NUSANTARA BIOSCIENCE
Vol. 8, No. 1, pp. 14-17
May 2016

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n080104

Short Communication: Antimycotic activity and phytochemical screening of fungal endophytes associated with *Santalum album*

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Manuscript received: 2 December 2015. Revision accepted: 3 February 2016.

Abstract. *Tapwal A, Pradhan S, Chandra S, Rashmi. 2016. Antimycotic activity and phytochemical screening of fungal endophytes associated with* Santalum album*. Nusantara Bioscience 8: 14-17.* The heartwood of *Santalum album* constitutes the central part of the tree is valued for its fragrance. The wood and oil are utilized in medicine. Sandalwood oil is extensively used in perfumery, cosmetics, aromatherapy, and pharmaceutical industry. The endophytic microorganisms inhabiting the plant tissues are expected to mimic some of the metabolites of its host. This study was aimed to isolate and screen the fungal endophytes inhabiting the *Santalum album* for antimicrobial activity and for the presence of important phytochemicals. Five fungal endophytes isolated from different parts of *S. album* have exhibited antimicrobial potential against *Fusarium oxysporum* in the range of 5.0-40.4%. The isolated endophytic fungi also indicated the presence of alkaloids, phenolics, and tannins, flavonoids, carbohydrates and glycosides, terpenoids, amino acids and proteins.

Keywords: Antimycotic activity, fungal endophyte, phytochemicals, *Santalum album*

INTRODUCTION

Sandalwood is a commercially and culturally important plant species belonging to the family Santalaceae and the genus *Santalum*. It is a hemiparasitic tree, native to semi-arid areas of the Indian subcontinent. It is now planted in India, China, Srilanka, Indonesia, Malaysia, the Philippines, and Northern Australia. Sandalwood is commercially known as the East Indian sandalwood and its oil as East Indian sandalwood oil. Sandalwood has great importance in Indian culture. The heartwood of the tree is valued for its aroma and also constitutes one of the finest natural materials for carving (Kumar et al. 2012). The fragrance is due to presence of α-and β santalols in sandalwood oil. The average yield of oil ranges from 4.5-2.5% (Sindhu et al. 2010). The oil is used for its therapeutic effects in Ayurveda, Chinese and Tibetan medicinal systems. The sandalwood tree has been widely used in folk medicine for treatment of common colds, bronchitis, skin disorders, heart ailments, general weakness, fever, infection of the urinary tract, inflammation of the mouth and pharynx, liver and gallbladder complaints. Plants are reservoir of a large number of phytochemicals, which are known to protect the human body from diseases and the damaging effect of free radicals. Sandalwood contains more than 200 constituents, the essential oil is emergent as an interesting and biologically valuable active source of phytochemicals (Misra and Dey 2013). Many fungal and bacterial species live inside the plant tissues without harming them are known as endophytes. Many of such endophytes mimic the secondary metabolism of host species and secret compound of human interest. These can be isolated from the host and can be commercially grown to get the compounds of human welfare. Endophytic fungi are able to produce a range of volatile organic compounds that are lethal to pathogenic fungi and bacteria. Endophytes may also produce chemicals, which inhibit the growth of competitors, including pathogenic organisms (Woropong et al. 2001). Sun et al. (2014) have isolated and identified 25 fungal endophytes associated with roots of *Santalum album* and *Kuhnia rosmarinifolia*. The most frequently isolated endophytes were species of *Penicillium* and *Fusarium*. Although the *S. album* is a root parasite of *K. rosmarinifolia*, but both the plants apparently do not share same endophyte isolates.

Beside the production of bioactive compounds, the endophytes are also known to release antibiotic substances which enable them to act as biocontrol agents (BCA) for management of various plant diseases. Endophytic fungi protect the plant from attack of insect-pest and diseases, and also being able to produce substances of biotechnological interest (Garcia et al. 2012). Mejia et al. (2008) screened the fungal endophytes of *Theobroma cacao* for antagonism against *Moniliophthora roreri*, *Phytophthora palmivora*, and *Moniliophthora perniciosa*, and recorded significant growth inhibition in laboratory conditions. The present study aimed at isolation of fungal endophytes associated with healthy *S. album* trees and their evaluation for antimycotic activity against *Fusarium oxysporum*. The fungal endophytes were also screened for the presence of important phytochemicals.

MATERIALS AND METHODS

Isolation of fungal endophytes

Different parts of *S. album* (bark, wood, leaves, and twigs) were collected and surface sterilized before inoculation. Four media [potato dextrose agar (PDA), czapek dox agar (CDA), yeast mannitol agar (YMA), and water agar (WA)] were tried. Pure cultures were raised by subsequent subculturing on PDA.

Screening of fungal endophytes against *Fusarium oxysporum* in dual culture

F. oxysporum, the common pathogen of *S. album* was procured from Forest Pathology Division, Forest Research Institute, Dehradun, India. Fungal endophytes were screened against *F. oxysporum* in dual cultures on PDA. For this, 5 mm discs of fungal endophyte and pathogen were co-inoculated 4 cm apart on Potato Dextrose Agar (PDA) in Petri plates. In control, only a disc of pathogen was inoculated. The plates were kept in incubator at 25±1°C. Radial growth of the pathogen was measured on the fifth day of incubation and compared with the growth of pathogen in control (Dennis and Webster, 1971**).** Whole of experiment was carried out in triplicates. Percent growth inhibition was determined by the formula:

$$\text{Percent inhibition} = (A_1\text{-}A_2)/A_1 \text{x} 100$$

Where A_1 is area covered by pathogen in control and A_2 is area covered by pathogen in dual culture.

Effect of non-volatile compounds released by fungal endophytes on the growth of *F. oxysporum*

Poisoned food technique (Nene and Thapliyal 1993) was followed to study the effect of non-volatile compounds released by the fungal endophyte on the growth of pathogen. The endophytes were grown in potato dextrose broth and incubated at 25±1°C. After 15 days of incubation, the media containing fungal endophyte were filtered through Whatman-I filter paper and finally passed through syringe filter (Ran Disc, PVD 0.45 µm) under aseptic conditions. The PDA was amended with culture filtrate (20%) just before pouring. Five mm discs of the pathogen were inoculated in the Petri plates amended with culture filtrate and incubated at 25±1°C. The colony diameter of the pathogen was measured and compared with control. PDA without culture filtrate served as control.

Extract preparation for phytochemical screening

Fungal endophytes were cultivated in potato dextrose broth for 15 days at 25±1°C in shaking conditions. Mycelial biomass was harvested by filtering through Whatman No. 1 filter paper. The filter papers containing mycelial mat were oven dried to get constant weight. The mycelial mat was crushed and mixed with distilled water and ethanol. Finally filtered and the filtrate was utilized for phytochemical screening.

Phytochemical screening

Preliminary phytochemical screening of the fungal endophytes for the presence or absence of important constitutes was carried out by adapting the following methodologies:

Test for alkaloids: Dragendorff's reagent test. Few mL of filtrate was taken in a test tube and 1-2 mL of Dragendorff's reagent was added. Appearance of yellow colored precipitate confirmed the presence of alkaloids.

Test for phenolics and tannins: Ferric chloride test. Extract was dissolved in the distilled water and few drops of 5% ferric chloride solution were added. A dark green color indicated the presence of Phenolics and Tannins.

Test for flavonoids. 5 mL of dilute ammonia solution was added to the portion of extract, followed by the addition of few drops of concentrated sulphuric acid. Appearance of yellow color confirmed the presence of flavonoids.

Test for carbohydrates and glycosides: Molisch test. 2 mL of filtrate was taken in the test tube followed by addition of 1-2 drops of alcoholic of α-naphthol, mixture was shaken well and concentrated sulphuric acid was added along sides of the test tube. Formation of a violet color ring confirmed the presence of carbohydrates.

Test for reducing sugars: Fehling's solution test. 1 mL of filtrate was boiled in water followed by addition of 1 mL of Fehling solution. Red colored precipitate indicated the presence of reducing sugars.

Test for terpenoids: Salkowski test. 5 mL of extract was mixed with 2 mL of chloroform and 3 mL of sulphuric acid was added from the sides of the test tube. Formation of reddish-brown coloration at interface indicated the presence of terpenoids.

Test for steroids: Liebermann burchard's test. Extract was dissolved in 2 mL of acetic anhydride and 1-2 drops of sulphuric acid were added along sides of the test tube. Blue-green ring appears, or the array of color changes indicates the presence of steroids.

Test for amino acids. 1-2 drops of phenolphthalein were added to the extract and of dilute sodium hydroxide solution was added drop by drop. Appearance of pink color confirmed the presence of amino acids.

Test for protein: Biuret test. 2 mL of filtrate was treated with one drop of 2% copper sulfate solution. To this, 1 mL of ethanol (95%) and excess of potassium hydroxide pellets were added. A pink color appears in the ethanolic layer confirmed the presence of proteins.

RESULTS AND DISCUSSION

Fungal endophytes

Temporary mounts of endophytic fungi were made in lactophenol cotton-blue and slides were observed under high magnification (10x45 lenses) using trinocular microscope. Fungi were identified on the basis of their cultural and microscopic characteristics (shape and size of spores, hyphae) by following standard monographs and taxonomic manuals. Five fungal endophytes of *S. album* were identified as *Fusarium oxysporum* (non-pathogenic),

F. solani, *Histoplasma* sp. and *Periconia* sp. and *Pestalotiopsis* sp. Out of these, three species (*F. oxysporum, Histoplasma* sp. and *Pestalotiopsis* sp.) were recorded on CDA and rest of two *Fusarium solani* and *Periconia* sp. have recorded their initial growth on YMA and PDA respectively (Table 1). Since all of identified fungal endophytes grow well of PDA, it was preferred as routine media for further experimentation.

In vitro antimycotic activity of fungal endophytes against *F. oxysporum*

The isolated endophytic fungi were screened for their antimycotic potential against pathogenic strain of *Fusarium oxysporum* by dual culture technique. The results revealed that the fungal endophytes restricted the growth of pathogen at variable rates and an inhibition zone has been observed at the point of contact. *Pestalotiopsis* sp. recorded maximum growth inhibition against *Fusarium oxysporum* (40.40%) followed by *Periconia* sp. (36.66%), *Fusarium solani* (22.38%) *Fusarium oxysporum* (non-pathogenic endophyte) (19.04%) and minimum 11.90% by *Histoplasma* sp. (Table 2).

Effect of non-volatile compounds released by fungal endophytes on the growth of *Fusarium oxysporum*

Growth inhibition of pathogen was observed on the PDA amended with culture filtrate of fungal endophytes (Table 2). It highest on the media amended with the culture filtrate of *Periconia* sp. (20.00%) followed by *Pestalotiopsis* sp. (16.66%), *Histoplasma* sp. (13.33%), *F. oxysporum* (5.0%) and lowest by *F. solani* (2.66%). It is assumed that during the growth of fungal endophyte in liquid media, may have released some secondary metabolites which have restricted the growth of pathogen. The growth inhibition was lower as compared to dual culture experiment, it may enhance with increase in the incubation period of fungal endophyte in broth or by increasing the concentration of culture filtrate used in PDA amendment.

Phytochemical screening of fungal endophytes

The hyphal biomass of fungal endophytes was screened for the presence of important phytochemicals. The results revealed that the amino acids, phenolic and tannins, proteins and steroids were present in all species, flavonoids were present in *Fusarium oxysporum*, *Periconia*, and *Pestalotiopsis* sp., the carbohydrates and glycosides were present in *Fusarium solani*, *F. oxysporum* and *Histoplasma* sp. while alkaloids and terpenoids were present only in *Fusarium solani* (Table 3).

Table 1. Fungal endophytes isolated on different media from *S. album*

Fungal endophytes	PDA	YMA	CDA	WA
Fusarium oxysporum	-	-	+	-
Fusarium solani	-	+	-	-
Histoplasma sp.	-	-	+	-
Periconia sp.	+	-	-	-
Pestalotiopsis sp.	-	-	+	-

Table 2. In vitro antimycotic activity of fungal endophytes against pathogen

Fungal endophytes	Growth inhibition (%)	
	Dual culture	Non-volatile compounds
Fusarium oxysporum	19.04	5.00
Fusarium solani	22.38	2.66
Histoplasma sp.	11.90	13.33
Periconia sp.	36.66	20.00
Pestalotiopsis sp.	40.40	16.66

Table 3. Phytochemical screening of fungal endophytes

Phytochemicals	Fungal endophytes				
	F. oxysporum	*F. solani*	*Histoplasma* sp.	*Periconia* sp.	*Pestalotiopsis* sp.
Alkaloids	-	+	-	-	-
Amino acids	+	+	+	+	+
Carbohydrates and glycosides	+	+	+	-	-
Flavonoids	+	-	-	+	+
Phenolics and tannins	+	+	+	+	+
Proteins	+	+	+	+	+
Steroids	+	+	+	+	+
Terpenoids	-	+	-	-	-

Discussion

Santalum album has been cultivated, processed and traded since ancient times for its essential oil due to good economic returns. It is hemiparasite to the roots of other tree species, but without major detriment to its hosts. East Indian sandalwood tree has been widely used in folk medicines for treatment of common colds, bronchitis, skin disorders, heart ailments, general weakness, fever, infection of the urinary tract, inflammation of the mouth and pharynx, liver and gallbladder complaints and other maladies (Misra and Dey 2013). Due to significance of this species in certain cultures and high market prices, it is facing large scale exploitation. To protect this species from overexploitation, alternates are required to get it's by-products. Use of fungal endophytes in this field is an emerging area of research. Endophytes are the microorganisms that live within a plant without causing apparent disease. Endophytes are also having an ability to produce novel secondary metabolites for medical, agricultural and industrial use. They may secrete the similar metabolite of their host plant. Endophytic fungi are known to be associated with plants for over 400 million years (Krings et al. 2007). Number of endophytic fungi are associated with medicinal plants can be cultured on artificial media to get variety of secondary metabolites and bioactive compounds valuable for the pharmaceutical industry (Krishnamurthy et al. 2008; Khan et al. 2010). It

will also reduce the harvesting pressure on the plant species. Earlier, many researchers have isolated number of fungal endophytes from different plant species and screened them for their antimicrobial and antioxidant activity and phytochemical potential (Deepake et al. 2012; Garcia et al. 2012; Ramesha and Srinivas 2014; Sadananda et al. 2014; Tapwal et al. 2015).

Sun et al. (2014) isolated and identified 25 endophytic fungi from the roots of *Santalum album* and *Kuhnia rosmarinifolia*. Most of isolated endophytes were species of *Penicillium* and *Fusarium* which were associated from the roots of *Santalum album.* Ting et al. (2010) isolated nine fungal endophytes from the stem tissues of *Musa* species and observed growth inhibition against *F. oxysporum* by eight fungal endophytes. Murthy et al. (2011) isolated endophytic species of *Fusarium*, *Aspergillus*, *Penicillium*, and *Mucor* from *Lobelia nicotianifolia* and evaluated them for different phytochemicals and antioxidant potential in methanolic extracts. They recorded positive correlation between the phenolic content and the antioxidant capacity by the extract of associated endophyte. Dhankhar et al. (2012) have isolated 27 fungal endophytes from different parts of *Salvadora oleoides* out of which the species of *Aspergillus, Penicillium*, and *Phoma* have exhibited considerable antioxidant activity and presence of alkaloids, flavonoids, saponins, carbohydrates, tannins, sterols and terpenoids. Sadrati et al. (2013) isolated 20 endophytic fungi and 23 endophytic actinomycetes from wheat (*Triticum durum*) and found considerable antimicrobial activity against twelve pathogenic bacteria, yeast, and two phytopathogenic fungi. In agreement with earlier works, we were also able to isolate and identify five fungal endophytes including two species of *Fusarium. In-vitro* experiments for screening of the endophytes for antimycotic potential against pathogenic strain of *F. oxysporum* revealed variable response. In both experiments, *Periconia* and *Pestalotiopsis* sp. have restricted the growth of pathogen at highest level and *Fusarium* species were less effective. The growth inhibition was more in dual culture as compared with non-volatile compound experiment. Inhibition zone was formed at the point of contact but hyphal coiling was not observed under microscopic examination. Therefore, the growth inhibition in dual culture may be due to releases of variety of phytochemicals by endophytic fungi on PDA. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties and are not essential for microorganism to sustain life. In this study, the isolated endophytic fungi were found positive for some important phytochemicals agreed with earlier findings (Devi et al. 2012; Govindappa et al. 2013; Ramesha and Srinivas; 2014; Tapwal et al. 2015). Further research on isolation and bioprospecting of endophytic fungi for important phytochemicals of *S. album* will definitely help in the conservation of this species.

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