Journal of Phytology 2011, 3(6): 20-22
ISSN: 2075-6240
www.scholarjournals.org

JOURNAL OF PHYTOLOGY

www.journal-phytology.com

Isolation of Endophytic *Colletotrichum gloeosporioides* Penz. from *Salacia chinensis* and its Antifungal Sensitivity

N. Bhagya, Sana Sheik, M. Samhitha Sharma, K. R. Chandrashekar*

Department of Applied Botany, Mangalore University, Mangalore-574199, Karnataka, India

Article Info

Article History

Received : 21-02-2011
Revised : 18-04-2011
Accepted : 19-04-2011

*Corresponding Author

Tel : +91-824 2287272
Fax : +91-824 2287367

Email: konambi@yahoo.com

Summary

Salacia chinensis L (Celestraceae), an endangered medicinal plant is well known for its antidiabetic activity. An attempt of *in – vitro* culturing to micropropagate the plant led to the discovery of an endophytic association of *Colletotrichum gloeosporioides* Penz. with both stem and leaf of the plants. The fungus did not respond to the lower concentrations of Amphotericin B and Nystatin (upto 60 µg/ml) in the culture medium. However, it was sensitive at a higher concentration of 100µg/ml.

Key Words: *Colletotrichum gloeosporioides* Penz., Endophyte, Nystatin, Amphotericin B, *Salacia chinensis* L

Introduction

The endophytes reside in the living tissues of the host plant show a variety of relationships, ranging from symbiotic to slightly pathogenic [1]. About 6500 endophytic fungi isolated from herbaceous plants and trees were screened for biological activities and biologically active compounds followed by their isolation and structural determination [2]. Endophytic fungi form the promising source for the production of novel products with biological activity [3].

Sixty-four fungal morphotaxa were characterized from12 tree species from Iwokrama Forest Reserve, Guyana and showed the frequent presence of *Colletotrichum*, *Nodulisporium*, *Pestalotiopsis* and *Phomopsis* species [4]. *Withania somnifera*, a medicinal plant was screened for the presence of endophytic fungi and showed the presence of 24 different fungal species with a dominant endophyte, *Aspergillus alternata* [5]. A number of cosmopolitan endophytic species such as *Colletotrichum gloeosporioides*, *Guignardia* sp., *Glomerella cingulata*, *Pestalotiopsis* spp., *Phomopsis* spp. and *Phyllosticta* sp. etc were isolated from the Chinese medicinal plant, *Tripterygium wilfordii* [6]. Suryanarayanan et al [7] reported the presence of endophytic fungi such as *Cladosporium* sp. and *Colletotrichum* sp in *Cuscuta rejlexa*- an angiosperm parasite and its host plants. *Colletotrichum* and *Fusarium* were isolated from *Taxus mairei* trees by Wang et al [8].

The endophytes have been reported to produce a plethora of substances of potential use to modern medicine, agriculture and industry. Some medicinally important compounds like novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds have been isolated, purified and characterized from choice endophytes in the recent past [1].

Salacia chinensis L. (Celestraceae), a large woody climbing shrub with opposite, stipulate leaves up to 7.5x3cm, oblong or ovate, crenate-serrate, obtusely-acuminate at apex, coriaceous, glabrous [9] is very well known for its antidiabetic effect [10]. The culturing of the stem and leaf explants of *S. chinensis* in the medium for micropropagation revealed the association of a fungus with the tissue. Repeated attempts of culturing the explants even with the incorporation of a range of antibiotics such as Nystatin (50µg/ml) and Amphotericin B (30µg/ml) in the medium failed to suppress the growth of the fungi. Therefore, an attempt was made to isolate and identify the fungus.

Materials and Methods

The healthy plant parts of *S. chinensis* such as stem and leaf samples were collected from the Herbal garden of Mangalore University campus. The samples were collected during pre monsoon (Feb – May), monsoon (June-Sept) and post monsoon (Oct – Jan) seasons and subjected to surface sterilization. For this, the samples were washed under running tap water for 30-45min followed by 2% Bavistine treatment (2hrs), 70% alcohol (1min) and mercuric chloride (10min). After each treatment the materials were washed using sterile distilled water, cut in to ~0.5cm size and then inoculated to both MS medium [11] and PDA medium and incubated at 25±2°C. Twenty Petri dishes each containing MS medium and 20 Petri dishes each containing PDA medium were inoculated with 4-6 explants. The experiment was repeated in all the 3 seasons.

The hyaline fungal mycelia were inoculated on to PDA slants to obtain the pure culture of the fungus. The culture was identified using Barnett and Hunter [12]. The cultures were also sent to Agharkar Research Institute, Pune, India for morphotaxonomical identification.

The sensitivity of the cultures against selected broad-spectrum antifungal agents was tested on PDA medium. For this, 200µl suspension prepared from the 5d old culture was

incorporated into the PDA medium following spread plate technique. The antifungal agents such as Nystatin (100µg/ml), Amphotericin B (100µg/ml), Ketoconozole (50µg/ml), Itracanozole (30µg/ml) supplied by Himedia laboratory Pvt Ltd. in the form of discs of 0.7mm size were used for antibiotic sensitivity test. Four discs of one antifungal agent were placed on the spread plate. Similarly, 3 plates were used for each of the antifungal agents. The experiment was repeated thrice during all the 3 seasons.

Results and Discussion

The emergence of the fungal hyphae from the cut ends of the explants was observed within 3-4 days on MS medium and within 5-6 days on PDA medium (Fig 1).

The profuse growth of cottony fungal colony was observed with white to pale grey mycelium. The microscopic observation revealed the presence of hyaline, septate, highly branched mycelia with large number of more or less cylindrical spores (Fig 2). The culture was identified as *Colletotrichum gloeosporioides* Penz. and the identity was confirmed by the Agharkar Research Institute, Pune, India and is being maintained there with accession number NFCCI No. 2158.

Fig 1: Explants showing the *emergence of fungal hyphae*

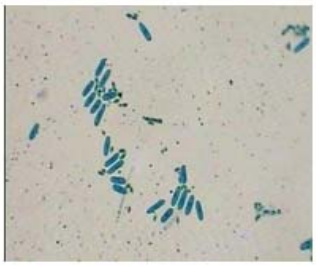

Fig 2: Microscopic view of *Colletotrichum gloeosporioides* Penz. spores (40x)

The presence of the *Colletotrichum gloeosporioides* Penz. as an endophyte has been reported in various plants such as *Justicia gendarussa* [13], *Artemisia mongolica* [14], *Tripterygium wilfordii* [6] and in many more plants. The fast growing, cottony, whitish to grey colored colony with orange conidial mass of the *Colletotrichum gloeosporioides* was observed by Photita et al. [15] and similar observations were made in the present study also. Muthukumar et al. [16] while investigating the roots of 107 medicinal and aromatic plants of the Western Ghats region of Southern India for arbuscular mycorrhizal (AM) and dark septate endophytic (DSE) associations reported the absence of AM or DSE in *S. chinensis* L.

The fungus showed extensive growth in MS medium which is rich in nitrogen and sucrose. The MS medium used for *in-vitro* culture technique provides 30% sucrose as the rich carbon source and ammonium nitrate and potassium nitrate as the major nitrogen sources. A study carried out by Sangeetha and Rawal [17] to check the suitable carbon and nitrogen for the growth and sporulation of *C. gloeosporioides* observed higher mycelial growth in the medium supplemented with mannitol, fructose and sucrose. They also reported the ammonium nitrate, potassium nitrate and sodium nitrate as suitable nitrogen sources. For *in – vitro* technique, the cultures were incubated at 25±2°C and this is the ideal temperature which favors the growth of endophyte. This is in agreement with the results of Nithya and Muthumary [18]. In MS medium, with sucrose as carbon source and ammonium/potassium nitrate as nitrogen sources the endophyte started growing earlier and faster than the explant.

Among the antifungal agents tested, Nystatin and Amphotericin B inhibited the growth of the endophyte at the concentration of 100 µg/ml with an inhibition zone of 0.86 ± 0.11 and 0.78 ± 0.18 cm respectively whereas, the remaining antifungal agents failed to suppress the fungal growth (Table 1, Fig 3). Kabir et al. [19] observed the inhibition of *Colletotrichum* sp against standard drugs like Ampicillin and Nystatin at the concentration of 100 µg/ml. Mohanan et al. [20] reported the sensitivity of cocoa pathogenic *Colletotrichum gloeosporioides* for nystatin.

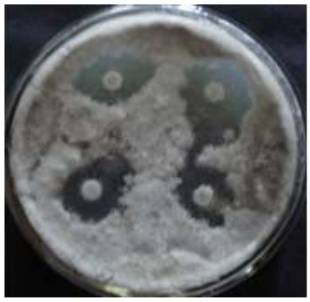

Fig 3: Antifungal sensitivity test with Nystatin

Table 1: Growth response of *Colletotrichum gloeosporioides* Penz. towards antifungal agents

Antifungal agent	Concentration (µg/ml)	Diameter of inhibition zone (cm)
Nystatin	100	0.86 ± 0.11
Amphotericin B	100	0.78 ± 0.18
Ketoconazole	50	-
Itracanazole	30	-

C. gloeosporioides is known for the production of anticancer drug taxol [13]. Medicinally and industrially important extra cellular lipase [21], an antimicrobial metabolite, colletotric acid [14] and colletoic acid, a 11*β*-hydroxysteroid dehydrogenase type 1 (11*β*-HSD1) inhibitor [22] were also isolated from *C. gloeosporioides.*

There is ample scope for the isolation of variants of *C. gloeosporioides* Penz. from different sources followed by the production, isolation, characterization and commercial applications of the metabolites produced from this endophyte.

Acknowledgement

Senior author (BN) is grateful to UGC, New Delhi for financial assistance under RFSMS scheme.

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