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Full Length Research Paper

Antifungal activity and molecular identification of endophytic fungi from the angiosperm *Rhodomyrtus tomentosa*

Juthatip Jeenkeawpieam[1], Souwalak Phongpaichit[1]*, Vatcharin Rukachaisirikul[2] and Jariya Sakayaroj[3]

[1]Department of Microbiology and Natural Products Research Center, Faculty of Science, Prince of Songkla University, Songkhla, Thailand.
[2]Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Songkhla, Thailand.
[3]Mycology Laboratory, National Center for Genetic Engineering and Biotechnology, Thailand Science Park, Pathumthani, Thailand.

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Problems associated with fungal resistance to antifungal agents are increasing worldwide. There is need to find sources of novel antifungal drugs and endophytic fungi could be another interesting source. In this study, 213 culturable endophytic fungi isolated from leaves and branches of *Rhodomyrtus tomentosa* from Thailand were screened for their ability to produce antifungal agents. Crude extracts from 177 out of 213 fungal isolates exhibited inhibitory activity against human pathogenic fungi, evaluated by a microbroth dilution method. Of the 349 active extracts out of 617 extracts tested, 43.5% inhibited *Penicillium marneffei*; 16.5 to 20.8% inhibited two strains of *Cryptococcus neoformans*; 6.0% *Microsporum gypseum*; 5.0% *Candida albicans*, with minimal inhibitory concentrations (MIC) of 1 to 200 μg/ml. 22 endophytic fungi showing strong antifungal activity (MICs of 1 to 8 μg/ml) were identified by morphological and molecular methods. They mainly belonged to the genera *Colletotrichum*, *Diaporthe*, *Guignardia* and *Phomopsis*. The results of this work indicate that endophytic fungi from *R. tomentosa* can be a good source of potential antifungal natural products.

Key words: *Rhodomyrtus tomentosa*, antifungal activity, endophytic fungi, molecular identification.

INTRODUCTION

Fungal infections pose an increasing threat to public health. Opportunistic infections, such as aspergillosis, candidiasis and cryptococcosis, have emerged as major problems worldwide in cancer patients, transplant recipients and other immunocompromised individuals, including those with acquired immune deficiency syndrome (AIDS) (Pitisuttithum et al., 2001; Patterson, 2005; Umeh and Umeakanne, 2010). There is a high incidence of penicilliosis in AIDS patients in Southeast Asia (Vanittanakom et al., 2006; Hai et al., 2010). Despite advances in antifungal therapy in the last decade and the increasing numbers of drugs available for treating fungal infections, a high prevalence of antifungal resistance and failures of clinical treatments have been reported (Bueid et al., 2010; Pfaller et al., 2010, 2011).

Since the discovery of penicillin, fungi have been regarded as a good source of antimicrobial natural products. Griseofulvin, a major antifungal agent is industrially obtained from the fungus *Penicillium griseofulvum* (Odds, 2003). Endophytic fungi of plants have been shown to produce a wide range of bioactive compounds (Zou et al., 2000; Strobel et al., 2004; Gunatilaka, 2006; Wang et al., 2007; Huang et al., 2008; Verma et al., 2009; Aly et al., 2010). Antifungal compounds have been reported from various fungal

*Corresponding author. E-mail: souwalak.p@psu.ac.th. Tel/Fax: +66 74 446661.

endophytes against both plant and human pathogens. Cryptocandin, a potent antifungal agent isolated from the endophytic fungus *Cryptosporiopsis cf. quercine* from the medicinal plant *Tripterigium wiflordii* showed excellent antifungal activities against *Candida albicans*, *Trichophyton metagrophytes* and *Trichophyton rubrum* with minimal inhibitory concentration (MIC) values of 0.03 to 0.07 µg/ml. It was also active against many plant pathogenic fungi (Strobel et al., 1999).

Four compounds produced from *Colletotrichum* sp. isolated from *Artemisia annua* were inhibitory to *C. albicans* and *Aspergillus niger* (MIC 50 to 100 µg/ml) and exhibited fungistatic activities towards plant pathogens (Lu et al., 2000). Silva et al. (2006) reported that the five cadinane sesquiterpenoids isolated from the endophytic fungus *Phomopsis cassiae* obtained from *Cassia spectabilis* exhibited antifungal activity against *Cladosporium cladosporioides* and *Cladosporium sphaerospermum*. Sordaricin, a compound produced by the endophytic fungus *Xylaria* sp. from the leaves of *Garcinia dulcis*, showed moderate activity against a broad range of human fungal pathogens (Pongcharoen et al., 2008). Two new cyclohexanone derivatives, pestalophone C and E produced by the endophytic fungus *Pestalotiopsis fici* showed significant antifungal activities against *Aspergillus fumigatus* (Liu et al., 2009).

Rhodomyrtus tomentosa (Aiton) Hassk is a flowering plant in the family Myrtaceae, native to southern and southeastern Asia. In Thailand *R. tomentosa* occurs most frequently in coastal sandy soils on both the east and west coasts of Peninsular Thailand (Winotai et al., 2005). It has been used in traditional medicine in the south of Thailand as an antipyretic, antidiarrheal and antidysentery agent (Chuakul, 2005). In the framework of a research program on new antimicrobial compounds of fungal origin, we isolated endophytic fungi from *R. tomentosa* and tested them for their ability to produce substances active against human fungal pathogens including *C. albicans*, *Cryptococcus neoformans*, *Microsporum gypseum*, and *Penicillium marneffei*. The isolates were then identified based on their morphology and the analyses of the large subunit (LSU) and the internal transcribed spacers (ITS1-5.8S-ITS2) of ribosomal deoxyribonucleic acid (rDNA) regions.

MATERIALS AND METHODS

Plant materials and isolation of endophytic fungi

Eight healthy *R. tomentosa* plants were collected from Songkhla and Phattalung provinces in the south of Thailand. The plant specimens were compared with the voucher specimens at Prince of Songkla University Herbarium. Small segments (0.5 × 0.5 cm^2) of leaves and branches were cut and surface-sterilized by sequential washes in 95% ethanol for 30 s, 5% sodium hypochlorite for 5 min, 95% ethanol for 30 s and finally in sterile distilled water for 1 min three times. After surface-drying with sterile filter paper, the leaf segments were placed onto corn meal agar medium (CMA) supplemented with antibiotics (penicillin plus streptomycin sulphate, each at 50 mg/L). Plates were incubated at 25°C for one week. Fungal growth was observed daily. Pure cultures were isolated, by hyphal tip isolation, on potato dextrose agar (PDA) plates without antibiotics and stored in 15% glycerol at -80°C.

Molecular identification and phylogenetic analysis

Endophytic fungi that produced good antifungal activity were identified based on morphological traits and the analysis of LSU and the ITS sequences of rDNA regions. Genomic DNA was extracted using the protocols described by Wang et al. (1993) and Jasalavich et al. (2000). The ITS regions were amplified by the polymerase chain reaction (PCR) with the universal primer pairs ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), or ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (White et al., 1990; Gardes and Bruns, 1993). For PCR amplifications of the 28S ribonucleic acid (RNA), we used the fungal primer set LROR (5′-ACCCGCTGAACTTAAGC-3′) and LR7 (5′-TACTACCACCAAGATCT-3′) or JS1 (5′-CGCTGAACTTAAGCATAT-3′) and JS8 (5′-CATCCATTTTCAGGGCTA-3′). Purification of the DNA fragment was performed utilizing the NucleoSpin® extract DNA purification kit Cat. No. 740 609.50 (Macherey-Nagel, Germany) as described by the manufacturer's protocol. DNA sequencing was performed using the primers aforementioned, at Macrogen, Korea. Both forward and reverse sequences were assembled and edited by Clustal W (Thompson et al., 1994) and BioEdit 7.0.7 programs (Hall, 2007).

Sequences of each endophytic fungal strain were aligned with nucleotide sequences obtained from the GenBank database. The alignment was inspected and adjusted manually where necessary in BioEdit 7.0.7 (Hall, 2007). Phylogenetic analysis was performed using maximum parsimony (MP), neighbor joining (NJ) and Bayesian inference approaches. MP with 100 random addition heuristic searches with a stepwise starting tree and tree bisection and reconnection (TBR) branch-swapping algorithm and NJ were carried out using PAUP*4.0b10 (Swofford, 2002). The stability of each clade was evaluated by bootstrap analysis with 1,000 replications. Bayesian phylogenetic inference was calculated with Mr Bayes 3.1.2 with a general time reversible (GTR) model of DNA substitution and a gamma distribution rate variation across sites (Huelsenbeck and Ronquist, 2001). Four Markov chains were run twice from random starting trees for 5,000,000 generations, trees were sampled every 100 generations and the first 5,000 trees were discarded as burn-in. The remaining trees were used to construct a majority rule consensus tree accompanied with posterior probability (PP) values.

Preparation of fungal extracts

Each endophytic fungal isolate was grown on PDA and incubated at 25°C for three to five days. Six mycelial plugs (1 × 1 cm^2) from the edge of the actively growing colony were inoculated into 500 ml Erlenmeyer flasks containing 300 ml potato dextrose broth (PDB) and incubated for three weeks at room temperature (RT) under stationary condition for production of possible antifungal agents. The culture broth was extracted with ethyl acetate (EtOAc) and a dry ethyl acetate extract from fermentation broth (BE) was obtained by evaporation to dryness under reduced pressure at 45°C using a rotary vacuum.

The fungal mycelia were extracted with methanol followed by hexane or EtOAc. Dry hexane extracts (CH) and ethyl acetate extracts (CE) from the mycelia were obtained by evaporation under vacuum.

Table 1. Antifungal activity of crude extracts from endophytic fungi from *R. tomentosa*.

Parameter	Antifungal activity				
	Ca	Cn12	Cn13	Pm	Mg
Active extracts/total extracts (%)	30/598 (5.0)	97/587 (16.5)	121/583 (20.8)	263/604 (43.5)	36/604 (6.0)
Active isolates/total isolates (%)	23/213 (10.8)	49/213 (23.0)	69/213 (32.4)	149/212 (70.3)	26/212 (12.3)
MIC (µg/ml)	1 - 200	8-200	1- 200	2-200	4-200
MFC (µg/ml)	2->200	64->200	32->200	16->200	64->200
Amphotericin B (MIC/MFC, µg/ml)	0.25/0.5	0.125/0.5	0.5/4	2/2	-
Miconazole (MIC/MFC, µg/ml)	-	-	-	-	2/4

Ca, *Candida albicans* ATCC90028; Cn12, *Cryptococcus neoformans* ATCC90112 (flucytosine sensitive); Cn13, *C. neoformans* ATCC90113 (flucytosine-resistant); Pm, *Penicillium marneffei* clinical isolate; Mg, *Microsporum gypseum* clinical isolate; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration.

Antifungal assay

The human pathogenic fungi used for testing antifungal activity were standard strains from the American type culture collection (ATCC) including *C. albicans* ATCC90028, *C. neoformans* ATCC90112 (flucytosine-sensitive), *C. neoformans* ATCC90113 (flucytosine-resistant), clinical isolates of *M. gypseum* from the Mycology Laboratory, Siriraj Hospital, Mahidol University and *P. marneffei* from Pathology Laboratory, Songklanagarind Hospital, Prince of Songkla University. Before testing, the isolates were kept in 15% glycerol at -80°C and subcultured on Sabouraud's dextrose agar (SDA) at 35°C for 24 h for *C. albicans* and at 25°C for 48 h for *C. neoformans* and seven days for *M. gypseum* and *P. marneffei*. Each isolate was subcultured at least twice to ensure purity. The dried endophytic fungal extracts were dissolved in dimethyl sulfoxide (DMSO) and screened for antifungal activity at a concentration of 200 µg/ml by a modification of the microbroth dilution methods described by the Clinical and Laboratory Standards Institute (CLSI) for yeasts (M27-A2 document) (CLSI, 2002a) and for filamentous fungi (M38-A) (CLSI, 2002b). Briefly, each extract was dissolved in DMSO to prepare a stock solution of 100 mg/ml and stored at -20°C. When tested, DMSO was used to dilute fungal extracts to 1:10 and Roswell Park Memorial Institute (RPMI) medium for further 1:25 dilution to obtain a concentration of 400 µg/ml. Triplicate 50 µl aliquots of crude extracts (400 µg/ml) were placed into sterile 96-well microtiter plates. 50 µl of fungal suspensions (10^6 CFU/ml for *C. albicans* and *C. neoformans*, 8×10^3 conidia/ml for *M. gypseum* and *P. marneffei*) were added to each well so that the final concentration of crude extract was then 200 µg/ml.

The final concentration of DMSO was 2%. Plates were incubated at 35°C for 24 h for *C. albicans*, 48 h at 25°C for *C. neoformans* and at 25°C for six days for *M. gypseum* and *P. marneffei*, then 10 µl resazurin indicator (0.18%) was added to each well and examined after further incubation for 5 h at 35°C for yeasts and one day for molds (Sarker et al., 2007). After incubation, a blue color of the wells indicated inhibition of growth (positive result) and a pink color indicated growth (negative). Amphotericin B and miconazole (10 µg/ml) were used as positive inhibitory controls and for comparison with the extract. DMSO and RPMI medium at the same concentration as in the test were used as solvent and growth controls, respectively.

Serial two-fold dilutions of active extracts were further tested by the same methods and the MIC was determined as the lowest concentration of extract that inhibited growth (blue color). Concentrations of extract at MIC and higher than the MIC was streaked onto SDA plates and incubated under appropriate conditions. The lowest concentration of extract that killed the test fungus (no growth on SDA) was recorded as the minimal fungicidal concentration (MFC).

RESULTS

Antifungal activity of endophytic fungal crude extracts

In total, 617 extracts from 213 fungal isolates from *R. tomentosa* were tested for antifungal activity against five strains of human pathogenic fungi. 349 extracts (56.6%) from which 177 isolates (83.1% of total isolates) showed antifungal activity against at least one test fungus. Among the three types of extracts, the CH extracts were the most active (65%) followed by CE (52%) and BE (52%). Antifungal activities of crude extracts are shown in Tables 1 and 2. Endophytic fungal extracts were most active against *P. marneffei* (43.5%) followed by *C. neoformans* (16.5 to 20.8%), *M. gypseum* (6.0%) and *C. albicans* (5.0%), respectively.

Among the 349 active extracts, 230, 72, 25, 12 and 10 extracts inhibited one, two, three, four and five test strains, respectively. Table 2 identifies 22 endophytic fungi that produced extracts with strong antifungal activity (MICs lower than 10 µg/ml). 14 and nine isolates strongly inhibited *C. neoformans* and *P. marneffei*, respectively. Only one isolate each strongly inhibited *C. albicans* and *M. gypseum*. The most active extracts such as RP22CE, RP42BE, RP42CH, RP43BE, RP43CE, RP44CE, RP62CE, RP83CH and RP202BE exhibited MIC values of 1 to 2 µg/ml, fully comparable to the MIC values of the standard antifungal drugs amphotericin B and miconazole. The antifungal compounds from these extracts will be further characterized.

Molecular identification of active endophytic fungi

Taxonomical identification was restricted to 22 fungal isolates that produced extracts with MIC values below 10 µg/ml (Table 2 and Figure 1). Phylogenetic analyses of

Table 2. Endophytic fungi showing strong antifungal (MIC < 10 µg/ml) and broad spectrum activity.

Endophytic fungi	rDNAregion	GenBank accession no.	Crude extract	MIC/MFC (µg/ml)				
				Ca	Cn12	Cn13	Pm	Mg
Guignardia mangiferae RP8[b]	ITS	JF441175	RP8CE			**8**/128		
	LSU	JF441204						
Guignardia mangiferae RP22[a,b]	ITS	JF441176	RP22CE	32/>200	16/>200	**2**/64	64/64	4/200
	LSU	JF441205	RP22CH		64/>200	**8**/128	128/128	
Phomopsis sp. RP42	ITS	JF441177	RP42BE		32/>200	**2**/128	200/>200	16/>200
	LSU	JF441206	RP42CE		16/>200	**4**/64		200/>200
			RP42CH		32/>200	**2**/64		200/>200
Diaporthe sp. RP43[a]	ITS	JF441178	RP43BE	200/>200	32/>200	**2**/200	200/>200	32/128
	LSU	JF441207	RP43CE	200/>200	8/200	**1**/64	32/>200	64/200
			RP43CH	200/>200	32/200	200/>200	128/>200	128/200
Diaporthe sp. RP44[a]	ITS	JF441179	RP44BE		32/>200	**4**/200		200/>200
	LSU	JF441208	RP44CE	200/>200	16/>200	**1**/64	128/128	128/200
			RP44CH	128/128	64/128	16/32	32/128	64/64
Diaporthe phaseolorum RP52	ITS	JF441180	RP52BE		128/>200	**8**/200		
	LSU	JF441209	RP52CE		32/200	16/128		
			RP52CH				128/>200	
Phomopsis sp. RP56	ITS	JF441181	RP56BE		200/>200	**8**/200		
	LSU	JF441210	RP56CE		64/>200	16/128	32/64	
			RP56CH		200/>200		64/>200	
Phomopsis sp. RP60	ITS	JF441182	RP60BE		**8**/>200	**4**/128		
	LSU	JF441211	RP60CE		**8**/>200	**4**/128	64/>200	
			RP60CH			200/>200	128/>200	
Diaporthe sp. RP62[a]	ITS	JF441183	RP62BE		**8**/>200	**4**/200		64/64
	LSU	JF441212	RP62CE	200/>200	**8**/>200	**2**/64	64/128	200/>200
			RP62CH	128/>200	32/128	16/64	32/32	64/128

Table 2. Contd.

Diaporthe sp. RP68	ITS	JF441184	RP68BE		32/200	**8**/200		200/>200
	LSU	JF441213	RP68CE		32/200	32/200	128/>200	
			RP68CH		200/>200	200/>200	200/>200	
Phomopsis sp. RP70	ITS	JF441185	RP70BE		**8**/200	**8**/200		
	LSU	JF441214	RP70CE		**8**/>200	**8**/200	128/>200	200/>200
			RP70CH			200/>200	32/>200	
Phomopsis phyllanthicola RP83[a]	ITS	JF441187	RP83BE		128/>200	**4**/128		
	LSU	JF441216	RP83CE		16/>200	**4**/128		
			RP83CH	**1/2**	128/>200	64/>200	64/>200	128/128
Phomopsis phyllanthicola RP84[a]	ITS	JF441188	RP84BE		16/>200	**4**/128		
	LSU	JF441217	RP84CE		32/>200	**4**/128		
			RP84CH	200/>200	200/>200	200/>200	200/>200	200/>200
Diaporthe phaseolorum RP91	ITS	JF441189	RP91BE		128/>200	16/>200		
	LSU	JF441218	RP91CE		64/>200	16/>200	**8**/200	
Colletotrichum gloeosporioides RP202[c]	ITS	JF441191	RP202BE				**2**/128	
	LSU	JF441220	RP202CE				200/>200	
			RP202CH				128/>200	
Colletotrichum gloeosporioides RP205[c]	ITS	JF441192	RP205BE				**8**/>200	
	LSU	JF441221	RP205CE				200/200	
			RP205CH				200/>200	
Colletotrichum gloeosporioides RP207[c]	ITS	JF441193	RP207BE				**4**/>200	200/>200
	LSU	JF441222	RP207CE				200/200	200/>200
			RP207CH				200/>200	
Colletotrichum gloeosporioides RP22[c]	ITS	JF441195	RP226BE				**4**/32	200/>200
	LSU	JF441224	RP226CE				200/>200	
			RP226CH				200/>200	
Colletotrichum gloeosporioides RP228[c]	ITS	JF441196	RP228BE				**4**/16	
	LSU	JF441225	RP228CE				200/200	200/>200
			RP228CH				200/>200	

Table 2. Contd.

Colletotrichum sp. RP250[c]	ITS	JF441200	RP250BE				**8**/>200	
	LSU	JF441228	RP250CE				200/>200	
			RP250CH				200/>200	
Colletotrichum gloeosporioides RP258[c]	ITS	JF441202	RP258BE				**8**/>200	200/>200
	LSU	JF441230	RP258CE				128/>200	
			RP258CH				200/>200	
Colletotrichum gloeosporioides RP262[c]	ITS	JF441203	RP262BE				**8**/>200	
	LSU	JF441231	RP262CE				200/>200	
			RP262CH				200/>200	
Amphotericin B				0.25/0.5	0.125/0.5	0.5/4	2/2	
Miconazole								2/4

BE, crude ethyl acetate extract from culture broth; CE, crude ethyl acetate extract from the fungal mycelia; CH, crude hexane extract of the fungal mycelia; Ca, *Candida albicans* ATCC90028; Cn12, *Cryptococcus neoformans* ATCC90112 (flucytosine-sensitive); Cn13, *C. neoformans* ATCC90113 (flucytosine-resistant); Pm, *Penicillium marneffei* clinical isolate; Mg, *Microsporum gypseum* clinical isolate; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; [a]Endophytic fungi that produced broad spectrum extract; [b]Endophytic fungi that produced pycnidiospores and ascospores; [c]Endophytic fungi that produced conidiospores.
Strong inhibitory activity is highlighted (bold).

LSU and ITS rDNA sequences were performed and 89 ITS sequences were analysed. *Lentinus tigrinus* AB478881, *Ganoderma fornicatum* FJ655476 and *Abstoma purpureum* GQ981488 were used as outgroups. Out of 552 total characters, 149 were constant and 348 were parsimony informative. 61 equally most parsimonious trees were obtained from the heuristic searches, the best of which is shown in Figure 1 (Tree length = 1481 steps, CI = 0.566, RI = 0.854, RC = 0.483; HI = 0.434). A similar topology was obtained from NJ and Bayesian analyses (trees not shown).

The results based on ITS sequence analysis revealed that the active endophytic fungi belong to the ascomycetous lineage of the Dothideomycetes and are distributed in three orders: the Botryosphaeriales, that is the sister group to the rest of the clade, the Glomerellales and Diaporthales (Figure 1). 12 isolates (RP42, RP43, RP44, RP52, RP56, RP60, RP62, RP68, RP70, RP83, RP84 and RP91) clustered with the genera *Phomopsis* and *Diaporthe* with strong bootstrap supports (94/100% MP/NJ). Although, genotypically closely related, the strains RP43, RP44, RP52, RP62, RP68, RP70 and RP91 segregated in a branch with relatively low MP/NJ (78/83%) bootstrap values. RP52 and RP91 grouped with several *Diaporthe phaseolorum* strains with 99.5% sequence identity. The isolate RP70 closely matched *Phomopsis* sp. GU066614 and *D. phaseolorum* GU066605 (99.2% sequence identity), whereas RP62 was closely related to *Diaporthe* sp. GU066695 and GU066697 (99.5 to 99.7% identity).

Based on the aforementioned data, the strains RP70 and RP62 are provisionally identified as *Phomopsis* sp. and *Diaporthe* sp., respectively. RP43, RP44 and RP68 sequences had high sequence identity (96.6 to 100%) with two *Diaporthe* sp. (AB505415 and GU066664). The isolates RP43 and RP44 showed 100% sequence identity with each other and with *Diaporthe* sp. GU066664. The isolates RP60 and RP42 were all placed in a subclade containing *Phomopsis* sp. (EU002931, HM999921, GQ407101, GU066650, FJ037768 and GU592007) and fungal sp. EU563599 with strong support (99/100% MP/NJ, 0.97 PP). RP60 and RP42 were found to have 99.5 and 100% sequence identity with *Phomopsis* sp. GU066650 and *Phomopsis* sp. FJ037768,

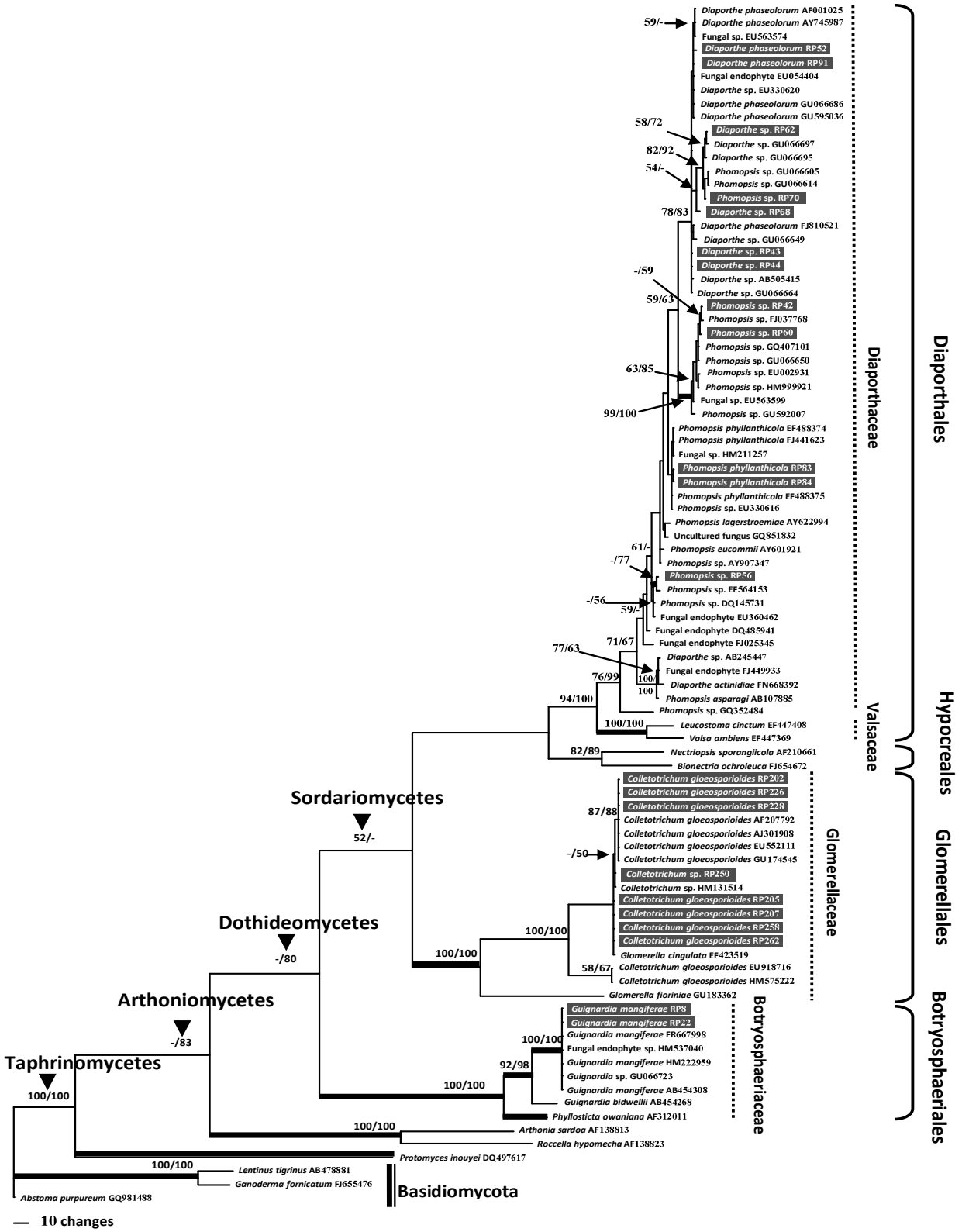

Figure 1. Phylogenetic tree based on ITS rDNA sequence data showing the taxonomic relationship of 22 fungal isolates from *Rhodomyrtus tomentosa* (labelled in black color) with reference taxa from GenBank. *Lentinus tigrinus* AB478881, *Ganoderma fornicatum* FJ655476 and *Abstoma purpureum* GQ981488 were used as outgroups. Branch support values (>50%) (n = 1000 replicates) are parsimony bootstrap and neighbor-joining bootstrap. Thickened branches represent clades with Bayesian posterior probabilities greater than 0.95. Scale bar represents 10 changes. ITS, Internal transcribed spacers.

respectively. Two of the fungal strains, RP83 and RP84, were grouped together into a subclade sister to *Phomopsis phyllanthicola* accessions and were 99.5 to 99.7% identical to *P. phyllanthicola* (EF488374, EF488375 and FJ441623). These taxa formed a well supported clade in all the analyses and therefore are provisionally identified as *P. phyllanthicola.* The isolate RP56 clustered with *Phomopsis* sp. (EF564153 and DQ145731) and the fungal endophyte EU360462 with 56% NJ bootstrap and 0.96 PP supports and had a relatively high sequence identity (98.3 to 98.5%) with both. Thus, RP56 is provisionally assigned to the genus *Phomopsis*.

On the basis of morphological and cultural characteristics for example, size and shape of conidia, type of conidiophores, presence of setae, growth rate, colony color and texture (Hyde et al., 2009), RP202, RP205, RP207, RP226, RP228, RP250, RP258 and RP262 were tentatively identified as *Colletotrichum* sp. Their colonies had dense, white to grey mycelium. Some isolates produced orange conidial masses. Conidium was hyaline, one-celled, cylindrical with one end rounded, slightly curved or dumbbell shaped. Melanized appressoria were also observed in some isolates. ITS sequence analysis confirmed that these isolates resided in the Glomerellaceae (100% MP and NJ bootstrap, 1.00 PP).

In agreement with the morphology-based diagnosis, the strains RP202, RP226 and RP228 clustered in a clade containing *Colletotrichum gloeosporioides* strains (AF207792, AJ301908, EU552111, and GU174545) with 87/88% MP/NJ bootstrap support. Nucleotide identity within this subclade ranged from 99.8 to 100%, with only one base pair difference, indicating that RP202, RP224, RP226 and RP228 could be assigned to *C. gloeosporioides.* RP250 was the only endophytic strain that clustered with *Colletotrichum* sp. HM131514, with a nucleotide sequence identity of 99.6%, supporting assignment of this strain to *Colletotrichum* sp. The endophytes RP205, RP207, RP258 and RP262 showed total sequence identity with *C. gloeosporioides* (AJ301986, AY266387, HM146134, AY266389, EU918716, and HM575222) and of 99.8 to 100% identity with *Glomerella cingulata*. They are therefore provisionally identified as *C. gloeosporioides*. Only two isolates (RP8 and RP22) produced pycnidiospores and ascospores. They showed their closest match with members of the Botryosphaeriales and formed a highly supported clade (100% MP and NJ bootstrap, 1.00 PP and 100% sequence identity with *Guignardia mangiferae*, *Guignardia* sp. and fungal endophyte. In this analysis, RP8 and RP22 are provisionally classified as *G. mangiferae.*

DISCUSSION

CH extracts showed higher antifungal activity than the CE and BE extracts from the same fungal isolates. This result support the conclusion by Buatong et al. (2011) that antifungal compounds present in fungal mycelia usually are either cell-bound components or extracellular components with low polarity. Crude extracts of endophytic fungi from *R. tomentosa* exhibited antifungal activity against important human pathogens. It is interesting to note that the flucytosine-resistant *C. neoformans* ATCC90113 was more susceptible to endophytic fungal extracts than the flucytosine sensitive *C. neoformans* ATCC90112. In addition, preliminary analysis of the chemical composition of endophytic fungal extracts has shown that tyrosol is the major compound in extracts produced by *Phomopsis* sp. RP56, RP60, RP70, *P. phyllanthicola* RP83, RP84 and *Diaporthe* sp. RP68. Tyrosol, 2-(4-hydroxyphenyl) ethanol is a well-known phenolic compound with antioxidant properties found in wine and olive oil (Bu et al., 2007). It has been reported to have antifungal (Slininger et al., 2004), antibacterial (Romero et al., 2007) and anticancer activities (Sommart et al., 2009). Interestingly, tyrosol is a possible quorum-sensing molecule in endophytic fungi (Guimarães et al., 2009). This compound may play an important role as an antimicrobial agent in extracts of these fungi.

A particularly relevant result of the present study is the discovery that crude extracts from endophytic fungi of *R. tomentosa* exhibited the highest inhibitory activity against *P. marneffei,* the etiological agent of penicilliosis in AIDS-affected patients. This is the first report of endophytic fungi active against *P. marneffei*. As the result of the identification of the inhibitory endophytic fungi, 22 isolates were distributed in various groups including *Colletotrichum* sp., *C. gloeosporioides*, *Phomopsis* sp., *P. phyllanthicola*, *Diaporthe* sp., *D. phaseolorum* and *G. mangiferae*. These are a highly diverse group of fungi that include endophytes and latent plant pathogens of trees and plants (Alves et al., 2008; Huang et al., 2008). It is well known that certain endophytic fungi may become pathogenic when trees and plants are under stress (Carroll, 1988; Old et al., 1990) but, at the same time, they are able to produce a diversity of secondary metabolites with antibacterial, antifungal and anti-tumoral activity (Liu et al., 2009; Ding et al., 2010).

Six isolates including *G. mangiferae* RP22, *Diaporthe* sp. RP43 and RP44, RP62, *P. phyllanthicola* RP83 and RP84 produced broad-spectrum metabolites that inhibited all the tested fungi. Some antifungal metabolites from members of these genera have been identified; these include phomopsichalasin (Horn et al., 1995), cytosporone B and C (Huang et al., 2008), 3, 11, 12-trihydroxycadalene (Silva et al., 2006) and 8α-acetoxymultiploide A (Wu et al., 2008) from *Phomopsis* sp., and colletotric acid (Ren et al., 2008) and colutellin A (Silva et al., 2006) from *Colletotrichum* sp. From this study, we were able to select many potential endophytic fungi from *R. tomentosa* that produced strong antifungal activity against various human pathogens. The results

indicate that *R. tomentosa* was a good plant source for endophytic fungi producing antifungal natural products. Of particular interest is the potential use of these endophytes as a source of active compounds against *P. marneffei.*

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