Short Communication

Isolation of endophytic actinomycetes from *Catharanthes roseus* (L.) G. Don leaves and their antimicrobial activity

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Abstract
Endophytic actinomycetes were isolated from surface sterilized leaves of *Catharanthes roseus* (L.) G. Don of family Apocynaceae. A total of 38 endophytic actinomycetes were recovered on Starch Casein Agar. Among the 38 isolates 20 morphologically different isolates were screened for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and for antifungal activity against fungi *Candida albicans, Botrytis cinerea, Curvularia lunata, Fusarium oxysporum, Fusarium solani* and *Rhizoctonia solani.* Sixty five percent of the isolates exhibited antimicrobial activity. Among the 20 isolates tested two isolates Cr-12, Cr-20 exhibited highest activity against the test organisms. The selective isolation of endophytic actinomycetes in the present study indicates the richness of microbial diversity in *Catharanthus roseus* and screening for antimicrobial activity should be investigated for a comprehensive identification and potential use as source of bioactive agents.

Keywords: Antimicrobial activity; *Catharanthes roseus*; endophytic actinomycetes; leaves

Natural products still remain the most important source for discovery of new and potential bioactive molecules that can be used to design new drugs to replace those against which pathogenic strains have rapidly acquired resistance. Large number of plants and microbes have been tested for the production of bioactive compounds. Though endophytes are an outstanding source of bioactive natural products inhabiting the higher plants growing in diverse environments, they are relatively less investigated group of microorganisms. Endophytes colonizing inner tissues of plants usually draw nutrition and protection from host plants and in return, confer enhanced fitness to the host by producing a variety of bioactive metabolites and providing protection for the plant (Strobel and Daisy, 2003; Tan and Zou, 2000). Growth stimulation of plant by endophytes can be a consequence of nitrogen fixation or the production of phytohormones (Nimnoi *et al*., 2010; Meguro *et al*., 2006; Igarashi *et al*., 2002), biocontrol of phytopathogens through production of enzymes, antibiotics or siderophores (Quecine *et al*., 2008; Castillo *et al*., 2007; Cao *et al*., 2004; Bacon and Hinton, 2002), induction of systemic disease resistance (Shimizu, 2007; Nishimura *et al*., 2002). Maitan (1998) observed rare endophytic actinomycetes isolated from *Solanum lycocarpum*. Matsumoto *et al*. (1998) isolated actinomycetes from fallen leaves and the genus *Microbispora* accounted for 44% of all isolates. Li *et al*. (2008) isolated 41 endophytic streptomycetes from pharmaceutical plants in rainforest for antitumor and antimicrobial activities, Chen *et al*. (2009) isolated a novel species *Streptomyces mayteni* from a Chinese medicinal plant. Bascom-Slack *et al*. (2009) isolated 14 actinomycete sp. from 300 plant stem samples with antimicrobial activity. Only limited attempts have been made to study endophytic actinomycetes and their metabolites in India (Verma *et al*., 2009).

This study was an attempt to isolate endophytic

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actinomycetes from the leaves of *Catharanthes roseus* and screen them for antimicrobial activity. *Catharanthes roseus* (L.) G.Don was selected for isolation of endophytic actinomycetes. Around 4-6 healthy leaves were collected from 10-15 individual plants of the same species from different locations in Puducherry. Leaf samples were placed in clean plastic bags and transported to the laboratory and processed within 4-5 hours. Leaves were washed in running tap water for 10 min and cut into small bits (1.0 × 0.5 cm). The leaf segments were surface sterilized following the method of Taechowisan and Lumyong (2003). Leaf bits were rinsed in 0.1% Tween 20 for a few seconds, then transferred to clean conical flask and sterilized by sequential immersion in 70% (v/v) ethanol for 60 seconds, followed by sodium hypochlorite solution (1% w/v, available chlorine) for 120 seconds and finally in 70% ethanol (v/v) for 30 seconds and then inoculated on starch casein agar (SCA) (Küster and Williams, 1964) at the rate of 10-15 leaf bits per plate. Nystatin and cycloheximide (50 µg/ml of each) were added to media to suppress fungal growth (Williams and Davies, 1965). All the plates were incubated under laboratory conditions at 26±2ºC for 15-30 days. Individual colonies with characteristics of Actinomycete morphology were isolated and pure culture of the respective isolates were obtained by repeated streaking on SCA plates. Pure isolates were transferred to freshly prepared PDA slants (Potato dextrose agar, pH 7) to establish stock cultures, code-numbered and incubated at 26±2ºC until sporulation. Well sporulated slant culture tubes were subsequently stored at 4ºC for further processing. Stock cultures were transferred every 4-5 weeks on to fresh PDA slants. For morphological observation, incubated leaf fragments on which the growth of actinomycetes was visible were selected for scanning electron microscopy (SEM). Specimens were mounted on stubs, sputter-coated with gold and viewed on SEM at an accelerating voltage of 5kV and images recorded.

Fifty ml yeast extract-malt extract (ISP2) (Shirling and Gottlieb, 1966) at pH 7 was inoculated with 0.5 ml spore suspension of selected isolates in a flask and incubated under static conditions at room temperature (28±2^{O}C) for 15 days. The contents of the flasks were filtered separately through a cotton pad to exclude spores and mycelial mass. The clear filtrate was centrifuged at 5000 rpm for 10 min. The clear supernatant was transferred to sterile conical flaks and stored in freezer for further processing.

Antimicrobial activity of the culture broth was tested by agar well method against four species of bacteria *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 424), *Proteus vulgaris* (MTCC 744). Bacteria were grown on nutrient broth (pH 7) and *Candida* grown on PDB (Potato dextrose broth, pH 6.5) for 24 hours were spread and inoculated onto sterile PDA agar in separate plates using sterile cotton swabs. Wells were made in each plate using sterile 6 mm diameter cork borer. Crude supernatant of each actinomycete strain (50µl) was added separately in to each well and incubated at room temperature for 24 hr, at the end of which bacterial growth was observed and the zone of inhibition was measured and recorded.

Five species of fungi *Botrytis cinerea*, *Curvularia lunata*, *Fusarium oxysporum* (MTCC 1755), *Fusarium solani, Rhizoctonia solani* were used for antifungal screening. Fungal mycelial discs (6 mm) of each of the five fungi were aseptically transferred and positioned in the centre of PDA (pH 6.5) plates. Streptomycin (50 µl/ml) was added to the medium to suppress bacterial contamination and wells were made in each plate using sterile 6 mm diameter cork borer 3cm away from the centre of the plate. Crude supernatant of each endophytic actinomycete strain was added separately (50 µl) into each well and incubated at room temperature for 3-5 days. The zone of inhibition between the edge of the fungal colony and the well was measured. The intensity of inhibition was noted as follows: ++ = 11-19 mm; + = 2-10 mm; ± = < 1 mm; - = 0 mm.

Selective isolation of actinomycetes from plants is important for obtaining new strains and diversity of microorganisms is an important factor for the screening and isolation of new bioactive compounds. A quantitative estimation of the presence of actinomycetes in the leaves of *Catharanthes roseus* is presented in Table 1. Around 70% of the fragments were colonized. Examination of leaf segments under SEM confirmed emergence of actinomycetes hypae from *Catharanthes roseus* (Fig. 1). The images were taken from leaf fragments that were incubated for 4 weeks on isolation plates. During this incubation, the green colour of leaves turned to brown and the colonies of actinomycetes emerged on the cut leaf margins. After 30 days of incubation a total of 38 endophytic actinomycetes were obtained from the leaves which indicated the richness of microbial diversity in *Catharanthus roseus*. Among the 38 isolates, 20 were selected based on colony morphology for antimicrobial activity screening against two Gram positive-*B. subtilis*, *S. aureus* and two Gram negative- *P. aeruginosa*, *P. vul-*

Table 1. Quantitative estimation of **e**ndophytic actinomycetes isolated from *Catharanthes roseus* leaf.

Plant	Starch-casein agar			
	n[a]	%[b]	Avg[c]	SD
Catharanthes roseus	30	70	2.26	1.94

[a]n, number of fragments, [b]Percentage of colonized fragments. [c]Average number of actinomycete colonies per fragment.

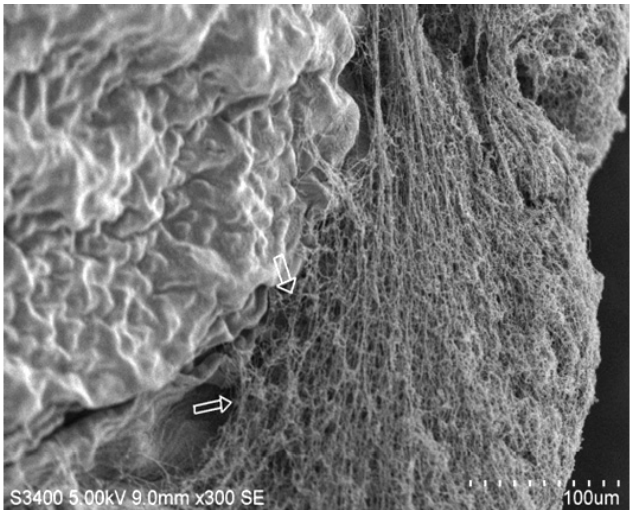

Figure 1. Scanning electron micrograph of aerial hyphae of actinomycetes growing on surface sterilized *Catharanthes roseus* leaf fragments.

garis, five filamentous fungi *F. oxysporum*, *F. solani*, *B. cinerea*, *R. solani*, *C. lunata* and one yeast *Candida albicans* (Table 2). Among the 20 isolates, 11 (55%) exhibited antifungal activity, where as 13 (65%) of the isolates evinced antibacterial activity (Fig. 2 A, B) and 35% were inactive. Eleven isolates inhibited both fungi and bacteria. Among the bacteria 11 isolates inhibited Gram positive bacteria and Gram negative bacteria. Interestingly, isolate Cr18 inhibited only prokaryotes and Cr19 inhibited only Gram positive bacteria but there was no activity towards Gram negative bacteria and fungi. Two isolates (Cr 12, Cr 20) inhibited all the filamentous fungi and thus exhibited broad spectrum activity. Out of the five filamentous fungi tested *C. lunata* was inhibited by 8 isolates followed by *B. cinerea* (6 isolates), *F. solani* (5 isolates),

Table 2. Antimicrobial activity of endophytic actinomycetes isolated from *Catharanthes roseus* leaf.

Actinomycetes Isolates	Fungi						Bacteria			
	Ca	Fo	Fs	Bc	Rs	Cl	Bs	Sa	Pv	Pa
Cr 01	-	-	-	-	-	-	-	-	-	-
Cr 02	-	-	-	-	-	-	-	-	-	-
Cr 03	-	-	+	+	-	++	++	++	-	-
Cr 04	+	-	-	++	-	-	++	++	++	-
Cr 05	-	-	-	-	-	-	-	-	-	-
Cr 06	-	-	-	-	-	-	-	-	-	-
Cr 07	-	-	-	-	-	-	-	-	-	-
Cr 08	-	-	-	-	-	-	-	-	-	-
Cr 09	-	-	-	-	-	+	-	++	++	+
Cr 10	-	+	+	-	++	+	++	-	++	-
Cr 11	-	+	-	-	++	+	++	-	++	+
Cr 12	-	+	+	++	++	++	++	++	++	-
Cr 13	+	-	-	-	-	-	-	-	++	+
Cr 14	-	-	-	-	-	+	-	++	-	++
Cr 15	-	-	-	+	-	+	+	-	+	-
Cr 16	-	-	-	-	-	-	-	-	-	-
Cr 17	+	+	+	+	-	-	-	-	+	++
Cr 18	-	-	-	-	-	-	++	-	+	+
Cr 19	-	-	-	-	-	-	++	++	-	-
Cr 20	-	+	+	++	+	+	++	++	++	-

Cr: *Catharanthes roseus,* Ca: *Candida albicans,* Fo: *Fusarium oxysporum,* Fs: *Fusarium solani,* Bc: *Botrytis cinerea,* Rs: *Rhizoctonia solani,* Cl: *Curvularia lunata,* Bs: *Bacillus subtilis,* Sa: *Staphylococcus aureus,* Pv: *Proteus vulgaris,* Pa: *Pseudomonas aeruginosa*. ++ = 11-19 mm, + = 2-10 mm; ± = < 1 mm; - = 0 mm

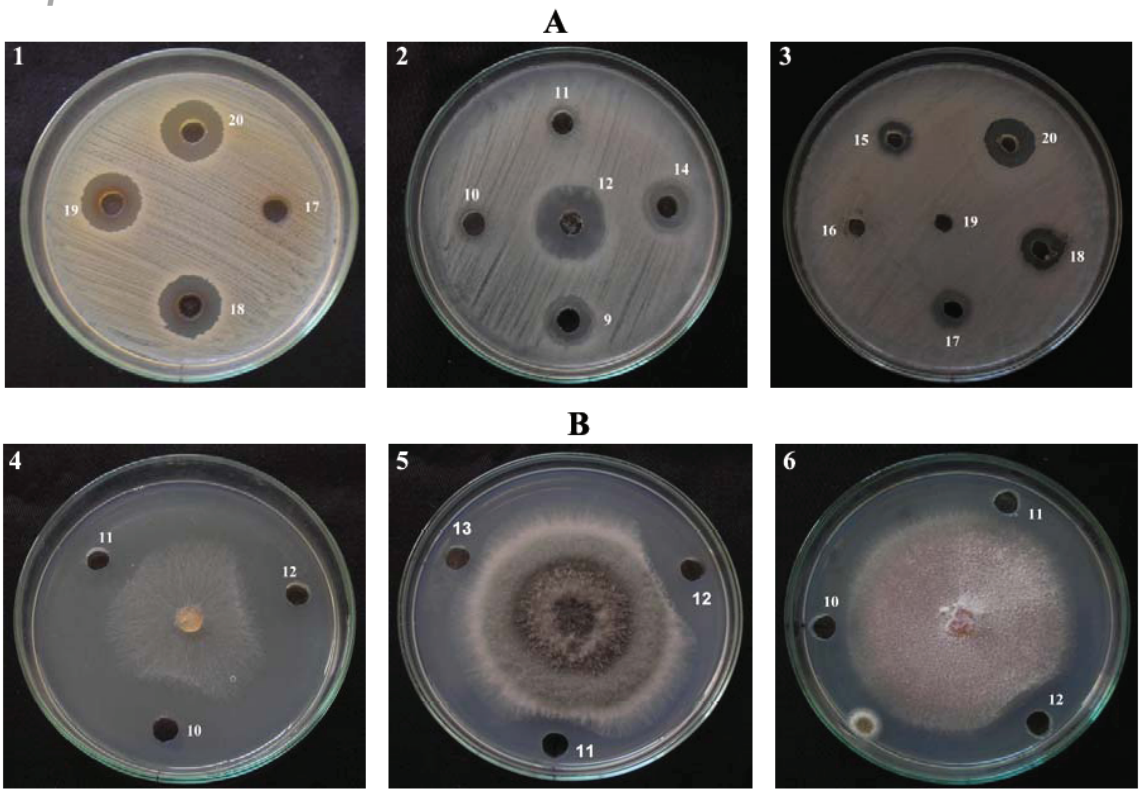

Figure 2. Antimicrobial activity of the isolates (refer table 2.) A: Antibacterial 1. *Bacillus subtilis* 2. *Staphylococcus aureus* 3. *Proteus vulgaris* B: Antifungal 4. *Rhizoctonia solani* 5. *Curvularia lunata* 6. *Botrytis cinerea*.

F. oxysporum (5 isolates) and *R. solani* (4 isolates). Only three isolates inhibited *Candida albicans*. Interestingly more number of isolates (10) inhibited *Proteus vulgaris*-a Gram negative bacterium, followed by *B. subtilis* (9 isolates), *S. aureus* (7 isolates), *P. aeruginosa* (6 isolates). Antibacterial screening indicated that six isolates inhibited a maximum of three test bacteria and seven isolates inhibited growth of two test organisms each. Seven isolates (35%) were inactive for bacteria. Correlating with present results, 53 endophytic actinomycete isolates were obtained from leaves and roots of maize (*Zea mays* L.) 43.4% showed antimicrobial activity (Araujo *et al*., 2000). Thirty isolates of endophytic actinomycetes from four medicinal plants indicated microbial inhibition against *Gardnerella vaginitis* and *Shigella boydii* and 27 isolates exhibited cytotoxic effect against *Artemia salina* (El-Shatoury *et al*., 2006). A few recent studies have highlighted the bioactive importance of endophytic actinomycetes, including bio-control of fungal plant pathogens (Taechowisan and Lumyong, 2003), production of plant growth regulators (Igarashi, 2004) and production of enzymes (Taechowisan *et al*., 2003; Stamford *et al*., 2002). *C. roseus* is well known producer of biologically active terpenoid indole alkaloids (TIAs) with over 130 compounds isolated and identified (Samuelsson, 1999; Verpoorte *et al*., 1997). Vinblastine and vincristine obtained from this plant are known anticancer drugs used in the treatment of acute leukemia and Hodgkin's disease (Noble, 1990). Actinomycetes of medicinal plants such as *Catharanthes roseus* have tremendous scope for the screening of novel bioactive compounds. Based on the screening results Cr-12 and Cr-20 which showed relatively remarkable intense activity against the test organisms, were selected for further studies. Among the selected isolates Cr12 had high level of sequence similarity to *Streptomyces cavourensis* subsp. *cavourensis* AB184264.1 (100% identity) by 16S rDNA sequence. Further study is in progress to identify the bioactive compounds of the selected isolates.

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