Brazilian Journal of Microbiology 46, 1, 49-57 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246120130657

Research Paper

Endophytic fungi from medicinal plant *Bauhinia forficata*: Diversity and biotechnological potential

Jadson D.P. Bezerra, Carlos C.F. Nascimento, Renan do N. Barbosa, Dianny C.V. da Silva, Virgínia M. Svedese, Eliane B. Silva-Nogueira, Bruno S. Gomes, Laura M. Paiva, Cristina M. Souza-Motta

Departamento de Micologia, Universidade Federal de Pernambuco, Recife, PE, Brazil.

Submitted: June 15, 2013; Approved: June 6, 2014.

Abstract

Bauhinia forficata is native to South America and used with relative success in the folk medicine in Brazil. The diversity, antibacterial activity, and extracellular hydrolytic enzymes of endophytic fungi associated with this plant were studied. Plant samples, which included leaves, sepals, stems, and seeds, were used. Ninety-five endophytic fungal were isolated (18 from leaves, 22 from sepals, 46 from stems, and nine from seeds), comprising 28 species. The most frequently isolated species were *Acremonium curvulum* (9.5%), *Aspergillus ochraceus* (7.37%), *Gibberella fujikuroi* (10.53%), *Myrothecium verrucaria* (10.53%) and *Trichoderma piluliferum* (7.37%). Diversity and species richness were higher in stem tissues, and Sorensen's index of similarity between the tissues was low. Eleven fungi showed antibacterial activity. *Aspergillus ochraceus*, *Gibberella baccata*, *Penicillium commune*, and *P. glabrum* were those with the greatest antibacterial activity against *Staphylococcus aureus* and/or *Streptococcus pyogenes*. Thirteen species showed proteolytic activity, particularly *Phoma putaminum*. Fourteen species were cellulase positive, particularly the *Penicillium* species and *Myrmecridium schulzeri*. All isolates tested were xylanase positive and 10 showed lipolytic activity, especially *Penicillium glabrum*. It is clear that the endophytic fungi from *B. forficata* have potential for the production of bioactive compounds and may be a source of new therapeutic agents for the effective treatment of diseases in humans, other animals, and plants. To our knowledge, this is the first study of endophytic fungi from different tissues of *B. forficata* and their biotechnological potential.

Key words: antibacterial agents, fungal endophytes, hydrolytic enzyme, symbiosis.

Introduction

Some microorganisms such as bacteria and fungi live as endophytes and inhabit the interiors of plants. According to Petrini (1991), they colonize healthy tissues of the aerial parts of the plant at some time in their life cycles without causing apparent damage, or producing visible external structures (Azevedo *et al.*, 2000).

Several studies have demonstrated the ability of endophytic fungi to produce various compounds such as enzymes (Teske and Trentini, 1995; Bezerra *et al.*, 2012b), antitumor substances (Chandra, 2012), antimicrobial substances (Souza *et al.*, 2004; Siqueira *et al.*, 2011; Pinheiro *et al.*, 2013), and plant growth hormones (Hwang *et al.*, 2011). The use of endophytic fungi in various industrial processes has aroused further study of these microorganisms, and led to the discovery of new compounds with industrial and pharmaceutical potential (Meng *et al.*, 2011; Wang and Dai, 2011). The initial production of `Taxol', an anticancer drug, for example, was from the medicinal plant *Taxus brevifolia*. A study of the endophytic fungi associated with *T. brevifolia* showed the ability of a new hyphomycete, *Taxomyces andreanae* Strobel, A. Stierle, D. Stierle & W.M. Hess, to produce Taxol in larger quantities than in traditional production (Stierle *et al.*, 1993; Strobel *et al.*, 1993). Besides knowing their biotechnological importance, the study of endophytic fungi has contributed to the

Send correspondence to C.M. Souza-Motta. Micoteca URM, Departamento de Micologia, Universidade Federal de Pernambuco, Av. Professor Nelson Chaves s/no, 50670-420 Cidade Universitária, Recife, PE, Brazil. E-mail: smotta@ufpe.br.

knowledge of diversity within this group, and new species have been reported with active extracellular metabolites (Siqueira *et al.*, 2008, 2011).

Medicinal plants have been studied from the point of view of potential endophytic interactions, and have shown many benefits, such as production of antibiotics, secondary metabolites of pharmacological interest, biomarkers of vitality, and biological control agents against pests and diseases (Sun *et al.*, 2008; Hilarino *et al.*, 2011; Bagchi and Banerjee, 2013; Pinheiro *et al.*, 2013). *Bauhinia forficata* Link is widely used in folk medicine in Brazil. This plant has pharmacological and/or biological properties, and has been used in herbal preparations for the treatment of diseases such as diabetes, infections, and as a painkiller (Gupta, 1995; Teske and Trentini, 1995). It has also been reported that that *Bauhinia* species have *in vitro* antimicrobial activity against fungi (*e.g. Aspergillus*, *Botrytis*, *Candida*, *Cladosporium*, and *Cryptococcus*,) and bacteria (*e.g. Salmonella*, *Staphylococcus*, and *Streptococcus*) (Silva and Cechinel-Filho, 2002). Despite the biotechnological potential of *Bauhinia*, to our knowledge no-one has examined the endophytic fungal community of this plant or the biotechnological potential of these microorganisms.

The aims of the present study were to: 1) investigate the endophytic mycobiota from leaves, sepals, stems and seeds of *B. forficata*; 2) determine the diversity and similarity of endophytic fungi from different tissues; 3) screen isolated endophytic fungi as potential agents against bacteria pathogenic to humans; and 4) detect the capacity of the endophytes to produce extracellular hydrolytic enzymes. To our knowledge, this is the first study of endophytic fungi from different tissues of *B. forficata* and their biotechnological potential.

Material and Methods

Isolation, identification, and frequency of endophytic fungi

The collection of leaves, stems, sepals, and seeds from healthy specimens of *Bauhinia forficata* was made randomly in the Didatic Garden of the Center of Biological Sciences, Federal University of Pernambuco, Recife, Brazil (8°3.047' S; 34°56.895' W), from September to October, 2008. Forty-five fragments were used from each plant tissue.

This botanical material was processed within 24 h, following Araújo *et al.* (2002) and Siqueira *et al.* (2011). After disinfection process, 6 mm disks were obtained from leaves and sepals, and 6 mm^2 fragments were obtained from stems and seeds. These were transferred to Petri dishes containing the potato dextrose agar (PDA) culture medium supplemented with chloramphenicol (100 mg L^1). The dishes were incubated at 28 ± 2 °C for up to 30 days in alternated periods of dark and light. Fungal colonies were isolated, purified, and maintained in PDA for identification. To check the efficacy of the surface sterilization, water samples (1 mL) from the last rinse were inoculated onto Petri dishes containing the same medium, using the same incubation conditions.

For identification of endophytic fungi, micro-cultivations were performed and the macro and micro morphological aspects of the somatic and reproductive structures were observed, using specific methodology and literature (Ellis, 1971; Sutton, 1980; Samson and Frisvad, 2004; Leslie and Summerell, 2006; Domsch *et al.*, 2007).

The absolute frequency was calculated as the total number of endophytes isolated. For the relative frequency, the number of isolates in each species was divided by the total number of isolates (Larran *et al.*, 2002).

Representative cultures of endophytic fungi isolated from *B. forficata* have been deposited into the URM Culture Collection of the Federal University of Pernambuco, Recife, Brazil (URM 5937, 5962, 5968, 5990, 5991, 5998-6001, 6011-6014, 6054-6059, 6229, 6234, and 6235).

Data analyses

The Simpson's diversity index, Shannon-Wiener index, and Evenness were calculated using PAST 1.7 software (Hammer *et al.*, 2001). To compare the frequency and richness of endophytic fungi on leaves, sepals, stems and seeds of *B. forficata*, the experimental design was completely randomized. Data were subjected to analysis of variance using the F test (ANOVA) (ASSISTAT Program version 7.7) and then the means were compared by Tukey test at 1% probability. Sorensen's similarity coefficient was employed and a binary matrix was produced and used to calculate the similarity matrix (DICE coefficient, Sorensen) and to plot a UPGMA dendrogram using NTSYSpc 2.10.

Antibacterial activity of endophytic fungi

Human pathogenic bacteria were obtained from the Collection of Microorganisms, Department of Antibiotics (UFPEDA), Federal University of Pernambuco, Brazil. The endophytic fungi were subjected to an antibacterial assay using a solid medium (Ichikawa *et al.*, 1971); this permitted a rapid and qualitative selection of the bioactive microorganisms. Each fungus was cultivated on the PDA surface in Petri dishes at 28 °C for 7 days. Six diameter disks were transferred to the surface of nutrient agar and/or brain heart infusion media previously spread with a test microorganism: *Staphylococcus aureus* Rosenbach (UFPEDA02), *Streptococcus pyogenes* Rosenbach (UFPEDA07), *Mycobacterium smegmatis* (Trevisan) Lehmann & Neumann (UFPEDA71), *Bacillus subtilis* (Ehrenberg) Cohn (UFPEDA86), *Enterococcus faecalis* (Andrewes & Horder) Schleifer & Kilpper-Bälz (UFPEDA138), *Salmonella typhi* (Schroeter) Warren & Scott (UFPEDA478), *Pseudomonas aeruginosa* (Schroeter) Migula (UFPEDA735), *Enterobacter*

aerogenes Hormaeche & Edwards (UFPEDA739), *Proteus vulgaris* Hauser (UFPEDA740), and *Escherichia coli* (Migula) Castellani & Chalmers (UFPEDA224). Petri dishes were incubated at 37 °C for 24 h or 48 h. Antibacterial activity was confirmed visually and by measurement of inhibition zones. The halos obtained were compared with information from the table of the Clinical and Laboratory Standards Institute (CLSI).

Preliminary selection of endophytic fungi for enzyme production

Nineteen endophytic fungi representative of the taxa considered rare and more frequent were randomly selected to evaluate the capacity of production extracellular hydrolytic enzymes (Table 1). Fragments (5 mm) of the endophytic fungal cultures grown in PDA for 7 days were transferred to the centers of Petri dishes containing solid medium with substrates specific to respective enzymes: milk casein to test protease production (Lacaz *et al.*, 2002), carboxymethylcellulose to test cellulases (Neirotti and Azevedo, 1988), xylan to test xylanases (Sarath *et al.*, 1989) and sorbitan monolaurate (Tween 20) to test lipases (Hankin and Anagnostakis, 1975). The cultures were incubated at 28 °C for 7 days. The zone of activity (ZA) was expressed as the relationship between the average diameter of the colony growth (cm) and the average diameter of colony growth (cm) + the average diameter of the degradation halo (cm) (Serda and Yucel, 2002). The score for the production of each enzyme was based on the following criteria: ZA between 0.9 and 1: very weak; ZA between 0.80 and 0.89: weak; ZA between 0.70 and 0.79: strong, and ZA smaller than 0.69: very strong.

Results

Endophytic fungi from *Bauhinia forficata*

In total, 95 fungi (18 from leaves, 22 from sepals, 46 from stems, and nine from seeds) were isolated from 180 fragments of *B. forficata* and grouped into 28 fungal species. Only *Acremonium curvulum*, *Aspergillus ochraceus*, *Gibberella fujikuroi*, and *Penicillium glabrum* were isolated from more than two tissue types. The most frequent species were *Myrothecium verrucaria* (10.52%) isolated only from stems, *G. fujikuroi* (10.52%) isolated from leaves and stems, and *A. curvulum* (9.5%) isolated from sepals and stems. Sixteen endophytic species had lower frequency and were isolated only once or twice (1.05% or 2.1%, respectively) (Table 1).

Seven species were isolated only from leaves, six were exclusively from sepals, eight from stems, and four were isolated only from seeds. The colonization and frequency of endophytic fungi were higher in the stems (48.42% isolates) than in the other tissues of *B. forficata* (Table 1). The frequency of endophytes varied significantly between tissues ($F = 418.59$; $p < 0.01$), however, between leaves and sepals there was no difference. The highest frequency of endophytes was observed in stems and the lowest in seeds. The richness of endophytic fungi varied in different tissues of *B. forficata* ($F = 25.00$; $p < 0.01$). Six, seven, and eight species were found in seeds, sepals, and leaves, respectively. The largest number (11) of species was found in the stems ($p < 0.01$).

The diversity of the endophytic community isolated from different tissues of *B. forficata* was compared using indices of α-diversity. The Simpson's dominance of endophytic fungi was higher in the seeds. Both Simpson and Shannon-Wiener's diversity indices were higher in the stems. The species richness was also greater in the stems. There was little difference in species evenness among the tissues studied (Table 2). Sorenson's similarity index showed that although the similarity between stems and seeds may be considered low, only 25% similarity between these tissues was sufficient to make them cluster differently from the leaves and sepals (Figure 1).

Antibacterial activity of endophytic fungi

Thirty-two endophytic fungi isolated from *B. forficata* were tested for antibacterial activity by disc diffusion assay against 10 clinical isolates of human pathogenic bacteria. Of 32 isolates, 11 (34.3%) showed antibacterial activity against one or more bacteria. Gram-positive bacteria were more sensitive than gram-negative. *Penicillium commune*, *Gibberella baccata*, *P. glabrum*, and *Aspergillus ochraceus* exhibited a high range of antibacterial activity, and inhibited the growth of four out of 10 pathogenic bacteria. *Khuskia oryzae* only showed activity against *Salmonella typhi*; however, this fungus was the only one to inhibit this bacterium and it exhibited the maximum inhibition zone (38 mm) among the fungi tested (Table 3).

Screening to detection the capacity to produce enzymes

The 19 endophytes randomly screened for enzyme production were representative of the taxa rare and more frequent. Table 4 shows the results of cultivating the endophytic fungi in specific solid media for the purposes of detecting their ability to produce proteases, cellulases, xylanases, and lipases.

Among the species analyzed, 14 (73%) showed cellulolytic activity. Five of these showed a ZA between 0.9 and 1.0 (very weak). *Penicillium commune*, *P. glabrum* (from the stems and the seeds), *Myrmecridium schulzeri*, and *Ascotricha chartarum* showed very strong production of cellulases with ZAs < 0.69. Thirteen (68%) species were able to produce protease.. *Ascotricha chartarum*, which had a ZA of 0.79 and 0.70 (strong), and *Phoma putaminum*, with a ZA < 0.69, were very strong. All cultures assessed showed xylanolytic activity. The value of ZA varied from 0.83 to 0.29. Only one isolate showed low capacity to en-

Table 1 - Number of isolates, absolute (f) and relative (fr) frequencies of endophytic fungi isolated from the medicinal plant *Bauhinia forficata*.

Endophytic fungi	Plant tissues				f	fr (%)
	Leaves	Sepals	Stems	Seeds		
Acremonium curvulum W. Gams		5	4		9	9.5
Ascotricha chartarum Berk.				2	2	2.1
Aspergillus niger Tiegh.			5		5	5.26
A. ochraceus G. Wilh.			4	3	7	7.37
Cochliobolus australiensis (Tsuda & Ueyama) Alcorn	1				1	1.05
C. lunatus R.R. Nelson & F.A. Haasis	1		5		6	6.32
Cladosporium oxysporum Berk. & M.A. Curtis		6			6	6.32
C. sphaerospermum Penz.	5				5	5.27
Diplococcium spicatum Grove	1				1	1.05
Gibberella baccata (Wallr.) Sacc.		4			4	4.21
G. fujikuroi (Sawada) Wollenw.	5		5		10	10.53
Khuskia oryzae H.J. Huds.		3			3	3.15
Lasmenia balansae Speg.			1		1	1.05
Myrmecridium schulzeri (Sacc.) Arzanlou, W. Gams & Crous		1			1	1.05
Myrothecium verrucaria (Alb. & Schwein.) Ditmar			10		10	10.53
Nodulisporium Preuss			1		1	1.05
Penicillium aurantiogriseum Dierckx				1	1	1.05
P. commune Thom		2			2	2.1
P. corylophilum Dierckx				1	1	1.05
P. glabrum (Wehmer) Westling			2	1	3	3.16
P. implicatum Biourge			2		2	2.1
Phoma putaminum Speg.		1			1	1.05
Phomopsis diachenii Sacc.	2				2	2.1
Pithomyces atro-olivaceus (Cooke & Harkn.) M.B. Ellis				1	1	1.05
Spegazzinia tessarthra (Berk. & M.A. Curtis) Sacc.	1				1	1.05
Talaromyces funiculosus (Thom) Samson, Yilmaz, Frisvad & Seifert	2				2	2.1
Trichoderma piluliferum J. Webster & Rifai			7		7	7.37
Total	18	22	46	9	95	
Species richness	8	7	11	6	28	

zyme production (ZA = 0.83) while most of the fungi tested showed ZAs < 0.69 (very strong). Ten isolates (52%) were lipase positive. The fungi that stood out as good producers of this enzyme were: *Aspergillus ochraceus*, *A. chartarum*, *M. verrucaria*, *M. schulzeri*, and *P. glabrum.*

Myrmecridium schulzeri and *P. glabrum* were the best producers of all the fungi tested, and were classified as `very strong' for cellulases, xylanases, and lipases, among the four enzymes tested.

Discussion

We found highest frequency of colonization by endophytic fungi in the stems (46 isolates), and the most frequent species were *Gibberella* (14.74%), *Myrothecium* (10.53%), and *Acremonium* (9.5%) (Table 1). In contrast, Siqueira *et al.* (2001) studied the species composition of endophytic fungi from *Lippia sidoides*, and found that colonization of leaves (50.41%) was higher than that of stems (35.40%); the most frequently isolated species were *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (12.3%) and *Alternaria alternata* (Fr.) Keissl. (7.08%). Analyzing the diversity of endophytic fungi from the leaves and stems of *Nyctanthes arbor-tristis*, Gond *et al.* (2012) isolated a larger number of fungi in the leaves (281 isolates) than in the stems (126 isolates). In Brazil, Mussi-Dias *et al.* (2012), investigating the composition of endophytic fungi in the leaves of 11 medicinal plants, reported the isolation of only 20 endophytes. In the same study, the researchers also analyzed leaves of *B. forficata* and recovered only two isolates identified as *Colletotrichum* sp. and *Nigrospora* sp., thus showing differing results from ours.

In our study some species were specific to plant tissues, for example, *A. chartarum* to seeds, *A. niger* to stems, *C. australiensis* to leaves, and *C. oxysporum* to sepals. Endophytic assemblages tend to be distributed in specific

Table 2 - Diversity indices for endophytic fungi isolated from *Bauhinia forficata*.

Indices	Leaves	Sepals	Stems	Seeds
Simpson's dominance	0.1914	0.1901	0.1257	0.2099
Simpson's diversity	0.8086	0.8099	0.8743	0.7901
Species richness	8	7	11	6
Shannon-Wiener	1.842	1.772	2.206	1.677
Evenness (E)	0.7888	0.8401	0.8252	0.8916

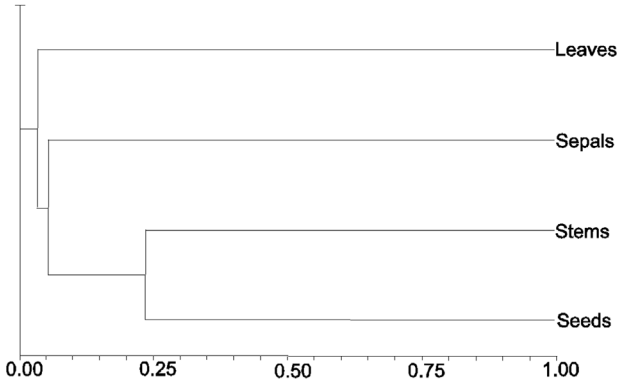

Figure 1 - Sorensen's similarity coefficient for the endophytic fungi isolated from *Bauhinia forficata*.

Table 4 - Enzymatic zone of activity (ZA) of the endophytic fungi isolated from *Bauhinia forficata*.

Endophytic fungi	Cellulase	Protease	Xylanase	Lipase
Leaves				
Cochliobolus lunatus		0.95	0.77	
C. australiensis	0.97	0.95	0.76	
Sepals				
Gibberella baccata	0.93		0.65	
Myrmecridium schulzeri	0.44	0.91	0.31	0.56
Penicillium commune	0.52	0.95	0.43	
Phoma putaminum	0.79	0.49	0.50	0.82
Stems				
Acremonium curvulum			0.67	
Aspergillus niger	0.86		0.70	
A. ochraceus	0.93	0.98	0.41	0.70
C. lunatus	0.89	0.96	0.76	0.90
G. fujikuroi		0.99	0.79	
Myrothecium verrucaria			0.83	0.73
Nodulisporium	0.95	0.97	0.44	
P. glabrum	0.67	0.95	0.47	0.74
Trichoderma piluliferum			0.53	0.92
Seeds				
A. chartarum	0.40	0.74	0.29	0.71
A. ochraceus	0.89	0.94	0.35	0.91
P. glabrum	0.64		0.19	0.68
Pithomyces atro-olivaceus	0.94	0.90	0.59	

ZA, diameter of the colony/diameter of the colony + precipitation zone.

Table 3 - Antibacterial activity (halo size in mm) of endophytic fungi isolated from *Bauhinia forficata*.

Endophytic fungi	Pathogenic bacteria (UFPEDA)[a]									
	02	07	71	86	138	478	735	739	740	224
Sepals										
Gibberella baccata	22	21		22	17					
Khuskia oryzae						38				
Penicillium commune	24	21		21	17					
Stems										
Aspergillus niger	11	15					15			
A. ochraceus									20	20
Cochliobolus lunatus		28			15					
Penicillium glabrum	24	37	21	21						
Seeds										
Ascotricha chartarum			10							
Aspergillus ochraceus	16	14	13	15						
P. corylophilum	15			15						
P. glabrum		11								

[a]Pathogenic bacteria: *S. aureus* (UFPEDA02), *S. pyogenes* (UFPEDA07), *M. smegmatis* (UFPEDA71), *B. subtilis* (UFPEDA86), *E. faecalis* (UFPEDA138), *S. typhi* (UFPEDA478), *P. aeruginosa* (UFPEDA735), *E. aerogenes* (UFPEDA739), *P. vulgaris* (UFPEDA740), and *E. coli* (UFPEDA224).

hosts and specific tissues (Siqueira *et al.*, 2011). Xing *et al.* (2010) observed tissue specificity for endophytic fungi in the roots, stems, and leaves of *Panax quinquefolium*. Indeed, Petrini *et al.* (1992) concluded that different plant tissues and organs may represent distinct microhabitats. Moreover, the host-plant age, associated vegetation, elevation, and exposure can all affect endophytic assemblages and frequency of colonization (Kusari *et al.*, 2013).

Although many species of fungi are commonly described as endophytes, others can be found occasionally colonizing the host tissue and are isolated only once or twice in several samples (Siqueira *et* al., 2011; Pinheiro *et al.*, 2013). The literature reports *Alternaria*, *Cladosporium*, *Colletotrichum*, *Guignardia*, *Phoma*, *Phomopsis*, *Phyllosticta*, and *Xylaria* as endophytes of various plant tissues (Costa *et al.*, 2012; Bezerra *et al.*, 2012a, 2012b, 2013). We found that *A. chartarum*, *C. australiensis*, *D. spicatum*, *L. balansae*, *P. corylophilum*, *P. glabrum*, *P. atro-olivaceus*, *M. schulzeri*, *S. tessarthra*, and *T. piluliferum* are reported as the first occurrence of these endophytes in Brazil.

Species of *Aspergillus*, *Gibberella*, and *Penicillium* that we isolated have been questioned as true endophytic fungi. However, several studies with various species of plants, including medicinal plants, have reported the isolation of species within these genera as endophytes (Bezerra *et al.*, 2012a, 2013; Kusari *et al.*, 2013).

The diversity of endophytic fungi was highest in the stems (Table 2). Kumar and Hyde (2004), analyzing endophytic fungi associated with *Tripterygium wilfordii*, reported the highest Shannon diversity index in the twig xylem followed by the leaves. In our study, species richness was higher in the stems (11 species) and the Sorensen similarity index was about 25% between the tissues of stems and seeds (Table 2). In contrast, Gond *et al.* (2012), studying medicinal plants of India, observed higher species richness of endophytic fungi in leaves than in stems, and a greater similarity in species distribution of fungi in the leaves. Studying the diversity and dynamics of fungal endophytes in leaves, stem and roots of Chinese medicinal plant *Stellera chamaejasme*, Jin *et al.* (2013) verified that Simpson's dominance (D) and Evenness (E) were greater in the roots (D = 0.99 and E = 0.34) than in the other studied tissues. Researching the endophytic fungal communities from *Madhuca indica* at different locations in India, Verma *et al.* (2013) observed a greater diversity in stem (D = 0.18) and in leaves (E = 0.6). These authors suggest that the highest frequency of colonization in stem may be due to spore abundance of a few dominant endophytes in stem tissue.

Endophytic fungi have shown potential in the synthesis of a wide range of biologically active metabolites (Aly *et* al., 2011), and are a diverse field of study and a valuable resource with enormous potential. New approaches must be designed efficiently and chemical study of this diversity needs to be intensified to discover and develop new medications (Schulz *et al.*, 2002). We screened the antibacterial activity of endophytic metabolites against clinical isolates of human pathogenic bacteria. A total of 34.3% of the isolates showed activity. Among these, *Penicillium commune*, *P. glabrum*, *Gibberella baccata*, and *Aspergillus ochraceus* stand out as the most effective against all gram-positive strains. Similarly, Shim *et al.* (2006) demonstrated that *Penicillium griseofulvum* Dierckx produce secondary metabolites, such as mycophenolic acid, active against this group of bacteria. Cui *et al.* (2011) studied the antimicrobial and antitumor activity of endophytic fungi isolated from the medicinal plant *Aquilaria sinensis* (Thymelaeaceae), and found that species within *Fusarium* [*Gibberella*] also demonstrated activity against *S. aureus* and *B. subtilis*.

Among the three fungi that showed activity against gram-negative bacteria, two belong to *Aspergillus*. This genus has been reported as a good inhibitor of microorganisms by Souza *et al.* (2004), who studied the antimicrobial activity of endophytic fungi isolated from toxic plants in the Brazilian Amazon, and by Sadananda *et al.* (2011), who tested endophytes from *Tabebuia argentea* against fungi and bacteria pathogenic to humans.

Although *Myrothecium verrucaria* was frequently isolated (10.41%) from *B. forficata*, it showed no antibacterial capacity. Some research has shown other capacities of *M. verrucaria*, such as potential to be a biological control agent of invasive plants from the inoculation of spores (Clarke *et al.*, 2007) and to be included in bioherbicide formulations (Hoagland *et al.*, 2007). However, some isolates of this species may produce mammalian toxins (Anderson and Hallett, 2004). Furthermore, this species has been reported to produce enzymes (Halliwell, 1961).

Khuskia oryzae [*Nigrospora oryzae*] produced the greatest inhibition zone (38 mm) against *S. typhi*, a causal agent of typhoid fever in humans. These results corroborate with recent studies that have shown the endophyte *K. oryzae* to be the main inhibitor of *S. paratyphi* (Gond *et al.*, 2012); however, the halo formed by this species (22 mm) in our work was larger. The antibiotic chloramphenicol is considered the drug for the treatment of typhoid fever (Pinheiro *et al.*, 2013) and according to the standard table of the *Clinical and Laboratory Standards Institute* (CLSI) a halo above 18 mm demonstrates that bacteria is sensitive to that antibiotic. Accordingly, *K. oryzae* was more efficient than the standard antibiotic and indicates the potential of the fungus for production of antibacterial compounds.

Enzymes are also among the wide variety of primary or secondary metabolites that can be produced by microorganisms, especially fungi (Stamford *et al.*, 1998). In our work, species of *Penicillium* and *Myrmecridium* were among the best producers of cellulase. Similarly, Ruegger and Tauk-Tornisielo (2004) highlighted *P. glabrum* as an excellent producer of this enzyme. *Myrmecridium*

schulzeri was found in leaf litter species from the Caatinga ecosystem in Brazil (Cruz and Gusmão, 2009), showing cellulase production ability even in extreme environments.

The proteolytic activity displayed by isolates was mostly considered low, and 31% of the fungi tested failed to produce this enzyme in culture. These results are confirmed by Silva *et al.* (2006), where 82% of the fungi studied showed no ability to degrade medium containing casein as the protein source. The only fungus with strong production of this enzyme, *Phoma putaminum*, is recognized for its potential as a herbicide, its ability to produce protease, and its production of putaminoxin, a substance with high phytotoxic effects (Evidente *et al.*, 1995).

In contrast, we found that all isolates tested were xylanase positive. This was also observed by Ruegger and Tauk-Tornisielo (2002); in their study, all isolates (*e.g. Aspegillus*, *Cladosporium*, *Penicillium*, and *Trichoderma* species) produced this enzyme, probably because they had been isolated from plant tissue, which has xylan in its cell walls. In our work, *Acremonium curvulum* was positive for xylanase only. However, Braz *et al.* (2009), studying different strains of *A. curvulum*, found that enzyme activity varied with the substrate used, emphasizing the view that the halo can be changed by the composition of the culture medium. This may explain the results we obtained.

Lipase production by microorganisms has no known function, but is thought to be related to the location and possibility of expansion of pathogenic fungal infection (Rivera-Orduña *et al.*, 2011). In our study, the fungi that were obviously good producers of this enzyme were *A. chartarum*, *A. ochraceus*, *M. schulzeri*, *M. verrucaria*, and *P. glabrum*. Different results were gained by Silva *et al.* (2006), where *Aspergillus* and *Penicillium* isolated from *Annona* spp. did not produce lipase; demonstrating that the enzyme production capacity varies among species and that it is therefore necessary to select isolates.

Our results demonstrate that different tissues of the medicinal plant *B. forficata* harbor distinct endophytic fungi communities. The wide richness of endophytes in different tissues of *B. forficata* seems to have an important ecological role in healthy plant tissues. This can also be suggested from the different endophytic species reported as the first occurrence as endophyte to Brazil, confirming that *B. forficata* is a reservoir of diversity of fungal endophytes. The bioactive compounds produced by these fungi can be a source of new therapeutic agents for the effective treatment of diseases in humans, other animals, and plants. In addition, endophytes can be used in the production of enzymes of industrial and environment interest. The species *A. ochraceus*, *F. lateritium*, *P. commune*, and *P. glabrum* are indicated for use in future experiments regarding antibacterial activity, and *M. schulzeri* and *P. glabrum* for enzymes production. Future studies of endophytic fungi from *B. forficata* are necessary to gain knowledge of its potential in the production of antimicrobial compounds and extracellular hydrolytic enzymes.

Acknowledgments

This manuscript is dedicated to Professors Débora M. M. Lima and Maria José S. Fernandes; taxonomists at the URM Culture Collection, Brazil. We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE), Financiadora de Estudos e Projetos (FINEP), and the Universidade Federal de Pernambuco (UFPE) for funding this project. We thank also Profa. Janete Magali for providing the bacteria, and the students of the Laboratório de Citologia e Genética de Fungos/UFPE for their technical help and processing of samples.

References

Aly AH, Debbab A, Proksch P (2011) Fungal endophytes: unique plant inhabitants with great promises. Appl Microbiol Biot 90:1829-1845.

Anderson KI, Hallett SG (2004) Herbicidal Spectrum and Activity of *Myrothecium verrucaria*. Weed Sci 52:623-627.

Araújo WL, Marcon J, Maccheroni Junior W, Elsas JDV, Vuurde JWL, Azevedo JL (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl Environ Microb 68:4906-4914.

Azevedo JL, Macheroni Jr W, Pereira JO, Araújo WL (2000) Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electron J Biotechnol 3:40-65.

Bagchi B, Banerjee D (2013) Diversity of fungal endophytes in *Bauhinia vahlii* (a lianas) from different regions of Paschim Medinipur district of West Bengal. IJSET 2:748-756.

Bezerra JDP, Lopes DHG, Santos MGS, Svedese VM, Paiva LM, Almeida-Cortez JS, Souza-Motta CM (2012b) Riqueza de micro-organismos endofíticos em espécies da família Cactaceae. Bol Soc Latin Carib Cact Suc 9:19-23.

Bezerra JDP, Santos MGS, Barbosa RN, Svedese VM, Lima DMM, Fernandes MJS, Gomes BS, Paiva LM, Almeida-Cortez JS, Souza-Motta CM (2013) Fungal endophytes from cactus *Cereus jamacaru* in Brazilian tropical dry forest: A first study. Symbiosis 60:53-63.

Bezerra JDP, Santos MGS, Svedese VM, Lima DMM, Fernandes MJS, Paiva LM, Souza-Motta CM (2012a) Richness of endophytic fungi isolated from *Opuntia ficus-indica* Mill. (Cactaceae) and preliminary screening for enzyme production. World J Microb Biot 28:1989-1995.

Braz SCM, Motta CMS, Massa DML, Neves RP, Magalhães OMC (2009) Viabilidade, confirmação taxonômica e detecção enzimática de espécies de *Acremonium* preservadas sob óleo mineral na Coleção de Culturas University Recife Mycology. Rev Soc Bras Med Trop 42:63-66.

Chandra S (2012) Endophytic fungi: novel sources of anticancer lead molecules. Appl Microbiol Biot 95:47-59.

Clarke TC, Shetty KG, Jayachandran K, Norland MR (2007) *Myrothecium verrucaria* - A potential biological control

agent for the invasive 'old world climbing fern' (*Lygodium microphyllum*). BioControl 52:399-411.

Costa IPMW, Assunção MMC, Lima TEF, Oliveira RJV, Cavalcanti MAQ (2012) Checklist of endophytic fungi from tropical regions. Mycotaxon 119, 494. http://www.mycotaxon.com/resources/checklists/costa_v119_checklist.pdf. Acessed August 11, 2012.

Cruz ACR, Gusmao LFP (2009) Fungos conidiais na Caatinga: espécies associadas ao folhedo. Acta Bot Bras 23:999-1012.

Cui J, Guo S, Xiai P (2011) Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. J Zhejiang Univ Sci B 12:385-392.

Domsch KH, Gams W, Anderson TH (2007) Compendium of Soil Fungi. IHW-Verlag, Eching.

Ellis MB (1971) Dematiaceus Hyphomycetes. Commonwealth Mycological Institute, Kew.

Evidente A, Lanzetta R, Capasso R, Andolfi A, Bottalico A, Vurro M, Zonno MC (1995) Putaminoxin, a phytotoxic nonenolide from *Phoma putaminum*. Phytochemistry 40:1637-1641.

Gond SK, Mishra A, Sharma VK, Verma SK, Kumar J, Kharwar RN, Kumar A (2012) Diversity and antimicrobial activity of endophytic fungi isolated from *Nyctanthes arbor-tristis*, a well-known medicinal plant of India. Mycoscience 53:113-121.

Gupta MP (1995) 270 Plantas Medicinales Iberoamericanas. Talleres de Editorial Presencia, Santafé de Bogotá.

Halliwell BG (1961) The action of cellulolytic enzymes from *Myrothecium verrucaria*. Biochem J 79:185-192.

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. Available at: http://palaeo-electronica.org/2001_1/past/issue1_01.htm. Accessed November 11, 2010.

Hankin L, Anagnostakis SL (1975) The use of the solid media for detection of enzyme production by fungi. Mycologia 67:597-607.

Hilarino MPA, Silveira FAO, Oki Y, Rodrigues L, Santos JC, Junior AC, Fernandes GW, Rosa CA (2011) Distribution of the endophytic fungi community in leaves of *Bauhinia brevipes* (Fabaceae). Acta Bot Bras 25:815-821.

Hoagland RE, Boyette CD, Abbas HK (2007) *Myrothecium verrucaria* isolates and formulations as bioherbicide agents for kudzu. Biocontrol Sci Techn 17:721-731.

Hwang J-S, You YH, Bae J-J, Khan SA, Kim J-G, Choo Y-S (2011) Effects of endophytic fungal secondary metabolites on the growth and physiological response of *Carex kobomugi* Ohwi J Coastal Res 27:544-548.

Ichikawa T, Date M, Ishikura T, Ozaki A (1971) Improvement of kasugamycin-producing strain by the agar piece method and the prototroph method. Folia Microbiol 16:218-224.

Jin H, Yan Z, Liu Q, Yang X, Chen J, Qin B (2013) Diversity and dynamics of fungal endophytes in leaves, stems and roots of *Stellera chamaejasme* L. in northwestern China. Antonie Leeuwenhoek 104:949-963.

Kumar DSS, Hyde KD (2004) Biodiversity and tissue-recurrence of endophytic fungi from *Tripterygium wilfordii*. Fungal Divers 17:69-90.

Kusari P, Kusari S, Spiteller M, Kayser O (2013) Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. Fungal Divers 60:137-151.

Lacaz CS, Porto E, Martins JEC (2002) Micologia Médica: Fungos, Actinomycetes e Algas de Interesse Médico. Sarvier-EDUSP, São Paulo.

Larran S, Perelló A, Simón MR, Moreno V (2002) Isolation and analysis of endophytic microorganisms in wheat (*Triticum aestivum* L.) leaves. World J Microb Biot 18:683-686.

Leslie J, Summerell BA (2006) The *Fusarium* Laboratory Manual. Blackwell Publishing, Iowa.

Meng L, Sun P, Tang H, Li L, Draeger S, Schulz B, Krohn K, Hussain H, Zhang W, Yi Y (2011) Endophytic fungus *Penicillium chrysogenum*, a new source of hypocrellins. Biochem Syst Ecol 39:163-165.

Mussi-Dias V, Araújo ACO, Silveira SF, Rocabado JMA, Araújo KL (2012) Fungos endofíticos associados a plantas medicinais. Rev Bras Pl Med 14:261-266.

Neirotti E, Azevedo JL (1988) Técnica semiquantitativa de avaliação da produção de celulases em *Humicola* sp. Rev Microbiol 19:78-81.

Petrini O (1991) Fungal endophytes of tree leaves. *In*: Andrews J, Hirano SS (eds) Microbial Ecology of Leaves. Springer Verlag, New York, pp 179-197.

Petrini O, Sieber TN, Toti L, Viret O (1992) Ecology, metabolite production and substrate utilization in endophytic fungi. Nat Toxins 1:185-196.

Pinheiro EAA, Carvalho JM, Santos DCP, Feitosa AO, Marinho PSB, Guilhon GMSP, Souza ADL, Silva FMA, Marinho AMR (2013) Antibacterial activity of alkaloids produced by endophytic fungus *Aspergillus* sp. EJC08 isolated from medical plant *Bauhinia guianensis*. Nat Prod Res 27:1633-1638.

Rivera-Orduña FN, Suarez-Sanchez RA, Flores-Bustamante ZR, Gracida-Rodriguez JN, Flores-Cotera LB (2011) Diversity of endophytic fungi of *Taxus globosa* (Mexican yew). Fungal Divers 47:65-74.

Rollof S, Hedstrom SA, Nilsson-Ehle P (1987) Purification and characterization of a lipase from *Staphylococcus aureus*. Biochim Biophys Acta 921:363-369.

Rowe B, Ward LR, Threlfall EJ (1997) Multidrug-resistant *Salmonella typhi*: a worldwide epidemic. Clin Infect Dis 24:106-109.

Ruegger MJS, Tauk-Tornisielo SM (2002) Produção de xilanase por fungos filamentosos isolados do solo da estação ecológica de Juréia-Itatins, SP, Brasil. Holos Environ 2:185-194.

Ruegger MJS, Tauk-Tornisielo SM (2004) Atividade da celulase de fungos isolados do solo da Estação Ecológica de Juréia-Itatins, São Paulo, Brasil. Rev Bras Bot 27:205-211.

Sadananda TS, Nirupama R, Chaithra K, Govindappa M, Chandrappa CP, Vinay Raghavendra B (2011) Antimicrobial and antioxidant activities of endophytes from *Tabebuia argentea* and identification of anticancer agent (Lapachol). J Med Plants Res 5:3643-3652.

Samson RA, Frisvad JC (2004). *Penicillium* subgenus *Penicillium*: new taxonomics schemes, mycotoxins and other extrolites. Studies in Mycology 49:1-260.

Sarath G, de La Motte RS, Wagner FW (1989) Protease assay methods. *In*: Beynon RJ, Bonde JS (eds) Proteolytics Enzymes: An Pratical Approach. Oxford University Press, Oxford, pp 25-54.

Schulz B, Boyle C, Draeger S, Rommert AK, Krohn K (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol Res 106:996-1004.

Serda SK, Yucel A (2002) Phospholipase and protease activities in clinical *Candida* isolates with reference to the sources of strains. Mycoses 45:160-165.

Shim SH, Gloer JB, Wicklow DT (2006) Penifulvins B-E and a silphinene analogue: sesquiterpenoids from a fungicolous isolate of *Penicillium griseofulvum*. J Nat Prod 69:1601-1605.

Silva KL, Cechinel-Filho VC (2002) Plantas do Gênero *Bauhinia*: composição química e potencial farmacológico. Quim Nova 25:449-454.

Silva RLO, Luz JS, Silveira EB, Cavalcante UMT (2006) Fungos endofíticos em *Annona* spp.: isolamento, caracterização enzimática e promoção do crescimento em mudas de pinha (*Annona squamosa* L.). Acta Bot Bras 20:649-655.

Siqueira VM, Conti R, Araújo JM, Souza-Motta CM (2011) Endophytic fungi from the medicinal plant *Lippia sidoides* Cham. and their antimicrobial activity. Symbiosis 53:89-95.

Siqueira VM, Souza-Motta C, Braun U (2008). *Corynespora subcylindrica* sp. nov., a new hyphomycete species from Brazil and a discussion on the taxonomy of corynespora-like genera. Sydowia 60:113-122.

Souza AQL, Souza ADL, Astolfi-Filho S, Pinheiro MLB, Sarquis MIM, Pereira JO (2004) Antimicrobial activity of endophytic fungi isolated from amazonian toxic plants: *Palicourea longiflora* (aubl.) rich and *Strychnos cogens* bentham. Acta Amaz 34:185-195.

Stamford TLM, Araújo JM, Stamford NP (1998) Atividade enzimática de microrganismos isolados do Jacatupé (*Pachyrhizus erosus* L. Urban). Ciência e Tecnologia de Alimentos 18:382-385.

Stierle A, Strobel GA, Stierle D (1993) Taxol and taxen production by *Taxomyces andreanae* an endophytic fungus of pacific yew. Science 260:214-216.

Strobel GA, Stierle A, Stierle D, Hess WM (1993) Taxomyces andreanae a proposed new taxon for a bulbilliferous hyphomycete associated with Pacific yew. Mycotaxon 47:71-78.

Sun JQ, Guo L-D, Zang W, Ping WX, Chi DF (2008) Diversity and ecological distribution of endophytic fungi associated with medicinal plants. Sci China Ser C 51:751-759.

Sutton BC (1980) The Coelomycetes: Fungi Imperfecti with Pycnidia, Acervuli and Stromata. Commonwealth Mycological Institute, Kew.

Tan RX, Zou WX (2001) Endophytes: a rich source of functional metabolites. Nat Prod Rep 18:448-459.

Teske M, Trentini AMM (1995) Compêndio de Fitoterapia. Herbarium Lab. Botânico, Curitiba.

Verma SK, Gond SK, Mishra A, Sharma VK, Kumar J, Singh DK, Kumar A, Goutam J, Kharwar RN (2013) Impact of environmental variables on the isolation, diversity and antibacterial activity of endophytic fungal communities from *Madhuca indica* Gmel. at different locations in India. Ann Microbiol. doi: 10.1007/s13213-013-0707-9.

Wang Y, Dai C-C (2011) Endophytes: a potential resource for biosynthesis, biotransformation, and biodegradation. Ann Microbiol 61:207-215.

Xing X, Guo S, Fu J (2010) Biodiversity and distribution of endophytic fungi associated with *Panax quinquefolium* L. cultivated in a forest reserve. Symbiosis 51:161-166.

Associate Editor: Lara Durães Sette