



Identification and evaluation of enzymatic ability of fungal endophytes from *Citrofortunella microcarpa* (Bunge) Wijnands

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Abstract

Citrofortunella microcarpa locally known as “kalamansi” belongs to the family Rutaceae is one of the most important marketable fruit crops grown in the Philippines. Aside from being a fruit crop, the plant also houses various endophytic microorganisms which exhibit various symbiotic relationships. Thus, this study isolated fungal endophytes from *C. microcarpa* stem and leaves and were identified culturally and morphologically. The identities of the fungal species were confirmed using ITS1 and ITS4 sequences. The identified fungal endophytes were tested for their ability to produce fungal enzymes such as amylase, cellulase, laccase and protease. Totally 11 fungal endophytes were isolated from stem and leaves of *C. microcarpa* namely, *Colletotrichum fructicola*, *Colletotrichum gloeosporioides*, *Colletotrichum siamense*, *Fusarium oxysporum*, *Lasiodiplodia theobromae*, *Nigrospora oryzae*, *Nigrospora rubi*, *Nodulisporium indicum*, *Phomopsis azadirachtae*, *Phyllosticta capitalensis*, and an unidentified species under the Order Pleosporales. All the identified endophytic fungal species showed production of amylase. For the cellulose assay, four species namely *L. theobromae*, *N. oryzae*, *C. gloeosporioides*, and the unidentified species had potential for cellulose degradation. *Fusarium oxysporum* and *P. azadirachtae* were found to be producers of laccase. Meanwhile, only the unidentified species showed extracellular protease activity.

Keywords – enzymes – extracellular – ITS primers – Pleosporales

Introduction

Citrofortunella microcarpa locally known as “kalamansi” belonging to the family Rutaceae, is one of the most important commercial fruit crops grown in the Philippines and used as a condiment in almost every famous dish (Morte et al. 2017). It is characterized as a small tree with smooth, oblong to broadly lanceolate, and narrowly winged short petioled leaves (Dulay & De Castro 2016). Aside from being a fruit crop, this plant also houses various endophytic microorganisms that are found in a wide variety of plant tissues such as seeds, ovules, fruits, stems, roots, leaves, tubers, buds, xylem and bark. Endophytic microorganisms are inhabitants of the internal part of plants, causing apparently no harm to their hosts. It has been also found that some endophytic fungi produce valuable enzymes that are relatively unexplored which are useful to pharmaceutical and agricultural industries producing valuable substances of biotechnological interest (Patel et al. 2013). Enzymes produced by microorganisms are considered as potential biocatalysts for a large number of reactions and are generally regarded as safe and functional at a wide range of temperature, pH, salinity or other extreme conditions (Priyadharsini & Dhanasekaran 2015). Thus, this study was conducted to evaluate the

enzymatic ability of endophytic fungi isolated from *C. microcarpa* stem and leaves that would provide information on their diversity and ability to produce fungal enzymes such as amylase, cellulase, protease and laccase that is of biotechnological interest.

Materials & methods

Isolation of fungal endophytes

Citrofortunella microcarpa stem and leaves were collected from calamansi plantations in Mayapyap Sur, Cabanatuan City, Nueva Ecija (15°31'05.0"N 120°57'48.9"E). Matured and healthy leaves and stem were cleaned of adhering debris and surface sterilized using 70% ethanol for 1 minute followed by sequential 1-minute washing with solutions containing 1% sodium hypochlorite, 70% ethanol and sterile distilled water and allowed to surface dry on filter paper. After proper drying, approximately 1x1 cm of sterilized leaf and 1cm length of stem were inoculated in a previously plated potato dextrose agar (PDA) and incubated at room temperature for 5 days. Then, individual colonies were inoculated onto new PDA plates and repetitive re-plating of the fungal colonies was continued until pure cultures were obtained (Patel et al. 2013). Pure cultures of the identified isolates were deposited at Biodiversity Conservation Laboratory, Department of Biological Sciences, College of Science, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines, 3120. Facesoffungi numbers were registered for the fungal cultures as per Jayasiri et al. (2015).

Cultural and morphological characterization of fungal species

Cultural characteristics such as colony appearances, mycelial textures and pigmentations on both obverse and reverse sides of PDA plates were observed after 7 days of incubation at room temperature. Growth rate via colony diameter were measured, initially standardized at 6 mm using a cork borer. Trials were repeated three times and in triplicates (Majid et al. 2015). For microscopic observation, slide culture was used to observe the different micro-morphological characteristics of the isolated fungal endophytes (Prakash & Bhargava 2016).

Molecular identification of fungal endophytes

Seven-day old cultures of the different fungal isolates grown on PDA medium on test tubes were sent to the Philippine Genome Center in Quezon City, Manila for DNA extraction and sequencing. DNA extraction was done using CTAB method. Gene was amplified by PCR and components include genomic DNA, Universal ITS1 (5'tccgtagtggaacctgccc-3') and ITS4 (5'-tcctccgcttattgatatgc-3') primers, Taq Buffer, DNA Polymerase, and dNTP Mix. Cycling parameters on thermal cycler: 95°C 5 min; 30 cycles of 95°C 1 min, 58°C 45 secs, 70°C 1 min; 72°C 10 min; hold at 4°C. Capillary sequencing was carried out on the ABI 3730xl DNA Analyzer using a 50cm 96-capillary array, POP7TM Polymer, and 3730xl. Data Collection Software v3.1 and base calling was done on Sequencing Analysis Software v5.4. Trimming and assembling of sequences was done using Codon Code Aligner V8.0.2. Sequencing was verified by BLASTn and aligned through CLUSTAL W.

Assessment of enzyme producing efficiencies of fungal isolates

Amylase activity

Fungal endophytes from *C. microcarpa* were grown on glucose yeast peptone agar (glucose: 1g; yeast extract: 1g; peptone: 0.5g; agar: 16g; d.w.:1000ml; pH 6) medium supplemented with 2% starch as substrate. Then, plates inoculated with fungal endophytes were incubated at room temperature for 7 days. After incubation, the plates were flooded with 1% iodine solution. The clear zone formed surrounding the fungal colonies were considered positive for the test (Patel et al. 2013).

Cellulase activity

Fungal endophytes from *C. microcarpa* were grown on yeast peptone agar medium (yeast

extract: 1g; peptone: 0.5g; agar: 16g; d.w. 1000ml) supplemented with 0.5% Na-carboxymethyl cellulose (CMC) as enzyme substrate. Then, plates were incubated for 7 days at room temperature. After incubation, the plates were flooded with 0.2% aqueous solution of Congo red and followed by destaining using 1M NaCl solution for 15 min. The clear zone surrounding the colony indicated positive for the test (Patel et al. 2013)

Laccase activity

Fungal endophytes from *C. microcarpa* were grown on glucose yeast peptone agar medium (glucose: 1g; yeast extract: 1g; peptone: 0.5g; agar: 16g; d.w.: 1000ml; pH: 6) amended with 0.005% 1-naphthol as enzyme substrate. Plates were the incubated at room temperature for 7 days. Oxidation of 1-naphthol by laccase, the culture medium changed from clear to blue. The color change indicated positive for the test (Patel et al. 2013).

Protease activity

Fungal endophytes from *C. microcarpa* were grown on glucose yeast peptone medium (glucose: 1g; yeast extract; 1g; peptone; 0.5g; agar; 16g; d.w.; 1000ml; pH: 6) supplemented with 0.4% gelatin as enzyme substrate. Then, plates inoculated with fungal endophytes were incubated at room temperature for 7 days. After incubation, saturated aqueous ammonium sulphate was used to flood the plates. The digested area surrounding the colonies appeared clear indicating positive for the test (Patel et al. 2013).

Data analysis

The enzymatic index (EI) were determined by dividing the measured colony diameter ($\text{\O}c$) and the formed enzymatic halo diameter ($\text{\O}h$) ($\text{EI} = \text{\O}h/\text{\O}c$). Samples with computed enzymatic index with 2.0 and higher are considered to have a high rate of enzyme production (Herculano et al. 2011). The halo presence or clear area around the fungal colony were the enzymatic activity indicator evidenced by the enzymes secretion made by fungi through the culture medium (Sharma & Sumbali 2014).

Statistical analysis

The enzymatic experiments were laid out on a Completely Randomized Design (CRD). Screening for enzyme activities were analyzed using analysis one-way Analysis of Variance (ANOVA). Comparison among means was done using Duncan multiple range test (DMRT) at 5% level of significance using IBM SPSS Statistics 2019 Ver. 26 (SPSS Inc., Chicago, USA).

Results

Cultural and morphological characteristics of fungal endophytes

A total of 11 fungal endophytes were isolated from stem and leaves of *Citrofortunella microcarpa* namely; *Colletotrichum fructicola*, *Colletotrichum gloeosporioides*, *Colletotrichum siamense*, *Fusarium oxysporum*, *Lasiodiplodia theobromae*, *Nigrospora oryzae*, *Nigrospora rubi*, *Nodulisporium indicum*, *Phomopsis azadirachtae*, *Phyllosticta capitalensis*, and an unidentified Pleosporales species. *C. fructicola* and *F. oxysporum* were isolated from leaves. *N. indicum*, *L. theobromae*, *P. azadirachtae*, *P. capitalensis* and the unidentified species were isolated from stem. Meanwhile, *N. oryzae*, *C. siamense*, *N. rubi* and *C. gloeosporioides* were isolated both from leaves and stem.

To confirm the identity of the fungal endophytes isolated from *C. microcarpa*, the ITS region was amplified using ITS1 and ITS4 primers and were sequenced. The PCR products of the ITS region in the eleven different fungi were confirmed to be in range of approximately 600-900bp which are characteristics of ITS1 and ITS4 region. Blast analysis revealed that fungal species were identified as *C. fructicola* FoF 06767 with (99.79%), *C. gloeosporioides* FoF 09424 (99.79%), *C. siamense* FoF 03599 (99.57%), *F. oxysporum* FoF 03824 (98.13%), *L. theobromae* FoF 00167 (99.02%), *N. oryzae*

FoF 06596 (99.58%), *N. rubi* FoF 06598 (99.38 %), *N. indicum* FoF 10588 (99.36%), *P. azadirachtae* FoF 10589 (98.31%), *P. capitalensis* FoF 06888 (99.64%), and unidentified species FoF10590 (95.83%), respectively (Table 1.)

Table 1 Identities of the cultured fungi based on BLAST search with NCBI Genbank accessions and Facesoffungi numbers

Isolate No.	Facesoffungii Number	Species	E value	Identity	Gen Bank Accession No.
LF01	FoF 06767	<i>Colletotrichum fructicola</i>	0.00	99.79 %	MK041513
LF02	FoF 03824	<i>Fusarium oxysporum</i>	0.00	98.13 %	MG543712
STM01	FoF 00167	<i>Lasiodiplodia theobromae</i>	0.00	99.02 %	KF814723
STM02	FoF 10588	<i>Nodulisporium indicum</i>	0.00	99.36 %	KJB26516
STM03	FoF 10589	<i>Phomopsis azadirachtae</i>	0.00	98.31 %	KR056296
STM05	FoF 06888	<i>Phyllosticta capitalensis</i>	0.00	99.64 %	MF153383
STM06	FoF10590	Unidentified species	0.00	95.83 %	HM751829
STM/LF01	FoF 03599	<i>Colletotrichum siamense</i>	0.00	99.57 %	MK569274
STM/LF02	FoF 06596	<i>Nigrospora oryzae</i>	0.00	99.58 %	KU360637
STM/LF03	FoF 06598	<i>Nigrospora rubi</i>	0.00	99.38 %	KX985948
STM/LF04	FoF 09424	<i>Colletotrichum gloeosporioides</i>	0.00	99.79 %	KP900290

LF = Isolated from leaf, STM = Isolated from stem

Assessment of enzyme producing efficiencies

Amylase activity

The eleven species of endophytic fungi tested were able to produce amylase (Table 2, Fig. 1). *P. capitalensis* had the highest computed enzymatic index which is 3.75 followed by the unidentified species and *N. oryzae* with 1.99 and 1.60, respectively. Computed values were significantly different from each other. Meanwhile, *N. indicum*, *F. oxysporum*, *L. theobromae*, *N. rubi*, *C. siamense* and *P. azadirachtae* all have computed enzymatic index of 1.0 which are significantly different from *C. fructicola*.

Table 2 Amylase activity of different fungal endophytes after seven days of incubation

Fungal endophytes	Amylase		
	Diameter of clear zone (mm)	Colony diameter (mm)	Enzymatic index (EI)
<i>Nodulisporium indicum</i>	85.00 ^a	85.00	1.00 ^d
<i>Fusarium oxysporum</i>	85.00 ^a	85.00	1.00 ^d
<i>Lasiodiplodia theobromae</i>	85.00 ^a	85.00	1.00 ^d
<i>Nigrospora rubi</i>	85.00 ^a	85.00	1.00 ^d
<i>Colletotrichum siamense</i>	85.00 ^a	85.00	1.00 ^d
<i>Phomopsis azadirachtae</i>	83.47 ^a	83.47	1.00 ^d
<i>Colletotrichum fructicola</i>	75.88 ^b	75.96	0.99 ^e
<i>Colletotrichum gloeosporioides</i>	65.80 ^c	65.80	1.00 ^d
<i>Nigrospora oryzae</i>	44.10 ^d	27.63	1.60 ^c
Unidentified species	40.90 ^e	20.53	1.99 ^b
<i>Phyllosticta capitalensis</i>	23.50 ^f	6.00	3.75 ^a

Values represent the mean measurements of the clear zones indicating amylase activity produced by the fungal endophytes from *Citrofortunella microcarpa*. Means with the same letter superscript are not significantly different at 5% level of significance using DMRT

***EI greater than 2.00 has high rate of enzyme production

Cellulase activity

A total of four species of endophytic fungi isolated from *C. microcarpa* namely *L. theobromae*, *N. oryzae*, *C. gloeosporioides* and unidentified Pleosporales showed cellulose degradation (Table 3, Fig. 2). The largest enzymatic index was unidentified species with an index of 2.02, second was *C. gloeosporioides* with 1.22 while *L. theobromae* and *N. oryzae* both having 1.06 computed enzymatic index respectively. Meanwhile, *L. theobromae*, and *N. oryzae* were comparable with each other. Only 25% of the endophytes tested in this study were able to produce cellulose.

Protease Activity

Among the tested organisms, extracellular protease activity was only observed in unidentified Pleosporales with a clear zone of 31.3 mm and computed enzymatic index of 1.47 (Fig. 3).

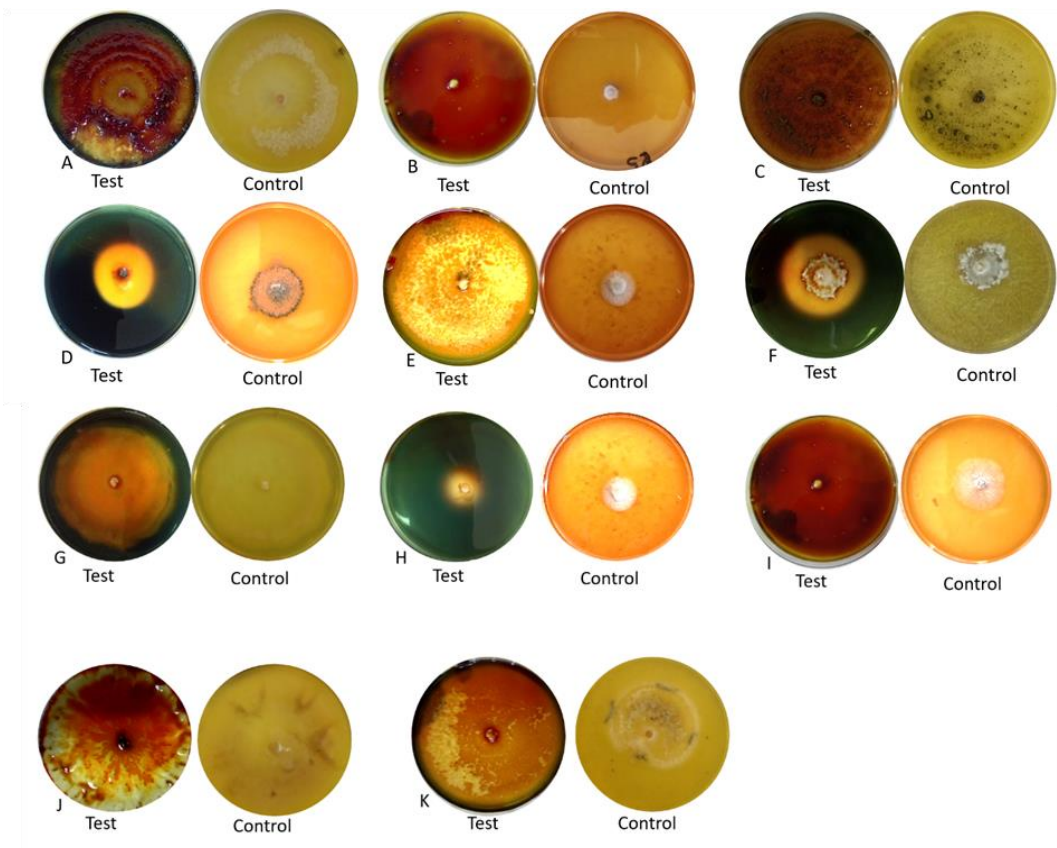


Fig. 1 – Clear zone produced by different fungal endophytes on amylase activity isolated from *C. microcarpa*. A *Colletotrichum fructicola*. B *Lasiodiplodia theobromae*. C *Nodulisporium indicum*. D *Phyllosticta capitalensis*. E *Colletotrichum siamense*. F *Nigrospora oryzae*. G *Colletotrichum gloeosporioides*. H unidentified species. I *Fusarium oxysporum*. J *Nigrospora rubi*. K *Phomopsis azadirachtae*.

Table 3 Cellulase activity of different fungal endophytes after seven days of incubation.

Fungal endophytes	Cellulase		
	Diameter of clear zone (mm)	Colony diameter (mm)	Enzymatic index (EI)
<i>Nigrospora oryzae</i>	80.0 ^a	75.60	1.06 ^c
<i>Lasiodiplodia theobromae</i>	78.4 ^b	74.20	1.06 ^c
<i>Colletotrichum gloeosporioides</i>	74.9 ^c	61.20	1.22 ^b
Unidentified species	25.2 ^d	12.70	2.02 ^a

Values represent the mean measurements of the clear zones indicating cellulase activity produced by the fungal endophytes from *Citrofortunella microcarpa*. Means with the same letter superscript are not significantly different at 5% level of significance using DMRT

***EI greater than 2.00 has high rate of enzyme production

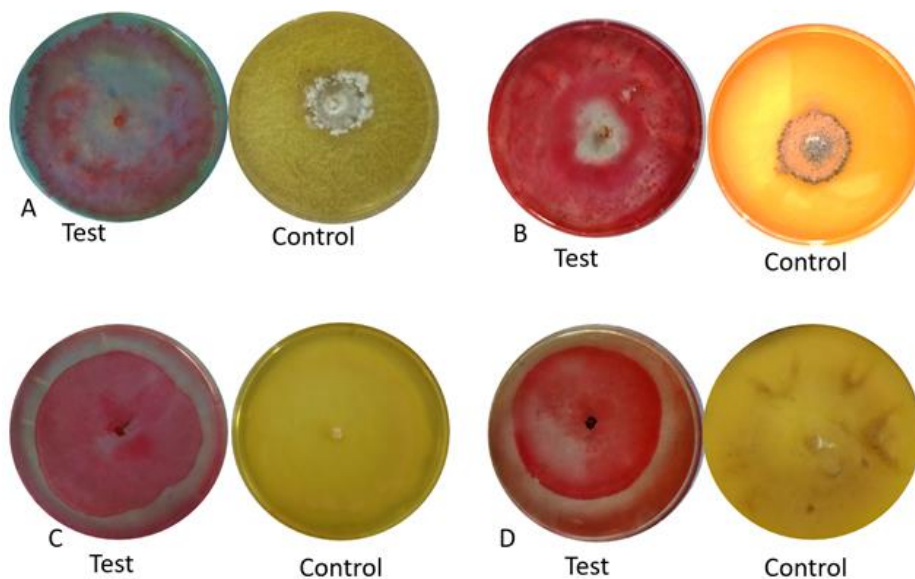


Fig. 2 – Clear zone produced by different fungal endophytes on cellulase activity isolated from *Citrofortunella microcarpa*. A *Nigrospora oryzae*. B unidentified Pleosporales. C *Colletotrichum gloeosporioides*. D *Lasiodiplodia theobromae*.

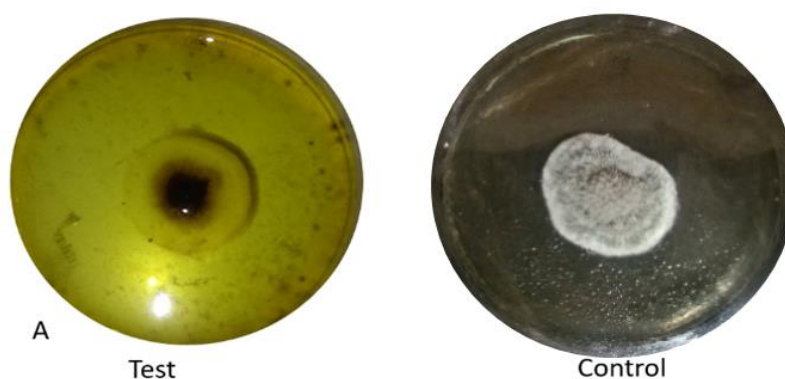


Fig. 3 – Clear zone produced by an unidentified Pleosporales on protease activity.

Laccase Activity

Fusarium oxysporum and *Phomopsis azadirachtae* were producers of laccase enzyme (Table 4, Fig. 4). Only two endophytic fungi in this study were able to produce laccase.

Table 4 Laccase activity of different isolated fungal endophytes after seven days of incubation

Fungal endophytes	Laccase
<i>Colletotrichum fructicola</i>	-
<i>Colletotrichum gloeosporioides</i>	-
<i>Colletotrichum siamense</i>	-
<i>Fusarium oxysporum</i>	+
<i>Lasiodiplodia theobromae</i>	-
<i>Nigrospora oryzae</i>	-
<i>Nigrospora rubi</i>	-

Table 4 Continued.

	Fungal endophytes	Laccase
	<i>Nodulisporium indicum</i>	-
	<i>Phomopsis azadirachtae</i>	+
	<i>Phyllosticta capitalensis</i>	-
	Unidentified species	-

(+) presence of color change, (-) absence of color change

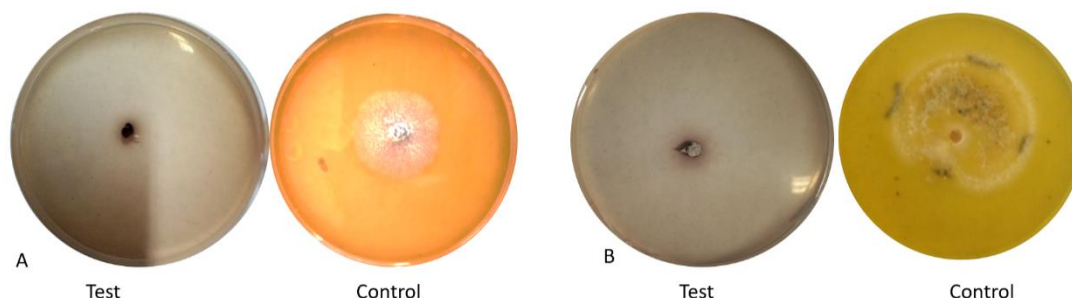


Fig. 4 – Color change produced by fungal endophytes on laccase activity. A *Fusarium oxysporum*. B *Phomopsis azadirachtae*.

Discussion

The role of endophytic fungi in plant decomposition has been demonstrated over several years, since these fungi are present in the senescent tissue, and they are the first ones to initiate plant decomposition (Wilson 2000). Furthermore, Sun et al. (2011) also indicated that endophytes generally decompose dead host leaves, not only as a single species, but also as communities. In this study, 11 fungal endophytes were isolated and identified from both leaves and stem of *Citronella microcarpa*. These were three species of *Colletotrichum*, two species of *Nigrospora* and species each for the genus *Fusarium*, *Lasiodiplodia*, *Nodulisporium*, *Phomopsis*, *Phyllosticta* and unidentified Pleosporales which were tested for their enzyme producing abilities.

Endophytes are fungi that asymptotically colonize plant tissues during some phase of their life cycle (Saikkonen 2007), but may turn pathogenic during host senescence (Rodriguez et al., 2009). The fungal endophytes isolated and identified were found to be associated from various diseases of *Citrus*. *Colletotrichum*, *Fusarium*, and *Lasiodiplodia* species were found to be associated with citrus diseases in Europe, Mediterranean and Florida (Yaseen & D’Onghia 2010, Zhang 2014, Guarnaccia et al. 2017a). Also, *Phyllosticta* and *Phomopsis* are causal agents of *Citrus* disease such as the stem-end rot and Citrus Black Spot Disease (Gopal et al. 2013, Guarnaccia et al. 2017b). Meanwhile, *Nodulisporium* produced antifungal volatile compounds that can control green mold decay on *Citrus limon* (Suwannarach et al. 2013) while *Nigrospora*, a ubiquitous endophytes, have been studied for the discovery of novel metabolites (Wang et al. 2017). Describing the niche and ecological role of endophytes are complicated because identical fungal species have been labelled as endophytic or pathogenic depending on the basis of the study and whether the study examines asexual or sexual stage of the fungal species (Ahlholm et al. 2002).

One of the most important enzymes used in biotechnology involved in starch hydrolysis are amylases. Amylases produced by various fungi have vast application in food and medicine production such as baking, brewing, preparation of digestive aids (Couto & Sanromán 2006). As a result of the amylase test, all fungal endophytes isolated and identified were able to degrade starch. Based on the studies of Venkatesagowda et al. (2012) they have found out that *Colletotrichum gloeosporioides*, *Fusarium oxysporum* and *Lasiodiplodia theobromae* produce amylolytic activity.

Cellulolytic microorganisms play an important role in the biosphere by recycling cellulose, the most abundant renewable carbohydrate produced by plants through the mechanism of photosynthesis (Qin et al. 2010). *N. oryzae*, and the unidentified species were able to show cellulose degradation

similar with the results of the previous study by Qin et al. (2010). Moreover, cellulase activity was also detected on *C. gloeosporioides* and *L. theobromae* (Toghueo et al. 2017).

Proteases are one of the major enzymes utilized widely as detergents, used in leather, food and pharmaceutical industries and for bioremediation processes (Najafi et al. 2005). Proteolytic enzymes play an important role in fungal physiology and development (Yike 2011).

Laccase is blue copper dependent oxidases which is a main ligninolytic enzyme produced by white rot fungus and catalyzing the oxidation of large numbers of phenolic compound (Poonkuzhali et al. 2011). Fungal laccases have boundless biotechnological functions such as discoloration and detoxification of industrial effluent, bleaching of pulp, phenolics elimination from wine and many others (Yaver et al. 2001). Only two endophytic fungi in this study were able to produce laccase, similar to the results of Sunitha et al. (2013), showing that species of *Fusarium* and *Phomopsis* showed laccase activity.

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