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Biomass destructuring enzymes of fungal endophytes of mangrove roots

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Abstract

Fungal endophytes of mangrove roots were found to be halotolerant and grew in the presence of different concentrations of NaCl in the growth medium (0, 1.5, 2.5, 3.5 or 4.5%). The growth of a few endophytes was reduced slightly with increasing salt (NaCl) concentration; the growth of *Lasiodiplodia theobromae* and *Pseudopestalotiopsis theae* was unaffected by salt in the medium. Interestingly, *Phomopsis* sp. 1 showed increased growth in the presence of salt. Some of the endophytes elaborated salt induced and salt tolerant cellulase and β-glucosidase enzymes. They also produced salt tolerant laccases. These results go to suggest that the halotolerant mangrove root endophytes could function effectively as biomass degraders in the submerged ecosystem of mangroves and play a role in nutrient cycling here.

Keywords – litter degradation – nutrient cycling – salt tolerant enzymes

Introduction

Rhizosphere microbes play an important role in recycling plant nutrients thereby contributing to the functioning of plants and maintenance of their ecosystem services (Liu et al. 2019). The rhizosphere of mangrove ecosystem is unique as it is characterised by high organic carbon content and redox potential, water logging and constant salinity changes (Liu et al. 2019). This ecosystem supports mostly diazotrophic and sulphate-reducing bacteria and fungi including mycorrhiza (Sengupta & Chaudhuri 2002).

The above and below ground tissues of plants including mangroves harbour non-pathogenic endophytic fungi within them. As in the case terrestrial plants, it is the endophytes of leaves of mangroves than those of the roots that have been studied in more detail (Li et al. 2016, Rajamani et al. 2018, Ramirez et al. 2020, Kumaresan et al. 2021). Mangrove leaf endophytes produce many novel bioactive compounds (Debbab et al. 2013, Ancheeva et al. 2018) and enzymes (Kumaresan & Suryanarayanan 2002, Maria et al. 2005, Govinda Rajulu et al. 2011) of pharmaceutical and industrial value. These fungi exhibit habitat-related adaptations such as tolerance to salinity which should enable them to survive in the harsh habitat (Kumaresan et al. 2002). However, there are only limited studies on fungal endophytes of the roots of mangroves (Ananda & Sridhar 2002). Generally, the root endobiome supports several bacteria and fungi as endophytes. Apart from a group of fungi termed the dark septate endophytes (DSE) (Newsham 2011), many other fungi including *Aspergillus*, *Fusarium*, *Gliocladium*, and *Phomopsis* occur as endophytes in roots

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(Suryanarayanan & Vijaykrishna 2001, Porras-Alfaro et al. 2008, Potshangbam et al. 2017). Here, we studied some of the non-DSE of mangrove roots for their salinity tolerance and production of salt tolerant biomass degrading enzymes to contemplate on their possible role in mangrove ecosystem.

Materials & Methods

Fungal source

Sixteen fungal endophytes isolated from roots of mangroves of Pichavaram and Andaman Islands mangrove forests of India and maintained by VINSTROM's culture collection were studied (Table 1). Since one these fungi produced salt tolerant chitin and chitosan modifying enzymes, we had identified it in our earlier study as *Talaromyces stipitatus* based on ITS sequence methodology; it has been deposited with National Fungal Culture Collection of India (NFCCI) (accession no. 4222) and the sequences were submitted to GenBank (Accession no. MG996147) (Paranetharan et al. 2018).

Table 1 List of mangrove root fungal endophytes obtained from VINSTROM for the study.

Fungus	Mangrove host	Location
Chaetomium globosum	*Avicennia marina*	Pichavaram Mangrove forest
Cladosporium sp.	*Rhizophora apiculata*	Pichavaram Mangrove forest
Fusarium sp.	*Rhizophora apiculata*	Pichavaram Mangrove forest
Lasiodiplodia theobromae	*Rhizophora apiculata*	Pichavaram Mangrove forest
Nodulisporium sp.	*Avicennia marina*	Andaman Mangrove forest
Phoma sp.	*Avicennia marina*	Pichavaram Mangrove forest
Phomopsis sp. 1	*Avicennia marina*	Pichavaram Mangrove forest
Phomopsis sp. 2	*Rhizophora apiculata*	Pichavaram Mangrove forest
Diaporthe biconispora	*Aegiceras corniculatum*	Andaman Mangrove forest
Pseudopestalotiopsis theae	*Avicennia marina*	Andaman Mangrove forest
Sterile form 1	*Avicennia marina*	Pichavaram Mangrove forest
Sterile form 2	*Rhizophora apiculata*	Pichavaram Mangrove forest
Sterile form 3	*Rhizophora apiculata*	Pichavaram Mangrove forest
Talaromyces stipitatus	*Avicennia marina*	Pichavaram Mangrove forest
Talaromyces sp.	*Rhizophora mucronata*	Andaman Mangrove forest
Xylaria sp.	*Rhizophora mucronata*	Andaman Mangrove forest

Test for salinity tolerance of endophytes

Each fungus was grown on Czapek Dox agar (CDA) medium for five days, the margin of the colony was cut with a sterile cork borer (5 mm dia) and this mycelial plug was placed (mycelial surface down) at the center of a Petri dish (9 cm dia.) containing 20 ml of CDA amended with different concentrations of NaCl (0, 1.5, 2.5, 3.5 or 4.5%) (Cantrell et al. 2006). Petri dishes were incubated at 26 ± 2ºC for three weeks and the colony diameter was measured every day with caliper. Three replicates were maintained for each treatment.

Detection of extracellular enzymes by plate assay

Cellulase **activity** (Rohrmann & Molitoris 1992)

Modified Glucose Yeast Peptone (GYP) medium where glucose was replaced with 0.5% Na-carboxy-methylcellulose (Na-CMC), pH 6.0, was used. After 3-5 days of colony growth, the plates were flooded with 0.2% aqueous Congo red solution and destained with 1 M NaCl. Appearance of a yellow zone around the fungal colony in an otherwise red medium indicated cellulase activity.

***Laccase* activity** (Rohrmann & Molitoris 1992)

GYP medium with 0.05 g 1-naphthol l^{-1}, pH 6.0, was used. As the fungus grew, the colourless medium turned blue indicating the oxidation of 1-naphthol by laccase.

***β-glucosidase* activity** (Saqib & Whitney 2006)

Fungi were grown in modified liquid GYP medium having 0.5% Na-CMC as the sole carbon source for seven days; the culture filtrate was centrifuged at 9,000 g for 10 min and the supernatant was used as follows.

One hundred ml of 4% agar in 0.2 M sodium acetate buffer (pH 5.0) was autoclaved and maintained at 50°C. One hundred ml of 0.2% esculin (Sigma) was mixed with 6 ml of 1% $FeCl_3$ solution and heated up to 50°C in a water bath; this was mixed with the agar solution and poured in Petri dishes (20 ml per plate). After solidification, 5 wells of 0.5 cm dia. were cut and 75 µl of a supernatant was poured into each well and incubated at 37°C for 5 h. The appearance of brown colour around the well indicated β-glucosidase activity.

For studying the salinity tolerance of the enzymes, the fungi were grown in medium containing different concentration of NaCl (1.5, 2.5, 3.5 or 4.5%) and tested for the enzymes as mentioned above.

Assay for cellulase enzyme

Sample preparation for cellulase enzyme

An endophyte was grown in liquid Czapek Dox medium containing Na-CMC (0.5%) as carbon source for nine days under static culture condition at 25 ± 1°C. The mycelium was removed by filtering through Whatman No.1 filter paper, the culture filtrate was centrifuged at 9,000 g for 10 min and the supernatant was used for testing the enzyme activity. For testing salinity tolerance of the enzyme, the above method was followed by growing the endophyte with different concentration of NaCl (0, 1.5, 2.5, 3.5 or 4.5%) in the growth medium.

Spectrophotometric assay of cellulase (Ghose 1987)

A one ml assay mixture containing 0.5 ml of 2% (w/v) Na-CMC in 50 mM citrate buffer (pH 5.3) and 0.5 ml of supernatant was prepared. The reaction was initiated by incubating the mixture at 50°C for 30 min followed by the addition of 3 ml of 1% DNS. It was incubated in a water bath at 100°C for 5 min and after which 1 ml 40% (w/v) potassium tartrate was added. The Na-CMC digestion by cellulase releases glucose which in turn reduces DNS to 3-amino, 5-nitrosalicylic acid; this absorbs 540 nm of the light. A standard curve was generated with known quantities of glucose to determine the amount of glucose released in the enzymatic assay. For each treatment, three replicates were maintained and the readings were taken using a spectrophotometer (UNICO Spectrophotometer, USA).

Protein estimation

Protein concentration was determined according to the method of Bradford (1976). Bradford reagent was prepared as follows. 20 mg of Coomassie Brilliant Blue G-250 (CBB) was dissolved in 10 ml of 95% ethanol; 20 ml of 85% (V/V) orthophosphoric acid was added and made up to 50 ml. This stock solution was diluted with distilled water (1:4) and filtered through Whatman No. 1 filter paper before use.

A mixture of 480 µl of 0.5 M sodium acetate buffer (pH 5.0), 20 µl of bovine serum albumin (BSA) [0.25 mg/ml] and 5 ml of Bradford reagent was incubated for 20 min and the absorbance read at 595 nm in a spectrophotometer to construct a standard graph.

The specific activity of the enzyme was calculated as follows:

Specific activity = ΔA_{550nm} / Incubation time × Protein (mg)

Results

The growth of none of the endophytes was inhibited even by the highest concentration of NaCl in the growth medium. The growth of 11 of the 16 endophytes including *Chaetomium globosum*, *Cladosporium* sp., *Fusarium* sp., *Nodulisporium* sp., *Phoma* sp., *Diaporthe biconispora*, Sterile forms 1 and 3, *Talaromyces stipitatus*, *Talaromyces* sp., and *Xylaria* sp., reduced with increasing concentration of NaCl (Table 2). While the growth of Sterile form 2 and *Phomopsis* sp. 2 was inhibited only by higher concentrations of NaCl, the growth of *Lasiodiplodia theobromae* and *Pseudopestalotiopsis theae* was not affected by salt concentration. Interestingly, *Phomopsis* sp. 1 showed increased growth in the presence of salt in the medium (Table 2).

Table 2 Growth of endophytes in medium amended with NaCl.

Fungus	**Concentration of NaCl in the growth medium (Growth on 10th day (dia in cm) ± SE**				
	0%	**1.5%**	**2.5%**	**3.5%**	**4.5%**
Chaetomium globosum	90±0.0	87.5±1.1	79.7±0.8	69.2±1.6	58±1.3
Cladosporium sp.	19±0.4	13.2±0.8	13±0.5	13±0.3	11±0.0
Fusarium sp.	61±0.4	47.5±0.2	36±0.0	26.5±0.2	23.5±0.2
Lasiodiplodia theobromae	90±0.0	90±0.0	90±0.0	90±0.0	90±0.0
Nodulisporium sp.	78±0.9	55±0.4	42±1.3	36.5±1.7	31±1.3
Phoma sp.	32.7±0.8	29.3±0.4	21.5±0.7	21±1.2	19.5±0.2
Phomopsis sp. 1	37.5±1.4	41±0.6	39±0.9	43.3±0.8	39.3±1.3
Phomopsis sp. 2	90±0.0	90±0.0	90±0.0	85.3±0.5	76.7±0.2
Diaporthe biconispora	19.5±1.4	18.3±2.3	15.3±0.6	15.2±0.2	11.5±1.4
Pseudopestalotiopsis theae	90±0.0	90±0.0	90±0.0	90±0.0	90±0.0
Sterile form 1	90±0.0	82±1.8	67.5±1.1	56.5±0.7	47.5±1.1
Sterile form 2	12.3±1.1	20.3±0.8	15±0.9	12.5±0.2	11±0.0
Sterile form 3	26±0.0	24±0.4	20±0.0	16±0.5	12.7±0.3
Talaromyces stipitatus	85.2±1.1	75.5±2.5	73.8±1.8	67.5±1.1	66±0.5
Talaromyces sp.	49.3±2.7	40±0.0	33.7±0.8	31±0.4	27±0.0
Xylaria sp.	44.7±2.8	35.7±3.0	29.8±1.6	20.7±1.0	16.2±1.0

Of the 16 fungi screened for cellulase (endoglucanase) production, 15 were positive for the enzyme (Table 3). Of these fungi, 14 produced cellulase in all concentrations of NaCl in the culture medium. *Nodulisporium* sp. failed to produce the enzyme at the highest concentration of NaCl (Table 3). A quantitative assay revealed that *Fusarium* sp., *L. theobromae*, *D. biconispora*, *P. theae*, *Talaromyces* sp., and *Xylaria* sp. had relatively more enzyme activity in the absence of NaCl (Fig. 1). Cellulase activity in *L. theobromae* was not affected by salt to any great extent. Salt at 3.5% and 1.5% concentrations induced maximum enzyme activity in *C. globosum* and *Phomopsis* sp. 1 respectively (Fig. 1).

Table 3 Effect of NaCl on the production of cellulase by fungal endophytes.

Fungus	**Cellulase activity with different concentrations of NaCl (%)**				
	0	**1.5**	**2.5**	**3.5**	**4.5**
Chaetomium globosum	+	+	+	+	+
Cladosporium sp.	+	+	+	+	+
Fusarium sp.	+	+	+	+	+
Lasiodiplodia theobromae	+	+	+	+	+
Nodulisporium sp.	+	+	+	+	–
Phoma sp.	–	–	–	–	–
Phomopsis sp. 1	+	+	+	+	+
Phomopsis sp. 2	+	+	+	+	+
Diaporthe biconispora	+	+	+	+	+

Table 3 Continued.

Fungus	Cellulase activity with different concentrations of NaCl (%)				
	0	**1.5**	**2.5**	**3.5**	**4.5**
Pseudopestalotiopsis theae	+	+	+	+	+
Sterile form 1	+	+	+	+	+
Sterile form 2	+	+	+	+	+
Sterile form 3	+	+	+	+	+
Talaromyces stipitatus	+	+	+	+	+
Talaromyces sp.	+	+	+	+	+
Xylaria sp.	+	+	+	+	+

+ = activity observed; – = no activity

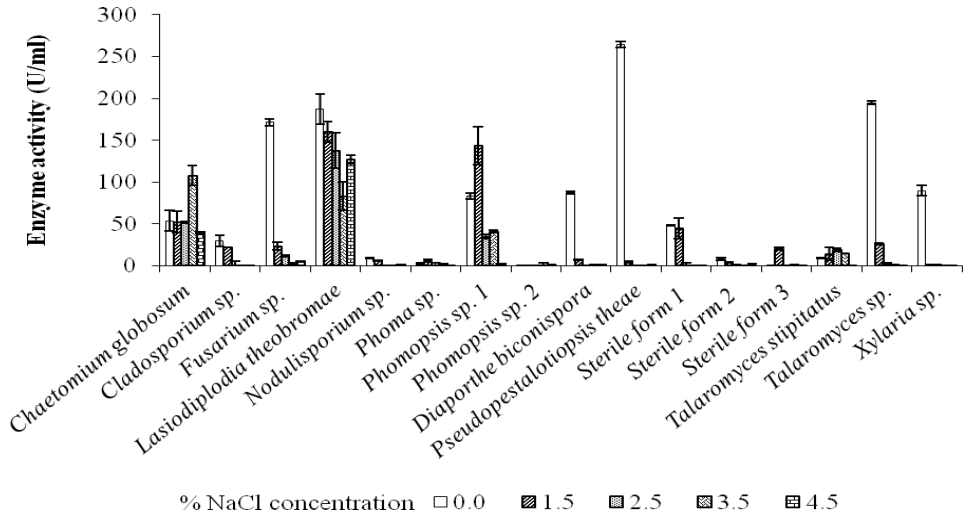

Fig. 1 – Cellulase activity of endophytes as influenced by different NaCl concentrations in the growth medium (Bars denote standard error).

Salt tolerant β-glucosidase was produced by *C. globosum*, *L. theobromae*, *Nodulisporium* sp., *P. theae*, *Phoma* sp., Sterile forms 1 and 2, and *T. stipitatus* (Table 4). Salt induced the production of the enzyme in Sterile form 2 (Fig. 2) and *Xylaria* sp. (Table 4). β-glucosidase production was inhibited by salt in *Phomopsis* sp. 1 and 2, Sterile form 3 and *Talaromyces* sp.

Of the 16 fungi tested, nine were positive for laccase enzyme; *C. globosum*, *Nodulisporium* sp., *Phoma* sp., and Sterile form 2 elaborated halotolerant laccase (Table 5). *Phomopsis* sp. 1, *P. theae*, and *Xylaria* sp. produced moderately salt resistant laccase. *Fusarium* sp. showed laccase activity only in the absence of NaCl.

Table 4 Effect of NaCl on the production of β-glucosidase by fungal endophytes.

Fungus	β-glucosidase activity with different concentrations of NaCl (%)				
	0	**1.5**	**2.5**	**3.5**	**4.5**
Chaetomium globosum	+	+	+	+	+
Cladosporium sp.	–	–	–	–	–
Fusarium sp.	–	–	–	–	–
Lasiodiplodia theobromae	+	+	+	+	+
Nodulisporium sp.	+	+	+	+	+
Phoma sp.	+	+	+	+	+
Phomopsis sp. 1	+	+	+	–	–
Phomopsis sp. 2	+	–	–	–	–
Diaporthe biconispora	–	–	–	–	–

Table 4 Continued.

Fungus	β-glucosidase activity with different concentrations of NaCl (%)				
	0	**1.5**	**2.5**	**3.5**	**4.5**
Pseudopestalotiopsis theae	+++	+++	+++	+++	++
Sterile form 1	+	+	+	+	+
Sterile form 2	–	+++	+++	+++	++
Sterile form 3	+	–	–	–	–
Talaromyces stipitatus	+	+	+	+	+
Talaromyces sp.	++	+	–	–	–
Xylaria sp.	++	+++	+++	+	–

+ = low activity; ++ = medium activity; +++ = high activity; – = no activity

Table 5 Effect of NaCl on the production of laccase by fungal endophytes.

Fungus	Laccase activity with different concentrations of NaCl (%)				
	0	**1.5**	**2.5**	**3.5**	**4.5**
Chaetomium globosum	+	+	+	+	+
Cladosporium sp.	–	–	–	–	–
Fusarium sp.	+	–	–	–	–
Lasiodiplodia theobromae	–	–	–	–	–
Nodulisporium sp.	+	+	+	+	+
Phoma sp.	–	–	+	+	+
Phomopsis sp. 1	+	+	+	–	–
Phomopsis sp. 2	–	–	–	–	–
Diaporthe biconispora	+	+	+	+	–
Pseudopestalotiopsis theae	+	+	–	–	–
Sterile form 1	+	+	+	+	–
Sterile form 2	+	+	+	+	+
Sterile form 3	–	–	–	–	–
Talaromyces stipitatus	–	–	–	–	–
Talaromyces sp.	–	–	–	–	–
Xylaria sp.	+	+	+	–	–

+ = activity; – = no activity

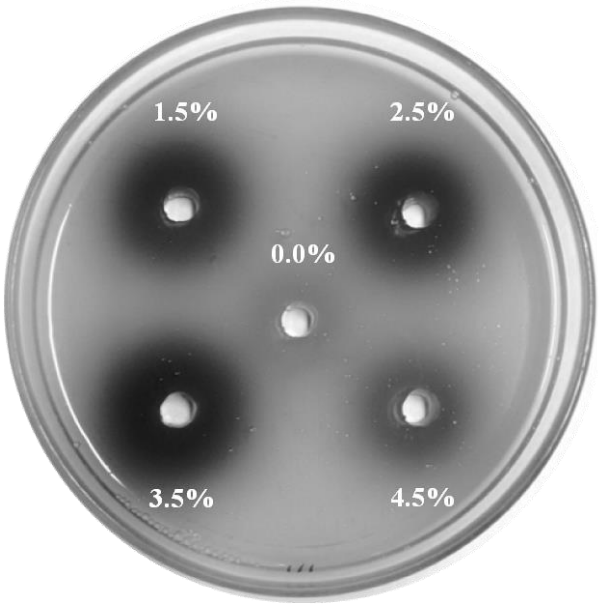

Fig. 2 – β-glucosidase enzyme activity in the secretome of Sterile form 2 grown in different concentrations of NaCl.

Discussion

Although there are many studies on the distribution, taxonomy, and ecosystem services of mangroves, little is known about the activities of microbes in mangrove rhizosphere and sediments (Holguin et al. 2001). Since these microbes contribute to mangrove productivity, carbon budget, and resilience of mangroves to climate change, the need for information on mangrove microbiomes and their role in nutrient recycling in mangrove ecosystem cannot be overstressed (Allard et al. 2020). Fungi and wood bores play a major role in the breakdown of plant detritus in mangrove forests thereby enabling transfer of organic carbon from land to the sea (Cragg et al. 2020, Hendy et al. 2022). Zones experiencing longer inundation and higher salinity inhibit the growth of basidiomycetes (Hendy et al. 2022) and hence, apart from the wood borers, the ascomycetes and bacteria contribute to detritus decay and carbon flux (Singh et al. 2022). It is felt that mostly bacteria play a role in the decomposition of both allochthonous and autochthonous detritus in mangrove ecosystems (Sherman et al. 1998, Rajendran & Kathiresan 2007). Our results show that ascomycetes, especially those that are endophytic in mangrove roots could be involved in this process. We show that although these endophytes are not halophilic, they can tolerate high levels of salt and produce salt tolerant biomass destructuring enzymes such as cellulase (endoglucanase), β-glucosidase and laccase. Indeed, salt induced or increased the activity of some of these enzymes. The fact that these fungi tolerated high salinity and produced salt tolerant and even salt induced biomass degrading enzymes suggest that mangrove root endophytes play a role in nutrient recycling in mangrove ecosystem.

Although it is fairly well established with terrestrial plants that fungal endophytes switch over to a saprotrophic mode and aid in biomass degradation (Voříšková & Baldrian 2013), the role of mangrove endophytes in nutrient recycling is not well understood. Kumaresan & Suryanarayanan (2002) showed that some of the leaf endophytes of mangroves are capable of growth after the leaves fall, and produce biomass degrading enzymes thus functioning as primary litter degraders. Our present study indicates that the root endophytes may play a role in litter degradation in mangrove ecosystem along with marine ascomycetes which also produce cellulase and xylanase enzymes (Bucher et al. 2004). Considering the key ecosystem services mangroves provide and the increasing rate of their loss, there is an urgent need to understand the functions of mangrove microbiome (Allard et al. 2020) including the root fungal endophytes. This would enable a holistic approach to re-establish and maintain mangrove cover. Furthermore, mangrove root endophytes appear to be a novel source of salt tolerant enzymes for technological use (Ruginescu et al. 2022) including biofuel production (Amoozegar et al. 2019).

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