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ANTIMICROBIAL POTENTIALS OF ENDOPHYTIC FUNGI INHABITING *RHODODENDRON ANTHOPOGON* D. DON

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ABSTRACT

Fungal endophytes have been studied from *Rhododendron anthopogon* D. Don Manaslu Conservation Area in Nepal. The endophytes were isolated from different parts of *Rhododendron* viz., root, stem and leaf using potato dextrose agar, malt extract agar and water agar. Altogether eighteen fungal endophytes belonging to nine genera were isolated. The endophytic species isolates belong to the genera *Stemphylium, Alternaria, Penicillium, Aspergillus, Trichoderma, Papulaspora, Hansfordia, Wardomyces* and *Geotrichum.* Out of 18 fungal isolates, 8 isolates could display antimicrobial activity inhibiting at least one of the test pathogens. Among the potent strains, 4 displayed both antibacterial and antifungal activities. Endophytic fungal isolates ERAA3, ERAA6 and ERAA8 displayed antimicrobial activity against all the tested bacterial (10) and fungal (5) pathogens. The endophytic strains were very effective against the bacterial pathogens and moderately active against the fungal pathogens. The study reinforced the assumption that endophytes of the high altitude medicinal plants could be a promising source of antimicrobial substances.

Key words: Antimicrobial, bacteria, diversity, endophyte, fungi.

INTRODUCTION

Endophytes are endosymbionts, mainly a bacterium or fungus living within the cells of a plant for atleast part of its life (sometimes for whole) without causing apparent harm to the host. They are ubiquitous and have been found in all the species of the plants studied so far. They have a unique role in preventing the access and colonization of pathogenic organisms in their host plants because certain endophytic associations lead to enhancement of the pathogen resistance of the plant (White and Cole 1985) and an increase in the vegetative growth (Clay 1987) when compared to similar uninfected plants. It is hypothesized that the endophytes, in contrast to known pathogens, generally have far greater phenotypic plasticity and thus more options to interact with their host than pathogens (Shultz and Boyle 2005). They act as a preventer and create a barrier effect where the local endophytes outcompete and prevent pathogenic organisms from taking hold. In the adverse conditions, they secrete metabolites, combat with pathogens and prevent the plants on which they reside. Endophytic fungi represent an important and qualifiable component of fungal biodiversity, and are known to affect plant community diversity and structure (Sanders 2004, Gonthier *et al.* 2006, Krings *et al.* 2007).

Rhododendron anthopogon D. Don (Ericaceae) is an evergreen small shrub that is native to Nepal growing up to the altitude of 4,500 m. This plant is widely used as incense for its aromatic properties. They have high medicinal value and the leaves and fresh flowers, are made into a tea by Himalayan healers and drunk to promote digestive heat, stimulate appetite and relieve liver disorders. Anthopogon tea is also drunk for sore throat, and to counteract water-earth illness, fire headaches, fire back pain, cold, blood disorders, bone disease, potato allergies, and vomiting (Siwakoti 2008). The aim of the current research was to investigate the diversity and antimicrobial potential of endophytic fungi from *R. anthopogon.*

MATERIALS AND METHODS

Study site and sample collection: The study area lies in the Kalchuman lake area of Prok VDC of Manaslu Conservation Area in Nepal. Bordering the Annapurna Conservation Area in the west and Tibetan Plateau on the east, the Manaslu region lies in Gorkha District of Nepal. Healthy (showing no visual disease symptom) and mature plants (roots, stems and leaves) of *R. anthopogon* were collected from Kalchuman lake area (sub alpine region) from an altitude of 3,690 masl (N 28°30'16.5", E 84°48'30") in April 2010. The collected samples were kept in sterile polythene bags, preserved in the cold ice boxes and processed as soon as it was brought to the laboratory.

Isolation and identification of endophytic fungal isolates: The sampling regime was designed with the intention of isolating as many endophyte species as possible from *R. anthopogon*. All the samples were washed twice in distilled water and then surface sterilized by immersion for one min in 70% ethanol, 4 min in sodium hypochlorite (3% available chlorine) and 30 sec in 70% ethanol and then washed 3 times in sterilized distilled water for one min each time to ensure complete sterilization. After surface sterilization the samples were blot dried on sterile blotting paper and then cut into 5-7mm pieces. These were then aseptically transferred to plates containing Potato Dextrose Agar (PDA), Malt extract agar (MEA), and Water agar (WA) media. Aliquots from the third wash were plated onto the medium to check that surface sterilization has been effective. A total of 319 fragments were plated, 96, 118 and 105 from root, stem and leaves, respectively. The plates were then incubated at 28°C and checked each day for up to 3 months.

Any fungi present was isolated, purified and then maintained at 4°C on PDA slopes for further identification. The morphological identification of endophytic fungal strains is based on morphology of the fungal culture colony or hyphae, characteristics of the spores and reproductive structures if these features were discernible (Wei 1979, Carmichael *et al.* 1980, Barnett and Hunter 1998). Induction of sporulation was done by inoculating the isolated fungi in different media. Measurements were taken properly noting the fungal characters in water mounts and the slides were subsequently mounted in lactophenol and sealed with nail vanish. The experiments and observations were performed in duplicate.

Colonization rate (CR): Colonization rate were calculated using the formula given by Taylor *et al.* (1999) and Li *et al.* (2007).

$$CR = \frac{\text{Total number of samples yielding} \geq 1 \text{ isolate} \times 100}{\text{Total number of samples in that trial}}$$

Antibacterial susceptibility testing: Different strains of bacteria used for antimicrobial assay were *Proteus mirabilis* (ATCC 49132), *Salmonella* Typhi, *Enterococcus faecalis* (ATCC 29212),

Bacillus subtilis (ATCC 6633), *Acinetobacter* sp., *Klebsiella pneumoniae, Shigella dysentriae, Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Dual culture technique was adopted for this. In a well grown colony of the endophytic fungus, a radial streaking of the respective bacteria was done followed by incubation overnight at 37^{o}C. The growth of bacteria against fungi and their zone of inhibition (ZOI) were recorded.

Antagonistic experimental design between endophytic strains and phytopathogens: Different pathogenic fungi employed for the test were *Fusarium oxysporum, F. moniliforme, F. proliferatum, Exherhilium turticum* and *Sclerotium rolfsii*. Dual culture technique was adopted for antifungal activity test against test pathogens on PDA plates. Five-day old disks (6 mm) of endophytes were placed on 4 different points of Petri plates containing PDA medium. Test pathogens were inoculated at the centre of PDA plates. Plates were incubated at 27^{0}C for 5-8 days. Antifungal activity was indicative as mycelial growth of test fungus prohibited in the direction of active endophytic fungus. The level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist colony from the fungal growth radius. The width of inhibition zones between the pathogen and the endophytes was evaluated as >10 mm (+++, strong inhibition), 2-10 mm (++, moderate inhibition) and <2 mm (+, weak inhibition) (Paul *et al.* 2007).

RESULTS AND DISCUSSION

A total of 18 endophytic fungi were isolated from 96 roots, 118 stem and 105 leaf fragments analyzed giving a colonization rate of 5.33%, 5.08% and 3.81%, respectively. The colonization rates (CR) of endophytic fungi in this study were higher in roots than in other tissue as shown by previous investigators (Stefan *et al.* 2001, Paul *et al.* 2007), but it has been reported elsewhere that leaves and stems were colonized more by endophytic fungi than roots (Huang *et al.* 2008a). The dissimilarity may be because the structure and substrate are different between different tissues. Altogether eighteen fungal endophytes belonging to at least nine genera were isolated. The endopytic species isolates belong to the genera *Stemphylium, Altenaria, Penicillium, Aspergillus, Trichoderma, Papulaspora, Hansfordia, Wardomyces, Geotrichum*. The endophytic fungal isolates belong to ten groups along with some *Mycelia sterilia*. *Mycelia sterilia* were distinguished on the basis of absence of any fruiting bodies (spores). The occurrence of fungal endophytes is mainly influenced by environmental factors and type of host tissue. Large numbers of isolates were found in underground parts than the aerial parts of selected medicinal plant which is evident from Table 1.

Colonization of the endophytic fungi is ubiquitous yet selective in nature. This selective colonization of the endophyte may lead to production of special compounds within the host plant (Huang *et al.* 2008b). The common endophytic fungi had a wide distribution in different tissues of the plant and hence a high isolate abundance. For example, *Mycelia sterilia*, *Alternaria* sp. and *Aspergillus* sp. were found in all parts of plant tested. Composition and abundance of the endophytes varied according to host tissues. The roots harboured more endophytic fungi than stems or leaves.

Isolation of only 18 endophytic fungal isolates shows that the medicinal property of the plant has some role to play in the colonization of endophytic fungi. This low rate of colonization may be attributed to secretion of the phyto-chemicals, since they contain certain antifungal and antibacterial components (Rajgopal *et al.* 2010).

Out of 18 fungal isolates, 8 isolates could display antimicrobial activity inhibiting at least one of the test pathogens. Among the potent strains, 4 displayed both antibacterial and antifungal activities. Endophytic fungal isolates *Alternaria* sp. (ERAA3) *Penicillium* sp. (ERAA8) and *Trichoderma* sp. (ERAA6) displayed antimicrobial activity against all the tested bacterial (10) and fungal (5) pathogens. The endophytic strains were very effective against the bacterial pathogens and moderately active against the fungal pathogens. The difference in antimicrobial activity is due to variation of structures of bacteria from that of fungi (Xuan *et al.* 2008). Antimicrobial activities of plant endophytic fungi have been reported by several groups (Paul *et al.* 2007, Li *et al.* 2005 and Naik *et al.* 2007). Presence of the endophytes within the plant tissues has a definite purpose. In fact, it can be said that there is a symbiotic relationship between the host and the endophyte. Endophyte helps in secreting different metabolites defending the host against the parasite while the host provides a sheltering mechanism and providing nourishment to the endophytes.

Table 1. Endophytic fungi isolates from *R. anthopogon*.

Isolates code	Isolation part	Identified fungus
ERAA1	Root	Sterile
ERAA2	Root	*Stemphylium* sp.
ERAA3	Root	*Alternaria* sp.
ERAA4	Root	*Hansfordia* sp.
ERAA5	Root	*Aspergillus* sp.
ERAA6	Root	*Trichoderma* sp.
ERAA7	Root	*Wardomyces* sp.
ERAA8	Root	*Penicilium* sp.
ERAB1	Stem	*Aspergillus* sp.
ERAB2	Stem	*Geotrichum* sp.
ERAB3	Stem	Sterile
ERAB4	Stem	Sterile
ERAB5	Stem	Sterile
ERAB6	Stem	*Papulaspora* sp.
ERAC1	Leaf	Sterile
ERAC2	Leaf	*Alternaria* sp.
ERAC3	Leaf	Sterile
ERAC4	Leaf	*Alternaria* sp.

Table 2. Antibacterial assay of the endophytic fungi isolates.

Endophytic fungi isolate	Antibacterial activity against the bacterial pathogens									
	B. subtilis	***S. aureus***	***E. coli***	***S. typhi***	***P. aeruginos***	***E. faecalis***	***S. dysentriae***	***Acinetobacter* spp.**	***P. miabilis***	***K. pneumoniae***
ERAA1	--	--	--	--	--	--	--	--	--	--
ERAA2	+	+	+	+	+	+	--	+	+	+
ERAA3	+++	+++	+++	+++	+++	++	+++	+++	+++	++
ERAA4	--	--	--	--	--	--	--	--	--	--
ERAA5	--	--	--	--	--	--	--	--	--	--
ERAA6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
ERAA7	--	--	--	--	--	--	--	--	--	--
ERAA8	++	+++	+++	++	+++	+++	+++	+++	+++	++
ERAB1	+	--	+	+	++	++	--	+	++	+
ERAB2	+	+	--	--	--	--	--	+	--	--
ERAB3	--	--	--	--	--	--	--	--	--	--
ERAB4	--	--	--	--	--	--	--	--	--	--
ERAB5	--	--	--	--	--	--	--	--	--	--
ERAB6	--	--	--	--	--	--	--	--	--	--
ERAC1	--	--	--	--	--	--	--	--	--	--
ERAC2	++	++	++	--	--	+	--	++	--	+
ERAC3	--	--	--	--	--	--	--	--	--	--
ERAC4	++	++	+	+	--	+	--	+	--	--

Note: (+) = weak antibacterial activity; (++) = moderate antibacterial activity; (+++) = strong antibacterial activity (--) = absence of antibacterial activity

Table 3. Antifungal assay of the endophytic fungi isolates.

Endophytic fungi isolates (Codes)	Antifungal activity against the fungal pathogens				
	F. oxysporum	***F. moniliforme***	***F. proliferatum***	***E. turticum***	***S. rolfsii***
ERAA1	--	--	--	--	--
ERAA2	--	--	--	--	--
ERAA3	+	+	+	++	++
ERAA4	--	--	--	--	--
ERAA5	--	--	--	--	--
ERAA6	++	++	++	++	++
ERAA7	--	--	--	--	--
ERAA8	++	+	+	++	+
ERAB1	--	--	--	--	--
ERAB2	--	--	--	--	--
ERAB3	--	--	--	--	--
ERAB4	--	--	--	--	--
ERAB5	--	--	--	--	--
ERAB6	--	--	--	--	--
ERAC1	--	--	--	--	--
ERAC2	--	--	--	--	--
ERAC3	--	--	--	--	--
ERAC4	--	+	--	++	+

Note: (+) = weak antagonistic activity; (++) = moderate antagonistic activity; (--) = absence of antagonistic activity

The fungal endophytes from medicinal plant *R. anthopogon* have great ability to synthesize natural products as they exhibit excellent antimicrobial properties (Tables 2 and 3). Endophytic microorganisms are excellent sources of bioactive natural products that can be used to satisfy demand of pharmaceutical, medicinal, agriculture and industries (Jalgaonwala *et al.* 2011). Plants have provided mankind with a source of medicinal agents, with natural products once serving as source of all drugs (Balandrin *et al.* 1993). Antibacterial resistance especially among Gram negative bacteria is an important issue that has created a number of problems in treatment of infectious diseases and necessitates the search for alternative drug or natural antibacterial agents (Gangadevi and Muthumary 2008). Fungal endophytes affect the early evolution of plants and probably impose selective pressure on plants creating an enormous diversity of endophytes that produce bioactive metabolites.

CONCLUSION

This study recorded diversity of endophytic fungi from a medicinal plant *R. anthopogon* found in high Himalayas of Nepal. The endophytic strains isolated were very effective against the bacterial pathogens and moderately active against the fungal pathogens. The study reinforced the assumption that endophytes of the high altitude medicinal plants could be a promising source of antimicrobial substances.

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