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ORIGINAL PAPER

Richness of endophytic fungi isolated from *Opuntia ficus-indica* Mill. (Cactaceae) and preliminary screening for enzyme production

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Abstract *Opuntia ficus-indica* Mill. (forage cactus) is farmed with relative success in the semi-arid region of the Brazilian northeast for commercial purposes, particularly as forage and food. Endophytic microorganisms are those that can be isolated inside plant tissues and can be a new source to production of enzymes with different potentialities. The objective of this study was to describe the richness of endophytic fungi from *O. ficus-indica* and to detect the capacity of these species to produce extracellular hydrolytic enzymes. Forty-four endophytic fungi species were isolated. Among them, the most commonly found were *Cladosporium cladosporioides* (20.43%) and *C. sphaerospermum* (15.99%). *Acremonium terricola, Monodictys castaneae, Penicillium glandicola*, *Phoma tropica* and *Tetraploa aristata* are being reported for the first time as endophytic fungi for Brazil. The majority of isolated fungi exhibited enzymatic potential. *Aspergillus japonicus* and *P. glandicola* presented pectinolytic activity. *Xylaria* sp. was the most important among the other 14 species with positive cellulase activity. All 24 isolates analysed were xylanase-positive. Protease was best produced by isolate PF103. The results indicate that there is a significant richness of endophytic fungi in *O. ficus-indica*, and that these isolates indicate promising potential for deployment in biotechnological processes involving production of pectinases, cellulases, xylanases and proteases.

Keywords Fungal endophytes · Taxonomy · Semi-arid · *Cladosporium* · Hydrolytic enzyme

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Introduction

Endophytic microorganisms are those that spend all or part of their life cycle colonizing the interior of tissues in host plants without causing them any apparent harm (Tan and Zou 2001). These microorganisms have been described as protectors against the attack of other microorganisms, insects, and herbivore animals, in addition to producing phyto-hormones, enzymes and other chemical compounds, providing advantages for the host plant (Azevedo et al. 2000). Endophytic fungi can also become a new and important resource for the degradation of polycyclic aromatic hydrocarbons (PAH), a toxic class of environmental pollutants (Dai et al. 2010). When colonization results in protection for plant tissues to biotic or abiotic stress, these fungi are called mutualistic (Latch 1993), as both benefit from this interaction (Wang and Dai 2011).

Studies on endophytic fungi of plants are necessary to provide fundamental information for the assessment of global fungal diversity and distribution, as well as for the discovery of new species (Stone et al. 2004; Siqueira et al. 2008). Currently, nearly all host plants studied have had endophytic microorganisms isolated (Wang and Dai 2011).

Endophytic microorganisms produce hydrolytic extracellular enzymes as part of their mechanism of resistance in overcoming the host's defenses against microbial invasion and/or to obtain nutrients from the soil (Tan and Zou

2001). These enzymes include pectinases, esterases, cellulases, lipases, proteases and xylanases (Petrini et al. 1992; Silva et al. 2006; Suto et al. 2002). As a way of establishing the functional role of endophytic fungi, among other factors, there is a need for a detection of extracellular enzymes (Carroll and Petrini 1983).

There are few studies in the literature regarding the endophytic mycobiota of Cactaceae (Bills 1996), except for a preliminary study of endophytic fungi associated with *Opuntia stricta* in the semi-arid regions of Australia (Fischer et al. 1994) and of species of cacti in Arizona (Suryanarayanan et al. 2005). *Opuntia ficus-indica* Mill., a forage cactus, is known for its broad usage in agriculture as a producer of edible fruit and cladodes, which can be used as food (fodder) for animal, including man (Scheinvar 1995). It has medicinal properties and is used as an antioxidant (Lee et al 2002), anti-inflammatory agent (Park et al. 2000) and in the prevention of ulcers (Galati et al. 2001). It is grown with relative success in the semi-arid region of the Brazilian northeast since the beginning of the twentieth century, similar to what happens in the arid and semi-arid regions of the United States, Mexico, South Africa, Australia, for displaying morphological and physiological features that render them appropriate for these regions, thereby becoming an important food base for cattle (Teixeira et al. 1999).

Due to the importance of the Caatinga, a biome unique to Brazil, and the scarcity of studies about the diversity of fungi in this environment, the present paper targeted investigating the richness of endophytic fungi in *O. ficus-indica* from the state of Pernambuco, northeast of Brazil, and their capacity to produce pectinases, cellulases, xylanases and proteases.

Materials and methods

Plant material and isolation of endophytic fungi

The samples of forage cactus were collected in the municipality of Itaíba, Pernambuco, Brazil (09°08.895S, 037°12.069 W) and processed within a maximum of 24 h. For the purpose of isolating endophytic fungi, 45 fragments of about 1 cm^2 were used. During processing, the cactus fragments were immersed in the following solutions: ethanol 75% for 20 s, sodium hypochlorite 4% for 90 s, ethanol 75% for 10 s, and rinsed three times in distilled and sterilized water (Fischer et al. 1994; Araújo et al. 2002; Suryanarayanan et al. 2005), and then transferred aseptically to the surface of the potato-dextrose-agar gel culture medium (PDA) supplemented with chloramphenicol (100 mg l^{-1}), to suppress bacterial growth in the Petri dishes. The dishes were then incubated at a temperature of 28 ± 2°C for up to 30 days. Dishes were inspected daily and any fungal colony found was isolated, purified and maintained in PDA for later identification. In order to check the efficacy of the surface sterilization, water samples (1 ml) from the last rinse were inoculated into Petri dishes containing the same medium, using the same incubation conditions.

Identification of endophytic fungi

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; Domsch et al. 2007).

Preliminary selection of endophytic fungi with screening for enzyme production

Fragments (5 mm) of the endophytic fungal cultures grown in PDA for 7 days were then transferred to the center of the Petri dishes containing the solid medium with specific substrates to each enzyme: citric pectin for pectinases (Uenojo and Pastore 2006), carboxymethylcellulose for cellulases (Neirotti and Azevedo 1988), xylan for xylanases (Sarath et al. 1989) and milk casein for proteases (Lacaz et al. 2002). The cultures were incubated at 28°C for 7 days. The zone of activity (ZA) was expressed by the relationship between the average diameter of the colony growth (cm) and the average diameter of the colony growth (cm) + average diameter of the degradation halo (cm) (Serda and Yucel 2002). The scores for the production of each enzyme were based on the following criteria: ZA between 0.9 and 1: very weak; ZA between 0.89 and 0.80: weak; ZA between 0.79 and 0.70: strong, and ZA smaller than 0.69: very strong. According to this system a lower ZA ratio corresponds with a higher enzyme activity.

Frequency of endophytic fungi

The absolute and relative frequency of endophytic fungi isolated was calculated. The absolute frequency was calculated as the total number of endophytic isolates and for the relative frequency, the number of isolates of each species was divided by the total of isolates (Larran et al. 2002).

Results and discussion

Endophytic fungi from forage cactus (Cactaceae)

There is a dearth of papers studying endophytic fungi in plants from arid to semi-arid regions (Bills 1996). Brazil

has an exclusive natural region, the Caatinga biome, which features a seasonally dry forest, being home to endemic species of plants, birds, mammals, fish and other species that ensure its importance. Little attention has been given to preserving the outstanding and varied landscape of the Caatinga, and the contribution made by its biota to the high level of biodiversity found in Brazil has been severely underestimated (Silva et al. 2004; Leal et al. 2005).

A total of 45 fragments of the cactus *O. ficus-indica* were analysed and 44 endophytic fungi were isolated, belonging to 12 genera and 13 species. Table 1 shows the frequency of endophytic fungi found in *O. ficus-indica*. Many of the genera identified in this study were found by Suryanarayanan et al. (2005), who isolated endophytic fungi associated with Arizona cacti, including *Cladosporium,* the most frequent species in this study, with a frequency of 36.42%. *Colletotrichum* and *Phyllosticta* have being commonly reported as endophytic fungi (Larran et al. 2002; Baayen et al. 2002; Glienke-Blanco et al. 2002; Hata et al. 2002; Santamaría and Bayman 2005; Chareprasert et al. 2006; Wang et al. 2007; Rakotoniriana et al. 2008; Xing et al. 2010; Juan Chen et al. 2011), but we did not isolate any species from these genera from *O. ficus-indica*. This was also observed by Fischer et al. (1994) who studied endophytic fungi from *Opuntia stricta* in the semi-arid regions of Australia and by Suryanarayanan et al. (2005) who analysed the endophytic fungi from cacti in Arizona.

Table 1 Frequency of endophytic fungi isolated in *Opuntia ficus-indica*

Endophytic fungi	f	fr (%)
Acremonium terricola (J.H. Mill., Giddens & A.A. Foster) W. Gams	2	4.54
Aspergillus japonicus Saito	1	2.27
Cladosporium cladosporioides (Fresen.) G.A. de Vries	9	20.43
Cladosporium sphaerospermum Penz.	7	15.99
Fusarium lateritium Nees	1	2.27
Monodictys castaneae (Wallr.) S. Hughes	1	2.27
Nigrospora sphaerica (Sacc.) E.W. Mason	1	2.27
Penicillium aurantiogriseum Dierckx	2	4.54
Penicillium glandicola (Oudem.) Seifert & Samson	2	4.54
Pestalotiopsis guepinii (Desm.) Steyaert	1	2.27
Isolate PF103	1	2.27
Isolate PF104	1	2.27
Isolate PF108	1	2.27
Isolate PF117	2	4.54
Isolate PF202	1	2.27
Isolate PF208	1	2.27
Isolate PF300	1	2.27
Isolate PF303	1	2.27
Isolate PF304	1	2.27
Phoma tropica R. Schneid. & Boerema	1	2.27
Phomopsis archeri B. Sutton	2	4.54
Tetraploa aristata Berk. & Broome	2	4.54
Xylaria sp. 1	1	2.27
Xylaria sp. 2	1	2.27

f: absolute frequency, fr: relative frequency

First reports of endophytic fungi for Brazil

Most species found in this study are commonly reported as endophytic to tropical, subtropical or temperate plants (Mariano et al. 1997; Pereira et al. 1999; Araújo et al. 2001; Photita et al. 2001; Kumar and Hyde 2004). However, *Acremonium terricola* (J.H. Mill., Giddens & A.A. Foster) W. Gams, *Monodictys castaneae* (Wallr.) S. Hughes, *P. glandicola* (Oudem.) Seifert & Samson, *Phoma tropica* R. Schneid. & Boerema and *Tetraploa aristata* Berk. & Broome are being listed for the first time for Brazil as endophytic species.

Acremonium terricola was reported by Gams (1971) in forest soil, and this species was isolated in leaves and litter of the sub-antarctic phanerogam *Poa flabellata* (Poaceae) (Hurst et al. 1983), from soil impacted by copper mining in Brazil (Costa et al. 2006) and in the rhizosphere of sugarcane (Braz et al. 2009); *Monodictys castaneae* was isolated in dead stem and rotting wood (Ellis 1971), in leaf surface of Labiatae, Solanaceae and Umbelliferae in Egypt (El Kady et al. 1997), home dust (El Bokhary 1999), as saprobe in Proteaceae and Restionaceae in a study in South Africa (Lee et al. 2004) and in soil of the Caatinga (Cavalcanti et al. 2006).

Penicilium glandicola was reported in a wide range of substrate types such as stored apples (Ates 1991), in soil of farm areas (Haliki and Dizbay 1997), foods (Topal 1998), forest soil (Paterson and Russell 2004; Kara and Bolat 2007), hospital air (Okten 2008), bat dung, mammal feces and earthworms (Nováková 2009) and in cereal products (Doolotkeldieva 2010); *Phoma tropica* was isolated from *Heliconia* sp. (Heliconiaceae) and from *Cordia colococca* (Boraginaceae) (Sutton 1980), isolated in water environment of the Atlantic Rain Forest (Schoenlein-Crusius and Milanez 1998), brown algae *Fucus spiralis* (Fucaceae) (Osterhage et al. 2002), necrotic spots in leaves of *Heliconia psittacorum* (Heliconiaceae) (Costa 2007) and in soil (Schoenlein et al. 2008), and as a phytopathogen in leaves of *Lablab purpureus* (Papilionoideae) in India (Patil et al. 2010);

Tetraploa aristata, usually found on the basis of leaves and stems just above soil surface (Ellis 1971), was registered as a saprophyte from *Syzygium* (Myrtaceae), *Cenchrus* (Poaceae) and in dead branches in South African provinces (Sinclair 1990); in dead stalks of *Elegia capensis*

(Restionaceae) Lee et al. (2004), in organic matter collected in the Santiago River in Argentina (Liberto and Saparrat 2005), decomposing leaf litter of *Caesalpinia echinata* (Fabaceae) (Grandi and Silva 2006), fruit of *Psidium guajava* (Myrtaceae) in decomposition (Pérez et al. 2003) and was also isolated in the dead trunk of *Alpinia formosa* (Zingiberaceae) in work in Japan (Tanaka et al. 2009).

Preliminary selection for production of enzymes

Fungi are important producers of enzymes, relatively easy to grow in controlled environments and highly sensitive to genetic alterations, enabling enhanced strains to be obtained in terms of production and quality of enzymes (Santos 2007).

Endophytic fungi have high capability for production of extracellular enzymes, such as pectinases, cellulases, proteases, phenol-oxidases, proteases and other catabolic enzymes (Oses et al. 2006; Tan and Zou 2001; Bischoff et al. 2009). Different studies have shown the efficacy of these enzymes in the degradation of residual plant matter (Wang and Dai 2011). Jordaan et al. (2006), in a study of endophytic fungi in pods of *Colophospermum mopane*, isolated species of the genera *Phoma, Phomopsis* and *Alternaria*, which displayed lignin cellulolytic activity accelerating significantly the dehiscence of pods, enabling effective germination of seeds in arid environments, when the conditions are favorable. Table 2 shows the results obtained after cultivating the endophytic fungi in specific solid medium for the purpose of detection the capacity to produce pectinases, cellulases, xylanases and proteases.

Table 2 Enzymatic zone of activity (ZA) of endophytic fungi isolated in *Opuntia ficus-indica*

Endophytic fungi	Pectinase	Cellulase	Xylanase	Protease
Acremonium terricola	–	0.49	0.80	0.39
Aspergillus japonicus	0.84	0.70	0.88	0.96
Cladosporium cladosporioides	–	0.46	1	0.46
Cladosporium sphaerospermum	–	–	1	1
Fusarium lateritium	–	0.89	0.90	1
Monodictys castaneae	–	–	1	–
Nigrospora sphaerica	–	0.77	1	0.52
Penicillium aurantiogriseum	–	0.62	0.88	0.44
Penicillium glandicola	0.61	0.52	0.88	1
Pestalotiopsis guepinii	–	0.64	1	–
Isolate PF103	–	–	1	0.31
Isolate PF104	–	0.57	1	1
Isolate PF108	–	–	1	1
Isolate PF117	–	0.61	1	0.87
Isolate PF202	–	0.65	1	1
Isolate PF208	–	–	1	–
Isolate PF300	–	0.35	1	1
Isolate PF303	–	–	1	0.85
Isolate PF304	–	0.60	0.73	0.61
Phoma tropica	–	–	1	0.36
Phomopsis archeri	–	–	1	1
Tetraploa aristata	–	–	0.72	0.36
Xylaria sp. 1	–	0.20	1	0.74
Xylaria sp. 2	–	–	1	0.75

ZA, diameter of the colony/diameter of the colony + precipitation zone. –, showed no activity zone

Pectinase

Of the 24 isolates tested, only *Aspergillus japonicus* and *P. glandicola* showed pectinolytic activity with ZA of 0.84 (weak) and 0.61 (very strong), respectively. Teixeira et al. (2000), in checking for pectinase production capability by *A. japonicus* in liquid medium with different concentrations of substrates, noted that the best enzymatic activity of this fungus was obtained under different concentrations of pectin. Yoon et al. (2007), using species of *Penicillium* for detection of enzymatic activity, noted that *P. glandicola* displayed weak or no pectinolytic activity, differing in the results found in this study where *P. glandicola* displayed very strong enzymatic action.

Cellulase

Among the cultures tested, 14 showed cellulolytic activity (53.84%). *Fusarium lateritium* showed ZA between 0.89 and 0.80 (weak), *Nigrospora sphaerica* and *A. japonicus* showed ZAs between 0.79 and 0.70 (strong), the other cultures showed ZA under 0.69 (very strong), with standout for *Xylaria* sp.1, isolate PF300 and *Cladosporium cladosporioides* that displayed, respectively, enzymatic indices of 0.20, 0.35 and 0.46. Species of the genera *Xylaria*, found growing in plant tissues are also reported as potential producers of cellulolytic enzymes (Wei et al. 1996), and also displayed lignin cellulolytic activity when isolated as endophytic in leaf litter of *Fagus crenata* (Osono and Takeda 2001, 2002). *C. cladosporioides* when isolated in the soil of an Ecologic Station did not show any cellulolytic activity (Ruegger and Tauk-Tornisielo 2004), differing from the isolate as endophytic in *O. fícus-indica*. On the other hand, Grandi and Silva (2006) in studies of fungi associated to *C. echinata* isolated *Cladosporium oxysporum* decomposing the leaf litter, thus evidencing the genera's cellulolytic capability.

Xylanase

All cultures assessed showed xylanolytic activity, but with very weak ZAs. The production of xylanases by fungi has been reported for a number of species of *Aspergillus*, *Penicillium*, *Trichoderma, Fusarium*, *Rhizopus*, *Cladosporium*, *Nigrospora* and *Myrothecium* (Saha 2002; Goulart et al. 2005; Butt et al. 2008). Some species of *Aspergillus* are known as producers of hemicellulolytic enzymes (Juven et al. 1985; Sakellaris et al. 1989). In literature, the majority of species of endophytic fungi are reported as producers of proteases, lipases, cellulases, amylases and pectinases (Silva et al. 2006). However no reports were found of these fungi's capacity to produce xylanases.

Protease

Of the isolates tested, 21 came out protease-positive (88%). Of these, eight isolates showed very strong ZAs, two strong, eleven isolates between weak and very weak and three showed no degradation halo. Isolate PF103, *Phoma tropica*, *T. aristata* and *A. terricola* stood out as the best producers of the enzyme with ZA varying between 0.31 and 0.39. Braz et al. (2009) detected protease activity in an *A. terricola* isolate from sugarcane rhizosphere, similar to our results in this study. No studies were found in the literature reporting proteolytic activity of *P. tropica* and *T. aristata*. Several fungi have been reported as good producers of proteases, such as *Aspergillus* and *Fusarium* (Vishwanatha et al. 2009). *A. japonicus* and *F. lateritium* were endophytic isolates of *O. fícus-indica* in this study, all of which displayed proteolytic activity, despite having very weak ZAs.

Conclusion

This is the first study on the endophytic fungi of forage cactus (*O. ficus-indica*) from the Caatinga, Brazil. The species *A terricola, M. castaneae, P. glandicola*, *P. tropica* and *T. aristata* are being reported for the first time as endophytic fungi for Brazil. The *Cladosporium* genus, and the species *C. cladosporioides* are the most commonly found endophytic fungi in *O. ficus-indica*. Endophytic fungi in *O. ficus-indica* show xylanolytic, proteolytic, pectinolytic and cellulolytic activity. The following species indicate possible use in biotechnological applications focused on enzyme production: *P. glandicola* for pectinases, *Xylaria* sp. 1 for cellulases, isolate PF103 for proteases and *T. aristata* for xylanases. This study has contributed to knowledge about the richness of endophytic fungi from Cactaceae of the Caatinga, which has been the subject of few studies, and provides information on the biotechnological potential of these fungi.

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