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Revealing the antimicrobial and enzymatic potentials of culturable fungal endophytes from tropical pitcher plants (*Nepenthes* spp.)

Lee JM, Tan WS and Ting ASY*

School of Science, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 46150 Petaling Jaya, Selangor, Malaysia.

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Abstract

In recent years, fungal endophytes from diverse host species have been extensively studied for valuable compounds (enzymes, antimicrobial compounds). Investigations on fungal endophytes from tropical *Nepenthes* spp. are however limited, despite hosting a wide range of macro- and microorganisms. In this study, we explored fungal endophytes from *N. ampullaria* and *N. mirabilis* for enzymatic and antimicrobial potential. A total of 26 isolates were obtained and they demonstrated a variety of enzymatic activities, mostly producing cellulase, chitinase, lipase and L-asparaginase. These endophytes also have antifungal activity. Sequencing results on 12 selected isolates revealed a diverse endophytic community, comprising mainly of the species complex of *Colletotrichum gloeosporioides* (and its teleomorph *Glomerella*) (3 isolates), *Phomopsis* sp. (2 isolates), *Trichoderma asperellum, Penicillium simplicissimum* and *Aspergillus terreus.* Several uncommon species were also revealed-*Lasiodiplodia, Isaria*, Sarcosomataceae and a yeast-like fungi *Meyerozyma guilliermondii*. We revealed the diversity and the novel profiling of enzymatic and antimicrobial potential of endophytes from *Nepenthes*.

Key words – antibacterial – antifungal – enzymes – *Nepenthes ampullaria* – *Nepenthes mirabilis*

Introduction

Fungal endophytes are defined as fungi that during any of their growth stages, asymptomatically colonize the internal tissues or organs of plant. They are found ubiquitously in various plants; from mosses and ferns to various gymnosperms and angiosperms (Arnold & Lutzoni 2007, U'Ren *et al.* 2012), including aquatic plants (Li *et al.* 2010). Their ability to co-exist and co-evolve with host plants is attributed to the balance achieved between the virulence of endophytes and the host's defenses (Schulz *et al.* 2006). In this symbiotic relationship, fungal endophytes receive 'shelter' and nutrients from the hosts while the hosts benefit from an array of beneficial attributes (White et al. 2002, Debbab et al. 2012) which include safeguarding against natural enemies of plants such as pathogens and herbivores (Tintjer & Rudges 2006, Vega *et al.* 2008; Singh et al. 2011); supporting plant growth through the production of phytohormones (Hamayun *et al.* 2010, Hoffman et al. 2014); and increasing the resistance of plant to multiple stresses such as salinity and heavy metal toxicity in soil (Rodriguez *et al.* 2008, Deng *et al.* 2011, Khan et al. 2014).

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Corresponding Author: Adeline S.Y. Ting – e-mail – adeline.ting@monash.edu

In recent years, fungal endophytes have been explored for diverse applications. This is attributed to the vast range of valuable compounds produced such as enzymes and secondary metabolites. Chitinase, lipase and cellulase are common enzymes produced by various fungal endophytes such as *Beauveria, Lecanicillium, Metarhizium* and *Trichoderma*, which contribute to their role in control of pest and diseases (Fang *et al.* 2005, St Leger & Wang 2010, Sandhu *et al.* 2012). Explorations of other enzymes such as asparaginase (Theantana *et al.* 2007, Thirunavukkarasu *et al.* 2011) and tyrosinase (Mandyam *et al.* 2010) have recently been intensified due to their valuable anticancer (anti-leukemia agent) (Appel *et al.* 2007) and medicinal value (treatment of Parkinson's disease, myocardial disease) (Raju *et al.* 1993). Hydrolytic enzymes (amylase, cellulase and laccase) with various industrial applications are also of major interest. Although hydrolyases are typically isolated from soil-borne *Aspergillus*, *Penicillium* and *Rhizopus* spp. (Pandey *et al.* 2000), endophytes producing these enzymes present an interesting alternative. In addition to enzymes, various secondary metabolites with antibacterial (Gunatilaka 2006, Ahmed *et al.* 2011, Praptiwi *et al.* 2013), anti-inflammatory (Zhang *et al*. 2014); anticancer (Hazalin *et al.* 2009, Mohana 2012), antituberculosis (Gordien *et al.* 2010) and antimalarial (Elfita *et al.* 2011) activities have been identified. Endophytes have also showed potential in the agriculture sector as plant growth promoters (Silva *et al*. 2012, Chujo and Scott 2014, Islam *et al*. 2014) and biocontrol agents (Li *et al.* 2008, Silva *et al.* 2012, Kusari *et al.* 2012).

In this study, the host plant of interest is the tropical pitcher plant, *Nepenthes* spp. or also known as "monkey cups". We selected this atypical host plant and explored the potential of the endophytes they harbor. Pitcher plants are interesting hosts of endophytes as they grow in hostile environments (in soils low in pH and nutrients); relying heavily on nutrient input from captured and digested prey in their pitchers (Mohan and Clarke, 2010). Digestion of prey is regulated by proteases (Athauda *et al.* 2004), lipases (Tokes *et al.* 1974), ribonucleases (Stephenson & Hogan 2006), and chitinases (Eilenberg *et al.* 2006). It is however, not known if the endophytes may have a role in secreting these extracellular enzymes although microbial-deriving lipase, acid phosphatase, glucosidase and glucosaminidase, have been detected in the fluids of the pitcher (Morohoshi *et al.* 2011, Takeuchi *et al.* 2011). Hence, pitcher plants are worthy alternative host plants to isolate beneficial fungal endophytes. For this study, we isolated fungal endophytes from pitchers and leaves of *N. ampullaria* and *N. mirabilis* and screened the endophytes for production of various enzymes and antimicrobial potential. Endophytes demonstrating strong enzymatic and antimicrobial activities were further identified by DNA sequencing of the internal transcribed spacer (ITS) region using universal primers of ITS1 and ITS4 to reveal their most probable identity. Our results here are novel; highlighting endophytes from unexplored host plants, the discovery of some uncommon endophyte species, and the documentation of their enzymatic and antimicrobial potential.

Materials & Methods

The chemicals, media and reagents used were products from various manufacturers and they are listed as the following, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4.7H_2O$ and K_2HPO_4 from Systerm; starch, Na-carboxymethyl cellulose (CMC), 1-napthol, Lactophenol Lotton Blue (LCB), potassium sodium tartarate, $CuNO_3.5H_2O$, $ZnSO_4.7H_2O$ and $CaCl_2.2H_2O$ from R & M Chemicals; chitin, Congo Red, N-acetylglucosamine (NAGA), L-asparagine, 2,2-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) and NaCl from Sigma; Potato Dextrose Agar (PDA), Nutrient Agar (NA), Tween 20, KCl and $NaNO_3$ from Merck; phenol red, glycine and K_2HPO_4 from Riendemann Schmidt; dinitrosalicylic acid (DNS) from Fluka; glucose from Kollin Chemicals; yeast extract from Becton, Dickson and Company; peptone from Lab M; agar powder from SRL Chemicals; and p-cresol from SAFC.

Isolation of endophytes

The pitchers and leaves of *N. ampullaria* and *N. mirabilis* were collected from Sungai Chilling waterfall in Selangor, Malaysia (3.599N 101.736E). The plant samples were surface-sterilized using the triple sterilization technique. Briefly, the plant tissues were sequentially

immersed in 95% ethanol (1 min), followed by 3.25% sodium hypochlorite (10 min) and finally 95% ethanol (30 sec), prior to rinsing with sterile distilled water (three times). The surface-sterilized tissues were then blot-dried, cut into smaller-sized segments (~1 × 1 cm^2) and placed onto Potato Dextrose Agar (PDA). All culture plates were incubated at 25±2^oC, with pure cultures subsequently established by culturing onto fresh PDA plates.

Screening for valuable enzymes from endophytes

Endophytes were screened for their production of amylases, cellulases, asparaginases, laccases, lipases, tyrosinases and chitinases. To detect amylase activity, a 0.5 cm mycelial plug of the endophyte was inoculated onto Glucose Yeast Extract Peptone (GYP) agar (1 g glucose, 0.1 g yeast extract, 0.5 g peptone, 16 g agar and 1 L of distilled water; pH 6) supplemented with 2% soluble starch, and incubated for 5 days at 25±2^oC. After incubation, the agar plate was overlaid with 1% iodine (2% potassium iodide). Amylase activity was confirmed by the formation of halo zone around the colony in an otherwise blue medium (Maria *et al.* 2005). Cellulase activity was detected by inoculating on GYP agar supplemented with 0.5% of CMC, in which after incubation, was overlaid with 0.2% aqueous Congo Red (15 min) and destained with 1M NaCl for another 15 min. Formation of halo zone indicate cellulase activity (Maria *et al.* 2005). Similarly, tyrosinase activity was detected by inoculating on GYP agar and after incubation, was overlaid with a mixture of 0.11% p-cresol and 0.05% glycine and left for 24 h. The appearance of a reddish-brown zone around the colony indicated positive tyrosinase activity (Maria *et al.* 2005).

Chitinase activity was detected by inoculating on chitin-based media (1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 2% agar, 0.5% crude chitin, and 1 L of distilled water, pH 5.5) (Sharaf *et al.* 2012) and incubated at 25±2^oC for 10 days. Isolates with chitinolytic activity grew on the agar while no growth indicates absence of chitinase activity as compared to the positive control using *Serratia marcescens* (Okay *et al.* 2008). Asparaginase activity was detected by inoculating onto modified Czapek Dox Agar (2 g glucose, 10 g L-asparagine, 1.52 g KH_2PO_4, 0.52 g $MgSO_4.7H_2O$, 0.52 g KCl, 0.01 g $FeSO_4.7H_2O$, 0.03 g $CuNO_3.5H_2O$, 0.05 g $ZnSO_4.7H_2O$, 20 g agar, 1 L distilled water) supplemented with 3 mL of phenol red (pH 6.2). After incubation, the formation of a pink zone around the colony in an otherwise yellow medium indicates asparaginase activity (Thirunavukkarasu *et al.* 2011). Laccase activity was assessed by inoculating on GYP agar supplemented with 0.005% 1-napthol (pH 6) and incubated at 25±2^oC for 5 days. Positive laccase activity was ascertained when a purple zone appears around the colony as a result of the oxidation of 1-napthol, on an otherwise clear agar (Maria *et al.* 2005). For lipase activity, mycelial plugs were inoculated onto peptone agar medium (10 g peptone, 5 g NaCl, 0.1 g $CaCl_2.2H_2O$, 16 g agar, 1 L distilled water; pH 6) supplemented with Tween 20 (separately filter sterilized, 1 mL to 100 mL medium) and incubated at 25±2^oC for 5 days. Lipase activity was confirmed with the detection of insoluble calcium crystal complex precipitates around the colony, as a result of the binding of liberated fatty acids with the calcium in the agar (Maria *et al.* 2005). For all enzyme activities, the diameters of the halo zones (or coloured zones in some assays) were measured and compared against the colony diameter to obtain the ratio of colony diameter to enzyme production (Bhagobaty & Joshi 2012).

Activities of key enzymes by selected endophytes

The activities of three key enzymes; amylase, cellulase and chitinase, were further determined quantitatively as these enzymes are related to their ability to colonize host-plants (amylase, cellulase) and their antifungal potential (chitinase). To initiate amylase assay, 25 mL of culture broth (3 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 5 g KCl, 0.01 g $FeSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 15 g starch, 1 L sterile distilled water) was first inoculated with a 0.5 cm mycelial plug and incubated at 25±2^oC for 7 days. The culture was then centrifuged (5000 rpm, 10 min, 4^oC) and the supernatant (crude enzyme) collected for analysis. Amylase activity was assayed by mixing 0.5 mL of crude enzyme with 0.5 mL of starch solution (1% starch in 0.1M phosphate buffer, pH 6.5), and incubated for 30 min at 25±2^oC (Hegde *et al.* 2011). The reducing sugar produced was determined using dinitrosalicylic acid (DNS) (Miller 1959) where 3 mL of DNS reagent was added to the reaction mixture, boiled at 90^oC for 10 min, followed by addition of 1 mL

of 40% potassium sodium tartrate. After cooling, the absorbance was read at 540 nm. The amount of glucose released was calculated from the glucose standard curve (0-35 µmole L^{-1}). One unit (U) of amylase activity was defined as the amount of enzyme needed to release 1 µmole of glucose per min under assay conditions described.

The cellulase activities were assayed based on methods by Gautam *et al.* (2011). It was conducted similarly to amylase assay, where cultures were first established in culture broth (3 g $NaNO_3$, 0.1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 1% CMC) leading to collection of supernatant. The supernatant (0.5 mL) was then mixed with 1.5 mL reaction mixture (0.5 mL of 1% CMC, 1 mL of 0.1 M sodium citrate buffer at pH 4.8) and incubated for 60 min at 50±2°C. The reducing sugar produced was determined using the DNS method as described in the previous section. One unit (U) of cellulase activity was defined as the amount of enzyme needed to release 1 µmole of glucose per min under assay conditions described.

For chitinase activity, cultures were first established in 25 mL culture broth (1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 5 g of $NaNO_3$, 0.5% crude chitin, 1 L sterile distilled water, pH 5) (Sharaf *et al.* 2012) and incubated at 25±2°C for 7 days. The culture was then centrifuged (5000 rpm, 10 min, 4°C), the supernatant collected and transferred (0.5 mL) into a 1.5 mL reaction mixture (containing 1 mL of sterile distilled water, 0.5 mL of 0.2% colloidal chitin in 0.05M phosphate buffer at pH 5.2). The mixture was incubated for 30 min at 50±2°C. The reducing sugar produced was determined using the DNS method as described in previous sections, with absorbance read at 575 nm. The amount of N-acetylglucosamine (NAGA) released was calculated from the NAGA standard curve constructed using concentrations of 0-30 µmole L^{-1}. One unit (U) of chitinase activity was defined as the amount of enzyme needed to release 1 µmole of N-acetylglucosamine per min under assay conditions as described.

Antimicrobial properties of endophytes from *Nepenthes* spp.

The antibacterial activities of endophytes were evaluated against five Gram Positive bacteria (*Bacillus cereus*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Enterococcus faecalis*) and five Gram Negative bacteria (*Escherichia coli*, *Salmonella* Typhi, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*). Plate assay based on methods by Kamalraj & Muthumary (2012) were adopted. Briefly, mycelial plug (5 mm) of fungal endophytes were inoculated onto Nutrient Agar (NA) seeded with test organisms (48 h prior). Agar plugs were used as negative control. The cultures were refrigerated at 4±2°C overnight to allow rapid diffusion of metabolites into the agar, and were subsequently incubated at 37±2°C for 24 h. The diameter of inhibition zone was then measured.

The antifungal activity of endophytes were tested against three major plant pathogens in the tropics; *Fusarium oxysporum* f.sp. *cubense* tropical race 4, and *Ganoderma boninense* and *G. miniatocinctum*, pathogens of the Fusarium wilt and Ganoderma Basal Stem Rot disease, respectively. The assay was carried out according to Rahman *et al.* (2009). Briefly, mycelial plugs (5 mm) of the endophytes and the test pathogen (*Fusarium oxysporum* f.sp. *cubense*) were placed on the same petri dish (paired-cultures), at an equi-distance of 3 cm from each other and from the periphery of the plate. Agar plugs were used as control. These paired-cultures were incubated at 25±2°C for 6 days. The percentage of inhibition of radial growth (PIRG) was determined as:

$$PIRG\ (\%) = R1\text{-}R2/R1 \times 100\%$$

where R1 represents the radial growth of pathogen in control; and R2 the radial growth of pathogen in paired-cultures. The procedure was repeated for all endophytes paired with various pathogens.

Identification of selected endophytes from *Nepenthes* spp.

Endophytes with strong enzymatic and antimicrobial activities were selected for identification based on DNA sequencing. Cultures were first established in Potato Dextrose Broth for 7 days, in which 300 mg fresh weight of the mycelia was harvested and used for genomic DNA extraction. The extraction was performed using the GF-1 Plant DNA Extraction kit (Vivantis, Malaysia) that is also suitable for fungi. Maxime PCR PreMix Kit (iNtRON Biotechnology, Korea)

was used for the PCR reaction mixture (20 µL), which was added with 5 µL DNA template, 1 µL universal forward primer ITS1 (5'TCCGTAGGTGAACCTGCGG3'), 1 µL universal reverse primer ITS4 (5'TCCTCCGCTTATTGATATGC3'), and 13 µL sterile distilled water. PCR was performed with the following temperature program, initial denaturation at 94^{o}C for 120 s; denaturation at 94^{o}C for 20 s; annealing at 60^{o}C for 10 s; extension at 67^{o}C for 45 s; final extension at 72^{o}C for 240 s. Denaturation, annealing and extension were repeated for 35 cycles. The PCR products were then purified using MEGA quick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology, Korea) and outsourced to First Base (Malaysia) for DNA sequencing. Once the DNA sequencing results were obtained, they were edited using the BioEdit (v7.1.3) program. The sequences were subjected to BLAST to determine the most probable species based on similarity (%) with existing isolates in the database.

Statistical analysis

All assays were performed in triplicates. The data obtained was subjected to One-way Analysis of Variance (ANOVA) with means compared using the Tukey's Studentized Test ($HSD_{(0.05)}$). All statistical analyses were carried out using SPSS statistical software package v. 20 (IBM).

Results

Isolation and identification of beneficial endophytes from *Nepenthes* spp.

A higher number of beneficial endophytes were recovered from *N. ampullaria* (9 isolates), from both leaves and pitchers, compared to *N. mirabilis* (3 isolates). Of the 12 endophytes sequenced, we obtained 11 filamentous fungi and one yeast-like fungi (Table 1). Majority of the isolates were common endophytic species, such as *Glomerella* sp. (isolate 10) or their anamorph *Colletotrichum gloeosporioides* (isolates 20, 23), *Phomopsis* sp. (isolates 5, 15), *Trichoderma asperellum* (isolate 3), *Aspergillus terreus* (isolate 24) and *Penicillium simplicissimum* (isolate 19) (Table 1). Several uncommon species such as *Lasiodiplodia*, *Isaria*, *Sarcosomataceous* and a yeast-like fungi *Meyerozyma guilliermondii* were also identified as probable species for isolates 13, 22, 25 and 9, respectively (Table 1). For isolate 20 and 23, we designated these isolates as *Colletotrichum gleosporiodes* species complex thereafter in this article as identification through ITS sequences alone is not sufficient to distinguish them and will require additional gene sequences (Weir *et al.* 2012).

Table 1 Most probable species of culturable endophytes from *N. ampullaria* and *N. mirabilis.*

Isolates	Location in plant	Query cover percentage (%)	Identity percentage (%)	E-value	Most probable identity
3	AL	99	99	0	*Trichoderma asperellum*
5	AL	98	99	0	*Phomopsis* sp.
9	AL	100	99	0	*Meyerozyma guilliermondii*
10	AP	100	100	0	*Glomerella* sp.
13	AP	100	99	0	*Lasiodiplodia* sp.
15	ML	99	97	0	*Phomopsis* sp.
19	MP	100	100	0	*Penicillium simplicissimum*
20	MP	100	100	0	*Colletotrichum gloeosporiodes*
22	AL	98	99	0	*Isaria* sp.
23	AL	99	100	0	*Colletotrichum gloeosporiodes*
24	AP	100	99	0	*Aspergillus terreus*
25	AP	98	96	0	*Sarcosomataceous* sp.

* AL, *N. ampullaria* leaf; AP, *N. ampullaria* pitcher; ML, *N. mirabilis* leaf; MP, *N. mirabilis* pitcher

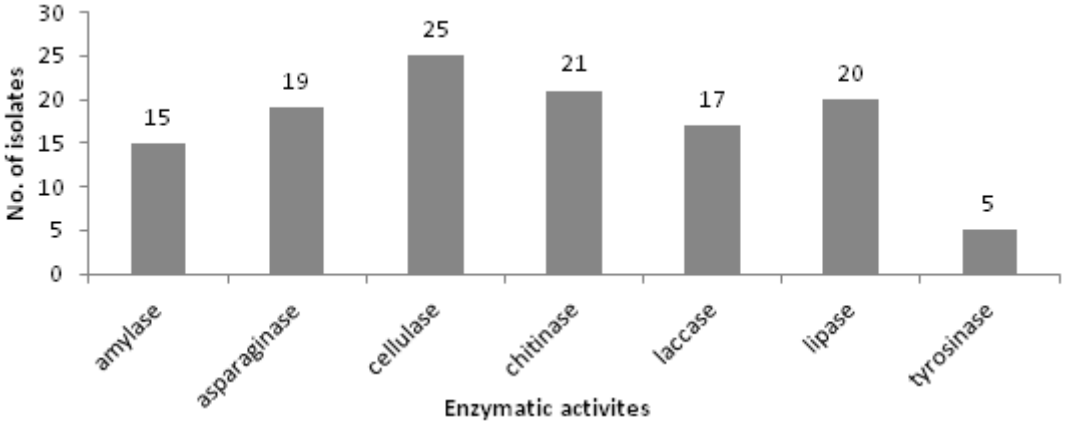

Fig. 1 – Total number of fungal endophytes expressing various enzymatic activities.

Enzymes produced by endophytes from *Nepenthes* spp.

The most common enzyme produced by the endophytes from *Nepenthes spp.* is cellulase, followed by chitinase, lipase, asparaginase, laccase, amylase and finally, tyrosinase, with 25, 21, 20, 19, 17, 15 and 5 positive isolates, respectively (Fig. 1). A comparison among endophytes revealed that isolates 9 (*M. guilliermondii*), 10 (*Glomerella* sp.) and 13 (*Lasiodiplodia* sp.) produced all enzymes assayed. The ratio of halo zone diameter to colony diameter for isolates 9, 10 and 13 were between 0.122 to 3.122, 0.242 to 1.438, and 0.64 to 1.315, respectively and were not necessarily the highest for each enzyme. Among the isolates, isolate 9 (*M. guilliermondii*) showed particularly strong lipase (ratio of 3.122) and asparaginase (ratio of 2.353) production. *Phomopsis* sp. (isolate 5) produced strong laccase activity (1.087) while strong amylase production was detected in *P. simpliscissimum* (isolate 19), *A. terreus* (isolate 24) and *Lasiodiplodia* sp. (isolate 13), each with a ratio of 1.0. Tyrosinase activity was only detected in four isolates; *Glomerella* sp. (isolate 10), *Lasidiplodia* sp. (isolate 13), *Sarcosomataceous* sp. (isolate 25) and *M. guilliermondii* (isolate 9), with ratios of 0.096, 1.0, 1.0 and 0.122, respectively.

Activities of key enzymes produced by selected endophytes

When comparing the two isolates (9 and 10) for amylase, cellulase, chitinase and laccase activities, isolate 10 (*Glomerella* sp.*)* showed better potential than isolate 9 (*M. guilliermondii*), with significantly higher cellulase, laccase and chitinase activities at 0.0276 U mL^{-1}, 0.0600 U mL^{-1}, and 0.0019 U mL^{-1}, respectively. Isolate 9 however, recorded significantly higher amylase activity of 2.1133 mL^{-1} compared to 0.01291 U mL^{-1} produced by isolate 10 (Figure 2). Isolate 13 was not tested as ratios derived from this isolate were relatively low compared to isolate 9 and 10, although all three isolates produced all seven enzymes assayed.

Antimicrobial properties of endophytes from *Nepenthes* sp.

Of the 26 isolates tested for antimicrobial properties, only one endophyte (isolate 17) showed antibacterial activity while 4, 7 and 10 isolates produced antifungal activities (>50% PIRG) towards *G. miniatocinctum*, *G. boninense* and Foc race 4, respectively (Figure 3). Isolate 17 inhibited the growth of *Streptococcus pyogenes* and *Enterococcus faecalis* with inhibitory diameter (mean±SD) of 25 ±0.58 mm and 29 ±1.7 mm, respectively (data not shown). However, this isolate did not produce strong antifungal activity (Fig. 3). Endophytes with antifungal activities towards pathogens were either via competitive exclusion (Fig. 4A), secretion of inhibitory metabolites resulting in a clear inhibition zone (Figure 4B), or a combination of both. Isolate 3 (*T. asperellum*) was consistently effective towards all three pathogens with significantly higher PIRG values of 77.55 % to 97.14 % (Fig. 3). The other isolates with antifungal activities towards all three pathogens were isolates 23 (*C. gloeosporiodes* species complex), 13 (*Lasiodiplodia* sp.) and 19 (*P. simplicissimum*), with PIRG values ranging from 68.75 % to 72.45 %, 49.44 % to 67.35 % and 60.00 % to 67.35 % respectively (Fig. 3). Mechanisms of inhibition were found to be similar for all of these three isolates, via competitive exclusion mechanism. Other isolates that were found to

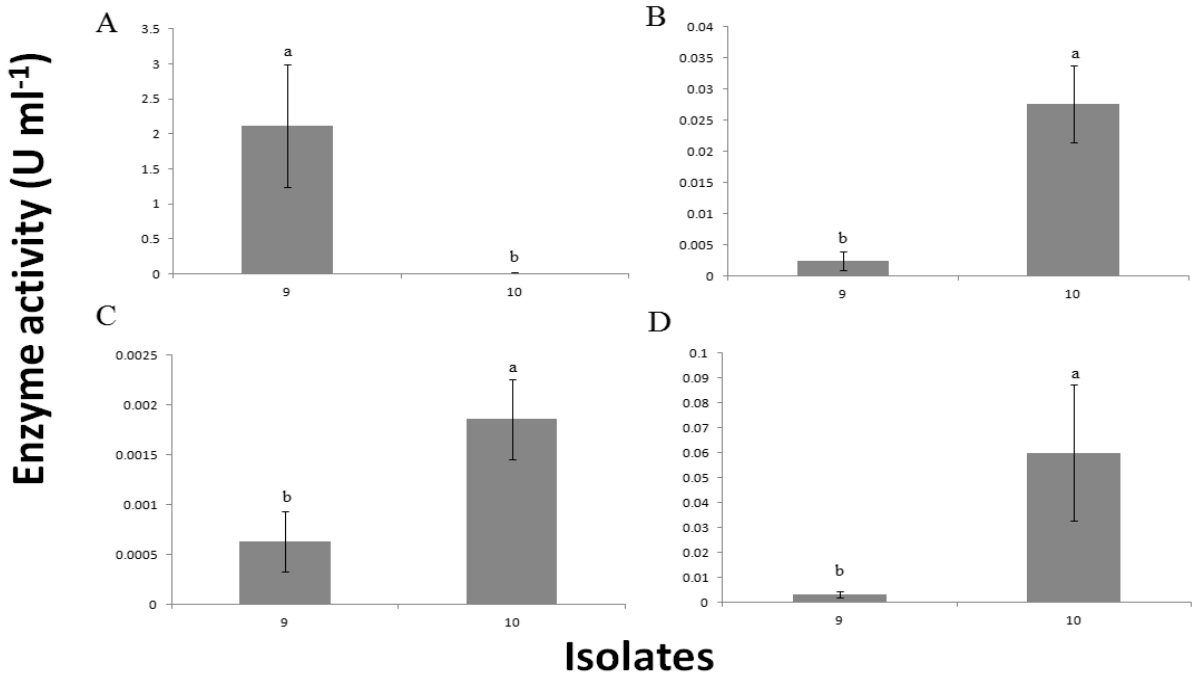

Fig. 2 – Enzymatic assay of A, amylase. B, cellulose. C, chitinase. D, laccase of selected isolates. Values are means of triplicates. Means with the same letters are not significantly different $HSD_{(0.05)}$. Bars are standard deviations of means.

inhibit *G. boninense* were isolates 22 (*Isaria* sp.) and 24 (*A. terreus*) while isolates 15 (*Phomopsis* sp.), 24 (*A. terreus*) and 25 (*Sarcosomataceous* sp.) have antifungal activities towards *G. miniatocinctum* as well (Figure 3). These isolates exhibit pathogens via competitive exclusion mechanism. Only three isolates, isolate 5 (*Phomopsis* sp.), 20 (*C. gloeosporiodes* species complex) and 25 (*Sarcosomataceous* sp.) displayed inhibition of fungal pathogen via secretion of secondary metabolites (Fig. 3).

Discussion

This report is the first to document the discovery of six different species (*Glomerella* sp., *Lasiodiplodia* sp., *P. simplicissimum, C. gloeosporioides* species complex, *A. terreus* and *Sarcosomataceous)* of fungal endophytes from the pitchers of *N. ampullaria* and *N. mirabilis*. Although fungal endophyte species have been found in other carnivorous plants such as the "trumpet pitchers" *Sarracenia* (Glen & Bodri 2012), "sundews" *Drosera* (Quilliam & Jones 2010) and "butterworts" *Pinguicula* (Quilliam & Jones 2012), this is the first report for tropical *N. ampullaria* and *N. mirabilis*. Of the many species, we find only *C. gloeosporioides* species complex that was recovered from both *N. ampullaria* and *N. mirabilis*. This species was also recovered from both pitchers and leaves (Table 1). Our finding here agrees with Glen & Bodri (2012) who also found *C. gloeosporioides* species complex as a true endophyte of *Sarracenia* due to their presence in different individuals, locations and years in this plant. We thereby postulate that *C. gloeosporioides* species complex could also be a possible true endophyte of *N. ampullaria* and *N. mirabilis.* These endophytic species, although is the first reporting for *N. ampullaria* and *N. mirabilis*, were common endophytes of rubber trees (Gazis & Chaverri 2010), banana plants (Xia *et al.* 2011), and medicinal plants such as *Eucalyptus nitens* (Fisher *et al.* 1993), *Justicia gendarussa* (Gangadevi & Muthumary 2008) and *Forsythia suspense* (Zhang *et al.* 2012). These species have limited documentation of their endophytic nature, having only reports of *M. guilliermondii* from leaves of nickel-hyperaccumulator plant *Berkheya coddii* (Postma *et al.* 2012), *Sarcosomataceous* sp. from the pearlwort *Colobanthus quitensis* (Rosa *et al.* 2010), and *Lasiodiplodia* sp. from the pepper plant *Piper hispidum* (Orlandelli *et al.* 2012), *Eucalyptus* and *Acacia* (Burgess *et al.* 2006, Zhao *et al.* 2010).

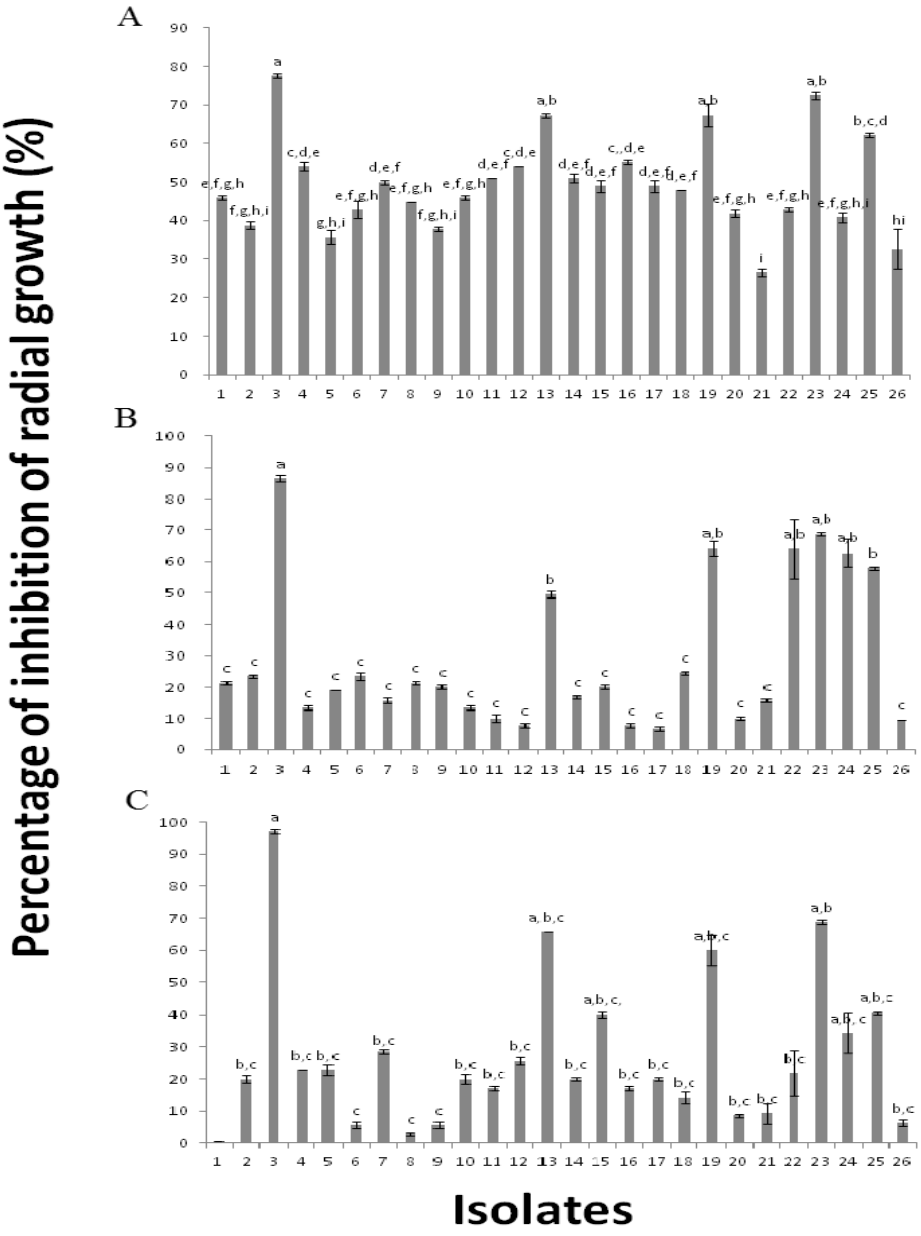

Fig. 3 – Percentage of inhibition of radial growth (PIRG %) of fungal endophytes against A, *Fusarium oxysporum* f. sp. *Cubense*. B, *Ganoderma boninense*, C, *Ganoderma miniatocinctum*, Values are means of triplicates. Means with the same letters are not significantly different $HSD_{(0.05)}$. Bars are standard deviations of means.

We note with interest that most of these endophytes have cellulase, chitinase and lipase activities; enzymes that are involved in the digestion of preys such as arthropods and leaf litters in pitchers (Mohan & Clarke 2010, Suderman *et al.* 2010, Pavlovic *et al.* 2011, Pavlovic, 2012). Although we cannot conclude the role of endophytes in prey digestion based on our results here, they support hypotheses by Morohoshi *et al.* (2011) and Takeuchi *et al.* (2011), linking enzyme production to the microbial community in the pitcher fluid rather than from the plant. Endogenous production of these enzymes by pitcher-plants is not known hence microbes, possibly including endophytes, have a vital role in prey digestion. Our results also strongly suggest that the prevalence of these enzymes may be aligned to the primary enzymes required by pitcher plants for physiological needs, possibly in response to co-evolution and host-selection pressure. Hence, pitcher plants which rely heavily on cellulases, chitinases and lipases for prey and leaf litter digestion, may drive the dominant expression of these enzymes in the endophytes.

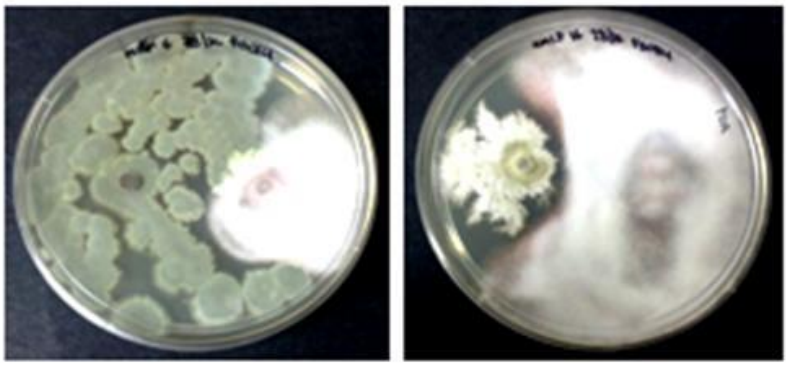

Fig. 4 – Mechanisms of antifungal activity by fungal endophytes (left) against fungal pathogen (right) either through (A) overgrowth or (B) secretion of secondary metabolites.

The other enzymes (amylases, laccases, asparaginases and tyrosinases) are not well-understood as either produced by *Nepenthes* spp. or their endophytes (Tokes *et al.* 1974). Our study here is the first to report on lipase and tyrosinase activity by endophytic *M. guilliermondii.* Other endophytes produced typical enzymes that has been reported elsewhere; *Phomopsis* for laccase production (Vedashree *et al.* 2013), *P. simplicissimum* and *A. terreus*for amylase (Zeng *et al.* 2006, Kuntal and Kumar 2012, Alhussaini 2013). Similarly, there are reports of *Lasiodiplodia* producing amylase (Salami and Akintokun 2008, Adejuwon 2011) but their production of tyrosinase has so far not been documented. A quick comparison with literatures revealed that amylase activities by endophytes from *Nepenthes* (isolate 9 and 10) were comparable to levels by endophytic *Pestalotiopsis microspora* strain VB5 and *Aspergillus oryzae* strain VB6 from mangroves (Joel & Bhimba, 2012). However, isolates 9 and 10 have inferior levels of laccase, cellulase, chitinase production compared to endophytic *Monotospora* sp. in *Cynodon dactylon* (Wang *et al.* 2006), *Phomopsis* sp. in *Bischofia polycarpa* (Dai *et al.* 2010), *P. glabrum* in *Espeletia* spp. (Cabezas *et al.* 2012), and endophytes from *Phragmites australis, Choiromyces aboriginum, Stachybotrys elegans* and *Cylindrocarpon* sp. (Cao *et al.* 2009).

Antimicrobial properties of the endophytes were attributed to the competitive exclusion mechanism whereby isolates were able to out-compete fungal pathogens for resources such as growing space and nutrient. It could also be attributed to the cell-wall degrading enzymes such as chitinase and β-1,3-gluconidase (Qualhato *et al.* 2013). The antimicrobial potential of endophytes from *Nepenthes* spp. is similar to endophytes from other various host species. *Phomopsis* sp. and *C. gloeosporiodes* species complex are known to produce bioactive inhibitory compounds such as alkaloids, terpenoids, phenolics (Silva *et al.* 2006, Huang *et al.* 2008, Fu *et al.* 2011, Brum *et al.* 2012).

To conclude, this study identified culturable endophytes from tropical pitcher plants, and highlighted their enzymatic and antifungal potential. Most of the beneficial endophytes belonged to the common genus of *Colletotrichum*, while atypical species such as *Lasiodiplodia, Isaria* and *Meyerozyma guilliermondii* were also identified. These species are interesting source of important enzymes and antifungal compounds. Our results here contribute to the understanding of endophytes from pitcher plants and their functional use.

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