Endophytic *Penicillium citrinum* Thom. from *Scoparia dulcis* Linn

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Abstract *Scoparia dulcis* of Scrophulariaceae is an annual herb distributed through out the tropics. *Penicillium citrinum* was obtained from apparently healthy roots, stem, leaves and fruits of this plant. Callus and multiple shoots produced during micropropagation from various explants were also symptomless but showed occurrence of *Penicillium citrinum* when cultured in Murashige & Skoog liquid medium for the production of secondary metabolites.

Keywords Endophyte · *Penicillium citrinum* · *Scoparia dulcis*

Abbreviations BA-N6 Benzylaminopurine;
K Ketakonazole; MS Murashige & Skoog medium(1962);
PD Potato dextrose; PDA Potato dextrose agar

Introduction

Endophytic fungi are known to colonize different living plant tissues such as leaves, bark, and roots [1] without causing any immediate negative effects [2]. They can live throughout or a part of a life cycle without causing any damage or disease and are transmitted to the next generation, through tissues of hosts, seeds, vegetative propagules; or by spores carried by air, insect or small animals [3]. These fungi can have effects on their hosts such as enhancement of stress, insect and disease resistance, [4–7] and also productivity improvement [8]. An endophytic fungus isolated from the inner barks of *Nothapodytis foetida* from the western coast of India produced a valuable alkaloid, viz., Camptothecin [9].

The present paper reports the isolation and identification of a fungal endophyte *Penicillium citrinum* from the living symptomless tissues of *Scoparia dulcis*. Endophytic Penicillia has been reported from many plants like *Azadirachta indica* [10], *Oryza sativa* [11] *Melia azedarach* [12] and *Prumnopitys andina* [13].

Penicillium citrinum produces a secondary metabolite, citrinin, which contaminates foods of animals [14]. This fungus is also reported to produce two other secondary metabolites, viz., tanzawic acid E and F [15].

Scoparia dulcis Linn. of the Scrophulariaceae (Sweet broom) is used in herbal medicine throughout the tropics. The plant is a tough glabrous leafy branched herb that grows up to 90 cms in height and is indigenous to India. This plant has antibacterial and anti-inflammatory effects and hence is used for respiratory and urinary infections. The antitumorous [16] and antiviral properties [17] have also been reported. Phytochemical screening has revealed

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the presence of mainly diterpenes, triterpenes, flavanoids and steroids [18–19].

Material and methods

Sample collection

Healthy plants were collected from ten different localities in Kerala, India. Voucher specimen have been deposited in the Herbarium of St. Thomas College Kozhencherry, Kerala, India.

Isolation & Culturing of Endophyte

The plants were surface sterilized with 1% laboline and washed under running tap water for 30 minutes. They were further treated with 10% aqueous sodium hypochlorite for 1–5 minutes, 70% ethyl alcohol for 1 minute and rinsed with sterile water. Ten fragments of meristematic tips, leaves from the top to the lowest senescent ones, stem, roots, and fruits were inoculated in potato dextrose (PD) liquid medium and Murashige-Skoog (MS) liquid medium with and without the antifungal agent

Ketokonazole (30 mg/L). The Petri plates were incubated at $30 \pm 2^\circ\text{C}$ and also at $25 \pm 2^\circ\text{C}$ for a period of 3 days. The fungal colonies were maintained on PD agar medium. The surface sterilized leaf explants were plated on MS agar supplemented with phytohormones, indole acetic acid 0.3 mg/L and benzyl adenine 0.3 mg/L for callus and multiple shoot production. Portions of callus and multiple shoots obtained after 12 days were inoculated on PD liquid and MS liquid medium at $30 \pm 2^\circ\text{C}$. Surface sterilized seeds were germinated on $\frac{1}{2}$ strength MS media to obtain seedlings.

Identification of the endophytic isolate

The fungal isolates were identified by studying their macroscopic characters such as the texture, pigmentation and

exudation of the colony. The microscopic characters were analysed by a slide culture technique using lactophenol cotton blue.

Histological methods

Histological examinations were done to confirm the presence of endophytes within host tissues. Leaves, stem, fruits, and roots, were kept in 5% KOH at $40\text{--}60^\circ\text{C}$ for seven days for clearing till the specimen turned to a golden straw colour. They were then rinsed in sterile water, bleached slightly with 3.5% hydrogen peroxide, and again rinsed in 2% HCl two times followed by staining in 0.5% acid fuchsin in lactic acid. The solution was changed daily till it remained colorless [20]. The sample was then observed for the presence of endophytic fungi.

Free - hand sections were made with fresh leaves as well as leaves with emerging endophytic mycelium obtained in MS liquid cultures. They were stained with safranin and lactophenol cotton blue and observed under a microscope.

Results and discussion

All samples of stem, leaf, seeds and multiple shoots inoculated in PD liquid medium produced a white fluffy mass of mycelium measuring approximately 0.5–15 mm in diameter. There was a change in the pH of the medium from 5.5 to 5.3. MS medium, supplemented with benzyl adenine inoculated with these samples and incubated at $30 \pm 2^\circ\text{C}$ showed the presence of white smooth ball-like masses of mycelia increasing in size with the pH of the medium changing from 5.6 to 7.2. The medium was clear and viscous. MS media for callus and multiple shoot production showed no visible symptoms of fungal growth at $25 \pm 2^\circ\text{C}$ (Table 1).

Germination of seeds on $\frac{1}{2}$ strength MS medium devoid of phytohormones showed no visible fungal growth while the plant segments of the seeds inoculated on PDA gave

Table 1 Occurrence of *Penicillium citrinum* in *Scoparia dulcis*

Sl. No.	No. of samples giving fungal growth in different media at different temperatures					
	Plant Part	No. of Samples	PD Liquid		MS Liquid + BA	
			$30 \pm 2^\circ\text{C}$	$25 \pm 2^\circ\text{C}$	$30 \pm 2^\circ\text{C}$	$25 \pm 2^\circ\text{C}$
1	Root	10	7	0	6	0
2	Stem	10	10	0	10	0
3	Leaves	10	10	0	10	0
4	Fruits	10	9	0	9	0
5	Callus	10	5	0	7	0
6	Multiple Shoot	10	10	0	10	0

BA Benzyl Adenine

MS Murashige & Skoog

PD Potato Dextrose.

visible fungal growth. The above media incorporated with Ketoconazole (30 mg/L) did not support the fungal growth. The fungal colony on PD was white at first and later became bluish green with a white margin. The reverse of the colony was light at first but turned yellow later. The colony produced golden brown exudates (Fig. 1). Examination of slide culture revealed the presence of septate hyphae, 2.5 μm in diameter, with branched compact verticils of phialides bearing globose green granular conidia (Fig. 2).

The entire structure showed the characteristic brush like appearance. The isolate was identified as *Penicillium citrinum* Thom and the identity was confirmed by the Institute of Microbial Technology, Chandigarh, India.

On histological examinations of fresh surface sterilized leaves, fungal mycelia were not observable, but when used in suspension cultures inter and intracellular hyphae were evident within the mesophyll tissue (Fig. 3). No sporulation was observed at this stage.

The above observations revealed that the tissues were excellent harbouring niches for the fungus which remained

latent, emerging later under favourable conditions. The MS media containing vitamins, amino acids, glucose, sucrose and hormones could be supposed to be favouring the emergence and the rapid growth of the latent endophyte. The large ball like growth of the fungus in MS medium could be due to this reason. The absence of fungal growth at $25 \pm 2^\circ\text{C}$ and its presence at $30 \pm 2^\circ\text{C}$ may be related to the temperature requirement by the particular fungus.

Most terrestrial plants, especially tropical plants are colonized by one or several species of endophytic fungi in healthy looking host tissue that do not cause visible symptoms. Reports of endophytic fungi living asymptotically in plant tissue and constituting a valuable source of secondary metabolites for potential therapeutic use threw light on this aspect [13]. Recent reports also claimed that endophytic microbes play a major role in the therapeutic properties of plants [10]. *Claviceps* [21] species were responsible for producing ergot alkaloids and they produce these alkaloids in pure culture. There was a possibility that the healing properties of *S. dulcis* might be due to the secretion of secondary metabolites by the endophytic *Penicillium citrinum* and that might be the reason for its high use in traditional herbal medicines. Preliminary screening of endophytic fungi from medicinal plants in Malaysia for antimicrobial and antitumour activity [22] also supported this view. Similarly the production of camptothecin from the suspension cultures of *Nothapodytis foetida* was found to be enhanced by the presence of endophytic fungi [19].

The frequency of isolation of *Penicillium citrinum* was more in the leaves of multiple shoot than in callus. Studies showed that only the young meristematic tip was devoid of the fungus. In *Scoparia dulcis* collected from different localities, *Penicillium citrinum* was the only endophytic fungus observed. The reason for such a specific mutualistic relationship between the plant and the fungus is to be further studied.

Fig. 1 Endophytic fungus colony, *Penicillium citrinum*

Fig. 2 *Penicillium citrinum* with conidia. Enhanced by X1000

Fig. 3 Leaves cultured on M.S medium with intercellular fungi. Enhanced by X1000

In conclusion, we suggest that *Penicillium citrinum* is an endophytic fungus in *Scoparia dulcis*. The biological activity of this endophyte, its possible role in secondary metabolite production, and its potential application as an elicitor in the secondary metabolite production *in vitro* etc. are under investigation.

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