, 169-71.

[24] Khan A, Anand V, Badrinarayanan V, Thirunethiran K, Natarajan P. In vitro Antioxidant and Cytotoxicity Analysis of Leaves of *Ficus racemosa*. Free Radicals and Antioxidants. 7(1), 2017, 8-12.

[25] Karthika K, Jamuna S, Paulsamy S. TLC and HPTLC Fingerprint Profiles of Different Bioactive Components from the Tuber of *Solena amplexicaulis*. Journal of Pharmacognosy and Phytochemistry.3 (1), 2014, 198-206.

[26] Hernawati H, Wiyono S, Santoso S. Leaf endophytic fungi of chili (*Capsicum annuum*) and their role in the protection against *Aphis gossypii* (*Homoptera: Aphididae*). Biodiversitas. 12(4), 2011, 187-191.

[27] Summerbell RC and Schroers HJ. Analysis of Phylogenetic Relationship of *Cylindrocarpon lichenicola* and *Acremonium falciforme* to the *Fusarium solani* Species Complex and a Review of Similarities in the Spectrum of Opportunistic Infections Caused by These Fungi. J Clin Microbiol. 40(8), 2002, 2866–2875.

[28] Amby DB, Thuy T, Ho BD, Kosawang C, Son TB, and Jørgensen

H. First Report of *Fusarium lichenicola* as a Causal Agent of Fruit Rot in Pomelo (*Citrus maxima*).APS Publications.2015. https://doi.org/10.1094/PDIS-10-14-1017-PDN

[29] Narayan Chandra Paul, Jian Xin Deng, Hyun Kyu Sang, Young Phil Choi and Seung Hun Yu. Distribution and Antifungal Activity of Endophytic Fungi in Different Growth Stages of Chili Pepper (*Capsicum annuum* L.) in Korea Plant Pathol. J. 28(1), 2012, 10-19.

[30] Rabha AJ, Naglot A, Sharma GD, Gogoi HK, Veer V. In Vitro Evaluation of Antagonism of Endophytic *Colletotrichum gloeosporioides* Against Potent Fungal Pathogens of *Camellia sinensis*. Indian J Microbiol. 54(3), 2014, 302–309.

[31] Jack, I.R. and Okorosaye-Orubite, K. Phytochemical analysis and antimicrobial activity of the extract of leaves of Fleabane (*Conyza sumatrensis*). J.Appl. Sci. Environ. Manage. 12(4), 2008, 63-65.

[32] Ates DA, Erdogrul OT. Antimicrobial activities of various medicinal and commercial plant extract. Turkish Journal of Biology. 27, 2003, 157–162.

[33] Corrado, Rodrigues. Antimicrobial evaluation of fungal extracts produced by endophytic strains of *Phomopsis* sp. J.Micro 44(1), 2004, 157-160.

[34] Rabha AJ, Naglot A, Sharma GD, Gogoi HK, Gupta VK, Shreemali DD, Veer V. Morphological and molecular diversity of endophytic *Colletotrichum gloeosporioides* from tea plant, *Camellia sinensis* (L.) O. Kuntze of Assam, India. Journal of Genetic Engineering and Biotechnology. 14(1), 2016, 181-187.