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Full Length Research Paper

Diversity and Biotechnological Potential of Endophytic Fungus from the Medicinal Plant Bauhinia for Ficata

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South American native Bauhinia forficata is utilized in Brazilian traditional medicine with varying degrees of efficacy. The endophytic fungus linked to this plant were examined for their variety, antibacterial activity, and extracellular hydrolytic enzymes. Samples of plants were used, including leaves, stems, seeds, and sepals. A total of 95 endophytic fungi, representing 28 species, were isolated (18 from leaves, 22 from sepals, 46 from stems, and 9 from seeds). Acremonium curvulum (9.5%), Aspergillus ochraceus (7.37%), Gibberella fujikuroi (10.53%), Myrothecium verrucaria (10.53%), and Trichoderma piluliferum (7.37%) were the most commonly isolated species. Stem tissues exhibited greater species richness and diversity, whereas the tissues' Sorensen's index of similarity was low. Eleven fungus exhibited antimicrobial properties. The microorganisms that shown the strongest antibacterial activity against Staphylococcus aureus and/or Streptococcus pyogenes were Aspergillus ochraceus, Gibberella baccata, Penicillium commune, and P. glabrum. Proteolytic activity was demonstrated by 13 species, especially Phoma putaminum. Fourteen species, including Myrmecridium schulzeri and Penicillium species, were cellulase positive. Ten isolates, particularly Penicillium glabrum, exhibited lipolytic activity, and all examined isolates were xylanase positive. It is evident that B. forficata endophytic fungi have the capacity to produce bioactive substances and could serve as a source of novel therapeutic agents for the effective management of illnesses in people, other animals, and plants. To the best of our knowledge, this is the first investigation into the biotechnological potential of endophytic fungi from various B. forficata tissues.

Key words: *A*ntibacterial agents, Fungal endophytes, Hydrolytic enzyme, Symbiosis.

INTRODUCTION

Food Some Certain microorganisms, such fungi and bacteria, live inside plants called endophytes. At some point throughout their life cycles, they colonize healthy tissues of the plant's aerial sections without appearing to cause damage or creating outwardly evident structures, according to Petrini (1991) (Azevedo et al., 2000).

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) are just a few of the compounds that have been shown in numerous studies to be produced by endophytic species. The utilization of endophytic fungi in a variety of industrial processes has stimulated additional research on these microorganisms and resulted in the identification of novel substances with potential applications in industry and medicine (Meng et al., 2011; Wang and Dai, 2011). For instance, the medicinal plant Taxus brevifolia was used to produce the first batch of the anticancer medication "Taxol." The ability of a new hypho-mycete, Taxomyces andreanae Strobel, A. Stierle, D. Stierle, & W.M. Hess, to create more Taxol than in conventional production was demonstrated by a study of the endophytic fungus associated with T. brevifolia (Stierle et al., 1993; Strobel et al., 1993). In addition to their industrial

significance, endophytic fungi have been studied to learn more about their variety, and new species with active extracellular metabolites have been identified (Siqueira et al., 2008, 2011).

Numerous advantages, including the production of antibiotics, secondary metabolites of pharmacological interest, biomarkers of viability, and biological control agents against pests and diseases, have been demonstrated by medicinal plants that have been investigated from the perspective of possible endophytic interactions (Sun et al., 2008; Hilarino et al., 2011; Bagchi and Banerjee, 2013; Pinheiro et al., 2013). Brazilian folk medicine makes extensive use of Bauhinia forficata Link. This plant has been utilized in herbal remedies to treat diseases like diabetes, infections, and pain because of its pharmacological and/or biological qualities (Gupta, 1995; Teske and Trentini, 1995). Additionally, Bauhinia species have been shown to exhibit in vitro antimicrobial action against bacteria (such as Salmonella, Staphylococcus, and Streptococcus) and fungi (such as Aspergillus, Botrytis, Candida, Cladosporium, and Cryptococcus) (Silva and Cechinel-Filho, 2002). To our knowledge, no one has investigated the endophytic fungal population of Bauhinia or the biotechnological potential of these microbes, despite the plant's biotechnological potential.

1) Examine the endophytic mycobiota from B. forficata leaves, sepals, stems, and seeds; 2) Identify the diversity and similarity of endophytic fungi from various tissues; 3) Screen isolated endophytic fungi as possible agents against human-pathogenic bacteria; and 4) Identify the endophytes' ability to produce extracellular hydrolytic enzymes. To the best of our knowledge, this is the first investigation into the biotechnological potential of endophytic fungi from various B. forficata tissues.

Material and Methods

Endophytic fungal isolation, identification, and frequency

The leaves, stems, sepals, and seeds of healthy Bauhinia forficata specimens were collected at random from September to October 2008 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 each plant tissue, 45 fragments were employed.

This botanical material was treated in a 24-hour period in accordance with Siqueira et al. (2011) and Araújo et al. (2002). Following the disinfection procedure, 6 mm2 fragments were extracted from stems and seeds, while 6 mm disks were extracted from leaves and sepals. These were put onto Petri dishes with the potato dextrose agar (PDA) culture medium and 100 mg L1 of chloramphenicol added. For up to 30 days, the dishes were incubated at 28 °C with alternating intervals of light and dark. For identification, fungal colonies were isolated, purified, and kept in PDA.

Water samples (1 mL) from the final rinse were inoculated into Petri dishes with the same medium under the same incubation conditions in order to assess the effectiveness of the surface sterilization.

To identify endophytic fungi, specific methodology and literature were used to perform micro-cultivations and observe the macro and micro morphological aspects of the somatic and reproductive structures (Ellis, 1971; Sutton, 1980; Samson and Frisvad, 2004; Leslie and Summerell, 2006; Domsch et al., 2007).

The total number of isolated endophytes was used to compute the absolute frequency. The number of isolates in each species was divided by the total number of isolates to get the relative frequency (Larran et al., 2002).

Representative cultures of endophytic fungi isolated from B. forficata have been deposited into the URM Cul- ture 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

PAST 1.7 software was used to calculate the Evenness, Shannon-Wiener, and Simpson's diversity indices (Hammer et al., 2001). The experimental design was fully randomized in order to compare the frequency and richness of endophytic fungi on B. forficata leaves, sepals, stems, and seeds. The F test (ANOVA) (ASSISTAT Program version 7.7) was used to analyze the data, and the Tukey test was used to compare the means with a 1% probability. Using NTSYSpc 2.10, a UPGMA dendrogram was plotted and the similarity matrix (DICE coefficient, Sorensen) was calculated using a binary matrix created using Sorensen's similarity coefficient.

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 as- say using a solid medium (Ichikawa et al., 1971); this per- mitted 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 mi- croorganism: 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. Antibacte- rial 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).

Initial selection of endophytic fungus for the synthesis of enzymes

To assess the ability to produce extracellular hydrolytic enzymes, 19 endophytic fungi representative of the taxa deemed rare and more common were chosen at random (Table 1). Endophytic fungal cultures that had been grown in PDA for seven days were broken up into 5 mm pieces and placed in the center of Petri dishes that contained solid medium with substrates specific to each enzyme: sorbitan monolaurate (Tween 20) to test lipases (Hankin and Anagnostakis, 1975), carboxymethylcellulose to test cellulases (Neirotti and Azevedo, 1988), xylan to test xylanases (Sarath et al., 1989), and milk casein to test protease production (Lacaz et al., 2002). For seven days, the cultures were incubated at 28 °C. According to Serda and Yucel (2002), the zone of activity (ZA) was defined as the relationship between the average colony growth diameter (cm) and the average colony growth diameter (cm) plus the average degradation halo diameter (cm). The following criteria were used to determine each enzyme's production score: ZA between 0.80 and 0.89 is weak, ZA between 0.70 and 0.79 is strong, ZA less than 0.69 is extremely strong, and ZA between 0.9 and 1 is very weak.

Results

Endophytic fungi from Bauhinia forficate

From 180 pieces of B. forficata, a total of 95 fungi were isolated (18 from leaves, 22 from sepals, 46 from stems, and 9 from seeds), which were then categorized into 28 fungal species. The only organisms isolated from more than two tissue types were Penicillium glabrum, Gibberella fujikuroi, Aspergillus ochraceus, and Acremonium curvulum. The most common species were A. curvulum (9.5%) isolated from sepals and stems, G. fujikuroi (10.52%) isolated from leaves and stems, and Myrothecium verrucaria (10.52%) isolated only from stems. Sixteen endophytic species were isolated only once or twice (1.05% or 2.1%, respectively) and had a lower frequency (Table 1).

Six species were recovered solely from sepals, eight from stems, four from seeds, and seven species were isolated solely from leaves. Compared to the other tissues of B. forficata, the stems had a higher colonization and frequency of endophytic fungus (48.42% isolates) (Table 1). There was no change in the frequency of endophytes between leaves and sepals, although there was a significant variation between tissues ($F = 418.59$; $p < 0.01$). Stems had the highest endophyte frequency, while seeds had the lowest. In several B. forficata tissues, the endophytic fungal richness varied ($F = 25.00$; $p < 0.01$). In seeds, sepals, and leaves, six, seven, and eight species were identified, respectively. The stems contained the greatest number of species (11) ($p < 0.01$).

Using indices of -diversity, the diversity of the endophytic population isolated from several B. forficata tissues was compared. The seeds had a greater Simpson's dominance of endophytic fungus. The stems had greater Shannon-Wiener and Simpson diversity indices. The stems also have a higher species richness. The species evenness of the tissues under study varied very little (Table 2). Even though stems and seeds may not be very similar, Sorenson's similarity score revealed that only 25% of these tissues were comparable enough to cluster differently from leaves and sepals (Figure 1).

Antibacterial activity of endophytic fungi

Using the disc diffusion technique, 32 endophytic fungi that were separated from B. forficata were examined for antibacterial activity against ten clinical isolates of human pathogenic bacteria. Eleven (34.3%) of the 32 isolates exhibited antibacterial activity against one or more microorganisms. Compared to gram-negative bacteria, gram-positive bacteria were more susceptible. Four out of ten harmful bacteria were inhibited in their growth by Penicillium commune, Gibberella baccata, P. glabrum, and Aspergillus ochraceus, which demonstrated a wide range of antibacterial action. The sole fungus that demonstrated efficacy against Salmonella typhi was Khuskia oryzae, which also had the greatest inhibition zone (38 mm) among the fungi examined (Table 3).

Detecting the ability to generate enzymes by screening

The 19 endophytes that underwent a random screening process for the synthesis of enzymes were typical of both rare and common taxa. The findings of growing the endophytic fungi in particular solid media to determine their capacity to produce lipases, xylanases, cellulases, and proteases are displayed in Table 4.

Fourteen (73%) of the species under analysis exhibited cellulolytic activity. Five of these had very weak ZAs, ranging from 0.9 to 1.0. Ascotricha chartarum, Myrmecridium schulzeri, Penicillium commune, and P. glabrum (from the stems and seeds) all demonstrated extremely high cellulase production with ZAs < 0.69. Protease was produced by 13 (68%) of the species. Ascotricha chartarum and Phoma putaminum were both quite strong, with ZA values of 0.79 and 0.70 (strong) and ZA < 0.69, respectively. Every culture that was evaluated

exhibited xylanolytic activity. The ZA value ranged between 0.83 to 0.29. Of the fungi tested, the majority had ZAs < 0.69 (extremely strong), while only one isolate had a modest capacity to produce enzymes (ZA = 0.83). A total of ten isolates (52%) had lipase. The fungi Aspergillus ochraceus, A. chartarum, M. verrucaria, M. schulzeri, and P. glabrum were notable for their high levels of this enzyme production. Out of all the fungi that were examined, Myrmecridium schulzeri and P. glabrum were the best producers. They were categorized as "very strong" for cellulases, xylanases, and lipases, the four enzymes that were evaluated.

Discussion

The stems (46 isolates) had the highest prevalence of endophytic fungal colonization, with Gibberella (14.74%), Myrothecium (10.53%), and Acremonium (9.5%) being the most common species (Table 1). Siqueira et al. (2001), on the other hand, examined the species composition of endophytic fungi from Lippia sidoides and discovered that the colonization of leaves was higher (50.41%) than that of stems (35.40%). The two most commonly isolated species were Alternaria alternata (Fr.) Keissl. (7.08%) and Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. (12.3%). When Gond et al. (2012) examined the variety of endophytic fungi found in Nyctanthes arbor-tristis leaves and stems, they found that there were more fungi in the leaves (281 isolates) than in the stems (126 isolates). Only 20 endophytes were isolated in Brazil, according to Mussi-Dias et al. (2012), who were examining the endophytic fungal composition in the leaves of 11 medicinal plants. The researchers' analysis of B. forficata leaves in the same study yielded different results from ours since they only recovered two isolates, which were identified as Colletotrichum sp. and Nigrospora sp.

A. chartarum was specific to seeds, A. niger to stems, C. australiensis to leaves, and C. oxysporum to sepals, among other species that were specific to plant tissues in our study. Endophytic assemblages are typically found in particular tissues and hosts (Siqueira et al., 2011). Tissue specificity for endophytic fungi was noted by Xing et al. (2010) in Panax quinquefolium roots, stems, and leaves. Indeed, Petrini et al. (1992) came to the conclusion that various plant tissues and organs might serve as unique microhabitats. Furthermore, endophytic assemblages and colonization frequency can be influenced by exposure, elevation, related vegetation, and host-plant age (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 tis- sues (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.

It has been questioned whether the Aspergillus, Gibberella, and Penicillium species we isolated are actually endophytic fungi. However, the isolation of species within these genera as endophytes has been documented in a number of investigations using a variety of plant taxa, including medicinal plants (Bezerra et al., 2012a, 2013; Kusari et al., 2013).

The stems had the greatest diversity of endophytic fungus (Table 2). In their analysis of endophytic fungi linked to Tripterygium wilfordii, Kumar and Hyde (2004) found that the twig xylem had the greatest Shannon diversity index, followed by the leaves. The stems in our study had a higher species richness (11 species), and the Sorensen similarity score between the stem and seed tissues was almost 25% (Table 2). In contrast, Gond et al. (2012), who studied Indian medicinal plants, found that the species distribution of endophytic fungi in leaves was more comparable to that of stems, and that the species richness of these fungi was higher in leaves. When Jin et al. (2013) examined the diversity and dynamics of fungal endophytes in the leaves, stem, and roots of the Chinese medicinal plant Stellera chamaejasme, they confirmed that the roots had higher Simpson's dominance (D) and Evenness (E) (D = 0.99 and E = 0.34) than the other tissues under investigation. Verma et al. (2013) investigated the endophytic fungal communities from Madhuca indica at several locations in India and found that there was more variety in the stem (D = 0.18) and leaves (E = 0.6). According to these scientists, the spore abundance of a few dominating endophytes in stem tissue may be the cause of the high-est frequency of colonization in stem.

Endophytic fungi are a large topic of study and a valuable resource with huge potential. They have demonstrated potential in the synthesis of a wide range of physiologically active metabolites (Aly et al., 2011). To find and create new drugs, new strategies must be created effectively, and chemical research of this variety must be increased (Schulz et al., 2002). We tested endophytic metabolites' antibacterial efficacy against clinical isolates of harmful bacteria that affect humans. 34.3% of the iso-lates were active overall. Of these, Aspergillus ochraceus, Gibberella baccata, P. glabrum, and Penicillium commune are the most potent against all gram-positive bacteria. Likewise, Shim et al. (2006) showed that Penicillium griseofulvum Dierckx produces secondary metabolites that are effective against this bacterial group, including mycophenolic acid. In their 2011 study, Cui et al. examined the antibacterial and anticancer properties of endophytic fungi that were isolated from the Thymelaeaceae medicinal plant Aquilaria sinensis. They discovered that species of Fusarium [Gibberella] also showed action against B. subtilis and S. aureus.

Two Aspergillus fungus were among the three that shown

action against gram-negative bacteria. Souza et al. (2004), who investigated the antimicrobial activity of endophytic fungi isolated from toxic plants in the Brazilian Amazon, and Sadananda et al. (2011), who tested endophytes from Tabebuia argentea against fungi and bacteria harmful to humans, both reported this gene to be a good inhibitor of microorganisms.

Despite being commonly isolated (10.41%) from B. forficata, Myrothecium verrucaria lacked antibacterial properties. Other abilities of M. verrucaria have been demonstrated by several studies, including the potential to be a biological control agent of invasive plants through spore inoculation (Clarke et al., 2007) and to be incorporated into bioherbicide formulations (Hoagland et al., 2007). However, according to Anderson and Hallett (2004), certain isolates of this species might create toxins that are harmful to mammals. Additionally, it has been shown that this species produces enzymes (Halliwell, 1961).

The largest inhibition zone (38 mm) against S. typhi, the causative agent of human typhoid disease, was produced by Khuskia oryzae [Nigrospora oryzae]. Although the halo generated by this species (22 mm) in our work was greater, these results are consistent with prior research that have demonstrated the endophyte K. oryzae to be the primary inhibitor of S. paratyphi (Gond et al., 2012). According to the Clinical and Laboratory Standards Institute (CLSI) standard table, a halo above 18 mm indicates that bacteria are susceptible to the antibiotic chloramphe-nicol, which is thought to be the medication used to treat typhoid fever (Pinheiro et al., 2013). As a result, K. oryzae outperformed the common antibiotic, suggesting that the fungus may be able to produce antibacterial compounds.

Microorganisms, particularly fungi, can create a vast array of primary or secondary metabolites, including enzymes (Stamford et al., 1998). Myrmecridium and Penicillium species were among the top cellulase producers in our study. In a similar vein, P. glabrum was noted by Ruegger and Tauk-Tornisielo (2004) as a superior generator of this enzyme. According to Cruz and Gusmão (2009), Myrmecridium schulzeri was discovered in leaf litter species from the Caatinga ecosystem in Brazil, demonstrating the capacity to produce cellulase even in harsh conditions.

31% of the fungi tested were unable to produce this enzyme in culture, and the isolates' proteolytic activity was largely regarded as low. Silva et al. (2006) supported these findings by finding that 82% of the fungi under investigation were unable to break down the medium that contained casein as a protein source. According to Evidence et al. (1995), Phoma putaminum, the sole fungus that produces a significant amount of this enzyme, is known for its potential as a herbicide, its capacity to make protease, and its synthesis of putaminoxin, a chemical with potent phytotoxic effects.

On the other hand, every isolate that we tested proved positive for xylanase. Ruegger and Tauk-Tornisielo (2002) also noted this; in their investigation, every isolate (including Aspegillus, Cladosporium, Penicillium, and Trichoderma species) generated this enzyme, most likely as a result of being isolated from plant tissue, which contains xylan in its cell walls. Acremonium curvulum only tested positive for xylanase in our investigation. Nevertheless, Braz et al. (2009), who examined various strains of A. curvulum, discovered that the substrate employed affected the enzyme activity, highlighting the idea that the culture medium's composition might alter the halo. This could account for our findings.

Although the function of lipase synthesis by microbes is unknown, it is believed to be connected to the location and potential for growth of pathogenic fungal infections (Rivera-Orduña et al., 2011). The fungi A. chartarum, A. ochraceus, M. schulzeri, M. verrucaria, and P. glabrum were clearly good producers of this enzyme in our investigation. Silva et al. (2006) obtained different results, showing that Aspergillus and Penicillium isolated from Annona spp. did not produce lipase. This highlights the need to choose isolates because the enzyme production capacity varies among species.

Our findings show that unique endophytic fungal populations are present in various tissues of the medicinal plant B. forficata. The diverse range of endophytes seen in B. forficata tissues appears to play a significant ecological role in the maintenance of healthy plant tissues. The fact that B. forficata is a reservoir of diversity of fungal endophytes is further supported by the several endophytic species that were reported as the first endophyte to occur in Brazil. These fungi's bioactive chemicals may provide novel therapeutic agents for the efficient treatment of illnesses in people, other animals, and plants. Furthermore, endophytes can be employed to produce environmentally and industrially useful enzymes. Future research on the antibacterial activity of A. ochraceus, F. lateritium, P. commune, and P. glabrum is recommended, while M. schulzeri and P. glabrum are recommended for the manufacture of enzymes. To learn more about B. forficata's potential for producing extracellular hydrolytic enzymes and antimicrobial chemicals, more research on its endophytic fungus is required.

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