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Community Analysis of Endophytic Fungi In Medicinal Plant Gloriosa Superba

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ABSTRACT

Aim: All plants are expected to host endophytes, but only 3% of plants have been explored for their fungal communities. Aim of the present study was to isolate the mycoflora of Gloriosa superba, an important medicinal plant of subcontinent.

Material and Methods: Different parts of G. superba were surface sterilized and internal tissues were aseptically exposed to the culture media unless the growth of mycelia was observed. Each fungal isolate was cultured separately to get the pure culture for further evaluation and analysis. Each culture was identified on the basis of its morphological characteristics and bioactive isolates were also characterized on the basis of their molecular profiling.

Results: 42 isolates belonging to 22 different genera were obtained and were identified. 5 isolates exhibited good antimicrobial and anticancer potential against human pathogenic bacterial strains and human cancer cell lines. These isolates were identified on the basis of their molecular characteristics. PCR was done using Primers for ITS region and PCR products of the isolates were sequenced and their sequences were BLASTED to NCBI. Sequences were also submitted to GenBank.

Conclusion: Results of the present study confirmed that plants are associated with microorganisms which are present in their internal tissues. These microorganisms survive in symbiotic relationship with their hosts and may provide assistance to their host to protect them against various pathogens.

Key words: Endophytes, Fungi, Polymerase chain reaction, Aspergillus, Penicillium.

INTRODUCTION

All higher plants appear to host endophytic microbes but to date only a few plants have been investigated for their endophytic biodiversity[1]. The most frequently encountered endophytes are the fungi; however, the existence of many endophytic bacteria has been documented as well[2]. It is important to isolate and characterize the endophyte diversity of medicinal plants as these strains are not only found to produce therapeutically active compounds but some novel microbial strains have also been isolated from plants. The introduction of molecular methods has led to significant advances in the characterization of these microbial strains. PCR-based methods, including real-time PCR have been successfully used to provide rapid, quantitative data

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on fungal species and ITS region is an area of particular importance to fungal diagnostics.

Gloriosa superba L (Fig.1) commonly known as "Kalihari" rubber, is a widely used medicinal plant in the Indian Sub-continent. It has long ethanopharmacological history and extensive uses in traditional medicine. *G. superba* belongs to the family Liliaceae. *G. superba* is a semi-woody herbaceous climber found throughout India up to an altitude of 6000 ft[3]. It is a native of tropical Africa and is now growing in many parts of tropical Asia including India, Burma, Malaysia and Srilanka. *G. superba* is an industrial medicinal crop in South India, for its high colchicine content, which is still collected from wild species of host[4]. Due to its over-exploitation in wild as well as problems faced during field cultivation, it was on the verge of extinction, and was one of the endangered species among the most valued medicinal plants. The present study was carried out to determine endophytic mycoflora in *Gloriosa superba,* a widely used medicinal plant.

MATERIALS AND METHODS

Plant sample collection

Fresh, mature, healthy parts of *Gloriosa superba* were collected and authenticated by Dr. K. Mahadeva Chetty, Head Department of Botany, S.V. University, Tirupati, India. Samples were transported in sterile plastic bags, stored overnight at 4°C and processed within 48 hrs. Plant selection was based on the ethanopharmacological uses of *Gloriosa superba* as an ayurvedic medicinal herb to cure diseases in various parts of Africa and Southeast Asia.

Isolation of endophytic fungi

Different plant parts were successively surface sterilized by method given by Petrini et al[5]. Sterilized plant pieces were blotted on sterile blotting paper and placed on Petri dishes containing Sabouraud Dextrose agar medium with Chloramphenicol. Petri dishes were sealed with parafilm and were placed in an incubator at 28°C until the outgrowth of endophytic fungi was detected. Most of the fungal growth was initiated within 7-10 days of inoculation. Fungi growing out of the segments were purified and pure cultures were then transferred to PDA and cultivated for 14 days at 28°C. Colonization Frequency was calculated as described by Suryanarayanan et al[6].

$$\text{Colonization frequency (\%)} = \frac{\text{Number of segments colonized by an endophyte}}{\text{Total no. of segments analyzed}} \times 100$$

Identification of fungal isolates

Each fungal isolate was identified on the basis of morphological and cultural characteristics[7]. Freshly growing mycelia of cultures was taken on microscopic slides and flushed with 2-3 drops of lacto phenol blue. Slides were observed under optical microscope at 40x. Few fungal strains, exhibiting good bioactive potential were selected and identified using ITS based primers.

Molecular characterization

Total cellular DNA of the selected fungal strains was isolated using method given by Saghai-Maroof[8]. PCR amplification was done using universal primers ITS regions of rDNA; ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3'), ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') against 18s and 28s rDNA. DNA of each sample was diluted to a working concentration of20 ng/µL for (PCR) analysis. The reaction mixture (25 µL) was comprised of 10X Taq buffer F, MgCl2 (25 mM), dNTPs (10 mM each), primers (20 pmol/µL each) and nuclease-free water. Initial denaturation was done at 95˚ C for 30 sec followed by primer annealing at 55˚ C for 40 seconds and final extension was carried at 72˚ C for 7 min. The process was repeated for 30 cycles. Amplified products along with appropriate DNA ladder were checked on 1.5% gel. The gel slice containing the amplified products was excised out using a sterile blade and DNA was eluted using SIGMA gel extraction kit as per manufacture's protocol. The eluted DNA was purified using PCR cleanup kit. Purified samples were again checked on agarose gel and were sent for sequencing. Sequences obtained in present study were also submitted to GenBank and their accession numbers were retrieved.

Phylogenetic analysis

A phylogenetic relationship between the sequenced culture and sequence obtained from BLAST search was first aligned using CLUSTALW software and Phylogenetic analyses were conducted

in MEGA4[9]. Ten other rDNA sequences; *Aspergillus oryzae* (JN561266), *Aspergillus oryzae* (HQ285579), *Penicilium pinophilum* (HM469418),

Fig 1: *Gloriosa superba* Linn.

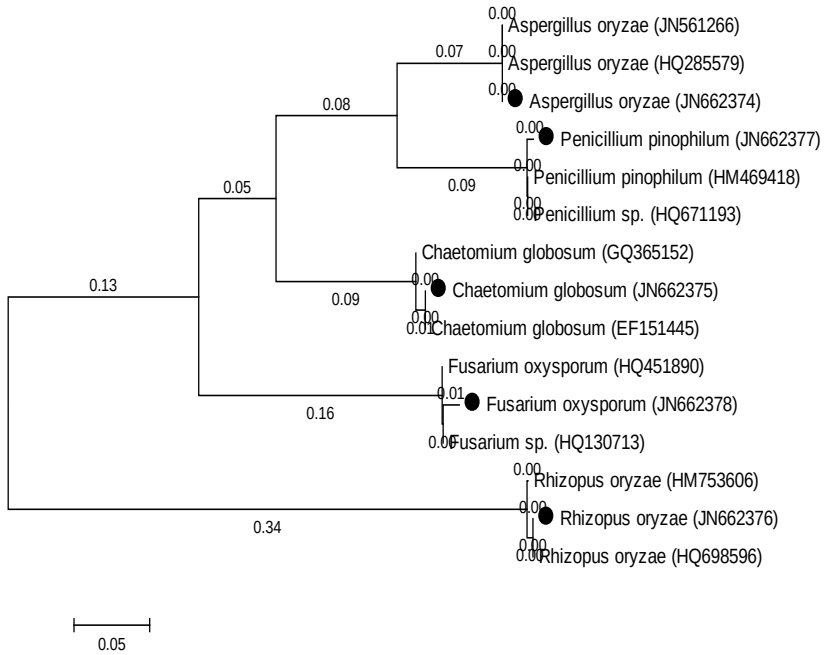

Fig 2: Phylogenetic trees of isolates identified on the basis of sequences of rDNA. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 432 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). *Penicilium pinophilum* (HQ671193), *Chaetomium globosum* (GQ365152), *Chaetomium globosum* (EF151445), *Fusarium oxysporum* (HQ451890), *Fusarium oxysporum* (HQ130713), *Rhizopus oryzae* (HM753606), *Rhizopus oryzae* (HQ698596) were also included in phylogenetic analysis.

RESULTS

Isolation of endophytes:

According to our knowledge and literature reviewed, the present study is the first report on the isolation and characterization of endophytic mycoflora from *Gloriosa superba*, a medicinal plant found in Africa, tropical Asia including India, Burma, Malaysia and Srilanka. A total of 105 segments including 42 of leaves, 28 of tubers and 35 of seeds were screened for the presence of endophytic fungi. Total 42 isolates belonging to twenty two different species were obtained during the present study. 15 isolates were obtained from surface sterilized leaves segments, 11 from tubers and 16 from seeds segments (Table 1)

Similar fungal species were isolated from the different parts of plant. Overall colonization frequency was 32.7%. *Aspergillus* was the most prominent genus followed by the occurrence of *Penicillium* species. FE-1 showed 100% similarity with *Penicillium pinophilum*. Colonization frequency of different fungal genus isolated in this study was calculated (Table 2)

Table 1: Number of isolates from different parts of *Gloriosa superba.*

Site of isolation	Number of samples	Number of fungi isolated
Leaves	42	15
Tubers	28	11
Seeds	35	16

Table 2: Colonization frequency of different isolates from different parts of G. superba.

S. No.	Fungi	% Frequency of colonization		
		Leaves	Tubers	Seeds
1.	*Penicillium pinophillum*	4.4		0.95
2.	*Curvularia lunata*		3.8	1.8
3.	*Rhizopus oryzae*	2.8	0.95	
4.	*Aspergillus awamori*	2.8		0.9
5.	*Penicillium bilaiae*		0.95	0.95
6.	*Geotrichum candidum*	1.9		0.95
7.	*Apergillius oryzae*	1.8		3.7
8.	Sterilia mycelia 1	2.8	1.8	
9.	*Phoma exigua*	0.9		1.9
10.	*Alternaria alternate*			2.8
11.	*Aspergillus flavus*		1.8	0.9
12.	*Fusarium oxysporum*	0.95	1.8	
13.	Sterilia mycelia 2		0.9	1.9
14.	*Chaetomium globosum*	1.9		0.95
15.	*Nigrospora sphaerica*	0.95		
16.	*Alternaria porri*		2.8	0.9
17.	*Aspergillus fumigates*	1.9		
18.	*Trichoderma viride*	1.9		0.9
19.	*Aspergillus niger*	2.8	0.95	2.8
20.	*Phoma* sp.	0.95	1.9	
21.	*Fusarium roseum*	0.95	0.9	
22.	*Rhizoctonia* sp.	0.95		0.95

Molecular Characterization

PCR based on rDNA gave an amplicon size of ~ 650 bps for all the isolates. PCR products of the isolates were sequenced and their sequences were BLASTED to NCBI. Sequences were also submitted to GenBank.

Accession numbers JN662374- JN662378 were retrieved from the GenBank for isolate FE-12 (*Aspergillus oryzae*), FE- 14 (*Chaetomium globosum*), FE-3 (*Rhizopus oryzae*), FE-1 (*Pencillium pinophilum*), FE-19 (*Fusarium oxysporum*), respectively.

Phylogenetic analysis

Sequences obtained after PCR amplification were BLASTED for their identity match with existing database of NCBI. The isolate FE-12, characterized as *Aspergillus oryzae* showed 99% similarity with the submission HQ285579. Isolate FE-1 showed 99% similarity with *Penicillium pinophilum* (HM469418) and 99% similarity with *Penicillium pinophilum* (HQ671193). Another isolate FE-14 of this study exhibited 99% similarity with *Chaetomium globosum* (GQ365152) and *Chaetomium globosum* (EF151445). FE-19 showed 100% similarity with submissions *Fusarium oxysporum* (HQ451890) and *Fusarium oxysporum* (HQ130713). FE-3, characterized as *Rhizopus oryzae* produced 98% similarity with *Rhizopus oryzae* (HM753606) and 99% similarity with *Rhizopus oryzae* (HQ698596). The evolutionary history was inferred using the Neighbor-Joining method[10]. The optimal tree with the sum of branch length = 1.03169638 is shown in Figure 2.

DISCUSSION

Microorganisms residing within the plant tissues not only receive their nutrition from the plant but they tend to reciprocate by providing the protection to their host by producing various antibiotics[11]. Many bioactive compounds have been isolated from these residents of plants but endophytes are still considered to be a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive compounds, which can be explored in wide area of applications[12]. An excellent example of biomolecules isolated from endophytes is the anticancer drug, taxol, which had been previously supposed to occur only in the plants[13]. Endophytic fungi from medicinal plants can therefore be used for the development of drugs. A major problem in exploring the bioactive potential of endophytes is rapidly diminishing because the volume of forests holding the greatest possible source for this poorly understood group of microorganisms is getting reduced. Recently studies have been carried out about the endophytic biodiversity, taxonomy, reproduction, host ecology and their effect on host[14]. The endophytic flora, both numbers and types, differ in their host and depends on host geographical position[15]. Majority of endophytic fungi belongs to Ascomycota and Deuteromycota[16]. It is not only the fact that these microorganisms have been proven to be novel sources of compounds with excellent bioactive potential but many times novel microbial species have been isolated from plants.

Gloriosa superba is a well known medicinal plant and its different parts are reported to have anti-inflammatory, antioxidant and anticancer activities. The medicinal properties of the plant could be attributed to the alkaloids present in different parts of this plant. Earlier an aerobic, Gram-stain-positive, non-acid–fast, nonmotile actinomycetes; Saccharopolyspora gloriosae sp. has been isolated from the stem of *Gloriosa superba* Linn[17]. Therefore, the present work was initiated to find out endophytic flora associated with this widely used medicinal plant. Diverse endophytic population was found to colonize this plant. During the present study, *Aspergillus, Penicillium* and *Phoma* sp. were prominently isolated as endophytic fungi. Deuteromycota fungi were largely prevalent (Table 2). A zygomycota; *Rhizopus oryzae* and an ascomycetes; *Chaetomium globosum* were also isolated in this study. An isolate in present study; FE-12 was found to produce a bioactive metabolite which appears to be biosynthetically similar to colchicine. The compound, identified on the basis of spectral data and named colchatetralene, possesses good antimicrobial and cytotoxic potential[18]. The results for molecular characterization of selected strains using primers for ITS region were very much in tune to the results for their morphological

characterization, confirming the utility of this method of identification.

CONFLICT OF INTEREST

Authors declare no conflict of interest

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REFERENCES

1. Sultan S, Shah SAA, Sun L. Bioactive fungal metabolites of 9pr2 isolated from roots of *Callophyllum ferrugineum*. Int J Pharm Pharm Sci 2011;3: 7-9.
2. Staniek A, Woerdenbag HJ, Kayse O Endophytes. Exploiting biodiversity for the improvement of natural product based drug discovery. J Plant Inter 2008; 3: 75-93.
3. Ghosh B, Mukherjee S, Jha TB, Jha S. Enhanced colchicine production in root cultures of *G. superba* by direct and indirect precursors of the biosynthetic pathway. Biotechnol Lett 2002; 24: 231–4.
4. Jana S, Shekhawat GS. Critical review on medicinally potent plant species: *Gloriosa superba*. Fitoterapia 2011; 82: 293–301.
5. Petrini O, Siaber TH, Toti L, Viret O. Ecology, Metabolite production and substrate utilization in endophytic fungi. Nat Toxins 1992; 1:185-96.
6. Suryanarayanan TS, Venkatesan G, Murali TS. Endophytic fungal communities in leaves of tropical forest trees: Diversity and distribution patterns. Curr Sci 2003; 85: 489-93.
7. Sutton BC. The Coelomycetes, Fungi Imperfecti with pycnidia Acervuli and Stromata. Robert Mac Lechose and Co. Ltd, England 1980.
8. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl. Acad. Sci. USA 1984; 81: 8014-18.
9. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 2004; 101: 11030–35.
10. Saitou N, Nei M. The neighbor-joining method, A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4: 406–25.
11. Gao F, Dai C, Liu X.. Mechanisms of fungal endophytes in plant protection against pathogens. Afr J Microbiol Res 4: 2010; 1346-51.
12. Strobel G, Daisy B, Castillo U, Harper J. Natural Product from endophytic microorganisms. J Nat Prod 2004; 67: 257-68.
13. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 2003; 1: 491-502.
14. Selosse MA and Scharl CL. Fungal endophytes of grass: hybrids rescued by vertical transmission? An evolutionary perspective. New Phytol 2007; 173: 452-58.
15. Gange AC, Dey S, Currie AF, Sutton BVC. Site- and species-species differences in endophyte occurrence in two herbaceous plants. J Ecol 2007; 95: 614-22.
16. Frohlich J, Hyde KJD. Biodiversity of palm fungi in the tropics: Are global fungal diversity estimated realistic. Biodiversity and Conservation 1999; 8: 977-1004.
17. Qin S, Chen HH, Klenk HP *et al*. *Saccharopolyspora gloriosae* sp. nov, an endophytic actinomycete isolated from the stem of *Gloriosa superba* L. Int J Syst Evol Microbio 2010; l 60: 1147–51.
18. Budhiraja A, Nepali K, Sapra S *et al*. Bioactive metabolites from an endophytic fungus of *Aspergillus* species isolated from seeds of *Gloriosa superba Linn*. Med Chem Res 2012; DOI 10.1007/s00044-012-0032-z.