Bacillus amyloliquefaciens, a Narra (*Pterocarpus indicus*) leaf endophyte, possesses antifungal activity against phytopathogenic fungi due to laminarinase activity and production of antimicrobial compounds

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Microbial endophytes are microorganisms that live within plant tissues but do not confer disease. Endophytes benefit their host plant by promotion of plant growth, reduction of disease severity, induction of the defense mechanisms of plants, secretion of products that prevent herbivory, fixation of nitrogen, and increasing nutrient uptake. The benefits such as resistance to pathogens and insect herbivory that they confer on plants make them ideal sources of antimicrobial compounds which can be developed into biocontrol agents against phytopathogenic fungi or biofungicides. Laminarin or 1,3-β-glucan is a major component of fungal cell walls; thus one approach in identifying potential antifungal agents is to determine whether they secrete the enzyme laminarinase which hydrolyses laminarin into its component 1,3-linked β-D-glucose residues. As an initial step in the development of a biofungicide, 249 endophytic bacteria and yeasts were screened for the production of laminarinase. One isolate, N2B2, recovered from the leaves of Narra (*Pterocarpus indicus*), was able to grow with laminarin as sole carbon source and exhibit antifungal activity against three phytopathogenic fungi, namely, *Cladosporium* sp., *Diaporthe* sp., and *Fusarium oxysporum.* In vitro assays showed that the antifungal activity was correlated with laminarinase production. However, antifungal ability of N2B2 was sustained when grown in a medium that did not contain laminarin, suggesting that antimicrobial ability of the isolate may be due to multiple mechanisms. Identification of N2B2 by sequencing of its 16s rDNA revealed it to be *B. amyloliquefaciens.* This is the first mention in the literature of the isolation of *B. amyloliquefaciens* from Narra.

KEYWORDS

Biocontrol, *Bacillus amyloliquefaciens*, Antifungal activity, Biofungicides, *Cladosporium* sp., *Diaporthe* sp., *Fusarium oxysporum*., Laminarinase, Laminarin

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INTRODUCTION

Most plants have a mutualistic relationship with microorganisms, in much the same way that animals such as invertebrates, insects,

Table 1. List of screened isolates for laminarinase activity.

Bacteria	Yeasts
35 endophytes of Narra (*Pterocarpus indicus*)	100 endophytes of Narra
5 endophytes of Balete (*Ficus* sp.)	16 endophytes from epiphytes of Narra
2 endophytes of Bagtikan (*Parashorea malaanonan*)	1 from soil of Narra
5 endophytes of Molave (*Vitex parviflora*)	9 from soil of Bagtikan
15 from rhizosphere of *Chrystella* sp.	2 from soil of Balete
21 endophytes of *Chrystella* sp.	6 from soil of Balilang Uak ((Roxb.))
15 from rhizosphere of *Synedrella nodiflora*	4 from soil of Salisi (*Ficus benjamina*)
10 endophytes of *Synedrella nodiflora*	3 from soil of Malaruhat na Pula (*Syzygium* sp.)
(Total of 108 bacterial isolates)	(Total of 141 yeast isolates)

and humans have beneficial symbiotic microbiomes. This relationship provides the plants a level of protection against pathogens such as bacteria, viruses, and fungi (Hardoim et al. 2015; Strobel 2003). Usually these microorganisms spend at least parts of their life cycle inside plants and are called endophytes (Miliute 2015; Azevedo et al. 2000). Endophytes are diverse microbial communities comprising bacterial, archaeal, fungal, and protistan taxa (Hardoim et al. 2015). This diversity is shaped primarily by climatic conditions and geographical location where the host plant grows (Dreyfuss and Chapela 1994). Because endophytes should be adapted to the specific plant environment which they colonize, the metabolic potential of these microorganims is likely to differ from their soil-dwelling counterparts (Strobel 2003). The variety in their metabolic properties produces a wide range of compounds useful for plants for their growth and for protection against environmental conditions and phytopathogens (Hardoim et al. 2008; Selosse et al. 2004). Some studies have shown that these endophytes affect the abundance, richness, and community of decomposer organisms (Nair and Padmavathy 2014). They also act as latent saprotrophs in abscised plant parts such that when decay occurs, they increase the rate of decomposition with implications on biogeochemical cycling (Saikkonen et al. 2015). Furthermore, several studies have determined that the inoculation of some strains of endophytic bacteria or the introduction of endophyte-derived bioactive compounds promotes plant growth and confers enhanced resistance to various pathogens (Santoyo et al. 2016; Deshmukh et al. 2015; Bandara et al. 2006; Bai et al. 2002).

Fungal cell walls are made up of 1,3-β-glucan or laminarin (60%), chitin (10%–20%), and other polymers and proteins (Adams 2004; Burns and Dick 2002; De Nobel et al. 2000). Laminarin is hydrolyzed by 1,3-β-glucanase, or laminarinase, while chitin is hydrolyzed by chitinase (Gohel et al. 2006). Researchers have developed cocktails of enzymes containing laminarinase and chitinase as main ingredients of biofungicides to combat plant fungal pathogens (Ait-Lahsen et al. 2001; Singh et al. 1999; Haran et al. 1995 as cited by Brzezinska 2014). Yeast laminarinases were able to inhibit *Bacillus cinerea* (Masih and Paul 2002; Jijakli and Lepoivre 1998), and laminarinase from *Trichoderma* was able to bind to cell walls of *Aspergillus niger*, *B. cinerea*, and *Colletotrichum* sp., among others (Ait-Lahsen et al. 2001). Encapsulated chitinase and laminarinase in soya lecithin liposomes maintained enzymatic activity for the control of *Fusarium oxysporum* (Ilyina et al. 2013). A biopesticide from the mycelia-free extracts of *Beauveria bassiana* was developed against TBLM (Tomato Black Leaf Mold), *Pseudocercospora fuligena*, and Eggplant Fruit Rot (*Phomopsis vexans*) (PCAARRD 2014). This study aims to identify microorganisms capable of producing laminarinase and other compounds that are effective against phytopathogenic fungi. This is the first step towards the development of a biofungicide.

MATERIALS AND METHODS

Sample source and maintenance

A total of 249 pure cultures of yeasts, and endophytic bacteria were obtained from the culture collection of the Microbial Ecology of Terrestrial and Aquatic Systems Research Laboratory of the Institute of Biology, University of the Philippines Diliman, Quezon City, Philippines (table 1). Bacterial cultures were maintained in Nutrient Agar (NA) (5g peptone, 30g beef extract, 5g NaCl, 15g agar per liter of water), while the fungi were maintained in Potato Dextrose Agar (PDA) (dehydrated media 36g Himedia(R) per liter of water). The yeasts were maintained in Yeast extract Peptone Dextrose Agar (YPD) (20g Bacto peptone, 10g yeast extract, 20g dextrose, 15g Bacto agar per liter of water). All cultures were grown at room temperature (28°–30°C) and then stored at 4°C until ready for use. All cultures were revived in broth or agar plates before use.

Screening for growth on laminarin

The isolation of a microorganism with laminarinase activity was based on the procedure of Kim et al. (2014) in screening bioactive microorganisms with a minor modification. Instead of carboxymethylcellulose, 0.1% laminarin from *Laminaria digitata* (L9634 Sigma) was used as sole carbon source along with 2% Bacto agar (BD Biosciences, USA) to selectively grow laminarin-degrading microorganisms. Pure isolates of microorganisms were point inoculated on minimal salts agar plates with and without laminarin. Isolates were also inoculated on agar alone. Yeast isolates were incubated at 28°C for five days while the bacterial isolates were incubated at 37°C for 18–24 hours. Isolates that formed colonies on the agar plate supplemented with laminarin were picked and screened for laminarinase activity.

Screening for laminarinase activity

The detection of laminarinase activity was done following a modified procedure in screening glucanase activity (Kasana et

al. 2008). Five microliters of overnight cultures of the isolates equalized to 0.5 McFarland turbidity standard were spot plated on Minimal Salts Agar (MSA) (Kasana et al. 2008) with laminarin (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05%

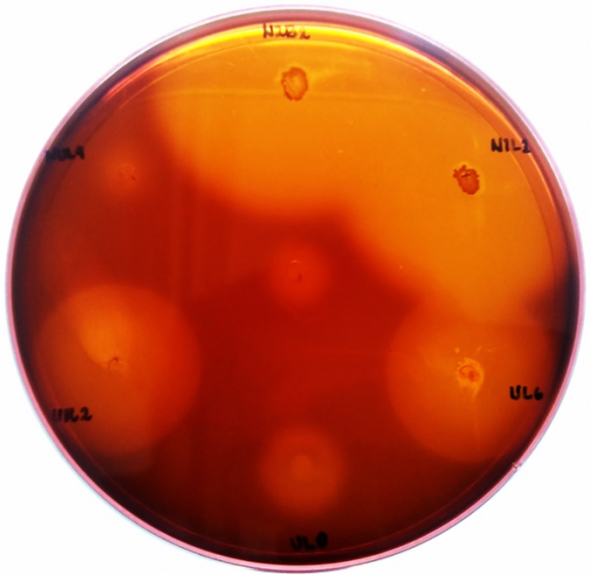

Figure 1: Qualitative assay for glucanase activity. Gram's iodine flooded plates after five minutes; zone of clearing exhibited by (clockwise) isolates N2B2, N1L5, UL6, UL8, UR2, and NUL4, *E. coli* was used as negative control (center).

KCl, 0.2% laminarin). Plates were incubated at 28°C for 48 hours and flooded with Gram's iodine (2.0g KI and 1.0g iodine in 300 mL distilled water) for three to five minutes and then observed for zones of clearing. Isolates possessing zones of clearing that were lower than 20 mm were not considered for further study.

Determination of growth in laminarin

Eighteen-hour cultures of isolates were inoculated in 150 mL of Minimal Broth (MSA without the agar) with or without 0.2% laminarin, with starting optical density (OD 600 nm) of 0.05 for isolates N1L5, NUL4, Be3, UL6, and 0.005 for N2B2. The setup was incubated at room temperature (28°–30°C) with continuous shaking at 150 rpm. The optical density was read every 6 hours for 48 hours.

Antagonistic activity

The screening for potential biocontrol activity was performed by using two cultivation techniques. In the modified cocultivation technique the fungal sample was inoculated two days prior to bacterial inoculation. Cell-free extracts of the isolates were prepared by the filtration of bacterial suspensions in 0.45 µm membrane filters. The extracts were then spot plated on PDA around the fungal block, and activity was observed three to five days after inoculation. The cocultivation technique involved the simultaneous inoculation of the bacterial isolates and the fungal samples. Twenty microliters of 18-hour nutrient broth culture of the bacterial isolate was spot plated adjacent to the 0.5 cm x 0.5 cm agar block of a 48-hour-old fungal sample.

Genomic DNA extraction

Bacterial genomic DNA was extracted by using a boil lysis method with a few modifications (Ahmed et al. 2014). One mL of 18-hour culture of the isolate was centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of sterile distilled water. The suspension was gently vortexed and boiled at 100°C for 10 minutes in a dry bloc (Premier®). The suspension was then centrifuged at 10,000 rpm for 5 minutes. The resulting supernatant was discarded, and the pellet was resuspended in 100 µL of sterile distilled water. The DNA extracts were kept at –20°C until polymerase chain reaction (PCR) amplification.

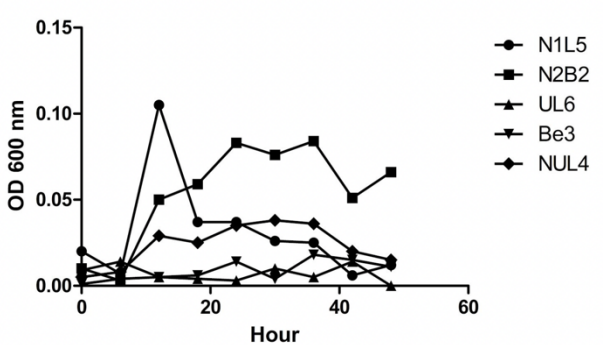

Figure 2: Determination of growth in laminarin. Isolates N1L5, N2B2, UL6, Be3, and NUL4 grown in minimal salts medium with or without laminarin (control) incubated at 28°C for a span of 48 hours with continuous shaking. Absorbance readings of the growth of the isolates in laminarin have been normalized to their respective negative controls. Measurements were taken every six hours at 600 nm.

Polymerase chain reaction

The 16S rDNA region of the isolates were amplified in a 12 µL reaction mixture containing 5 µL of GoTaq® Green Master Mix (Promega), 2 µL of extracted DNA, 9 µL of nuclease-free water, and 1 µL of each of 10 µm of universal forward and reverse bacterial primers (27F: AGAGTTTGATCMTGGCTCAG and 1492R: GGYTACCTTGTTACGACTT) (Weisburg et al. 1991). PCR was conducted by using a thermal cycler (Thermo Scientific™ Arktik™) with the following amplification conditions: 1 cycle of 95°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 4 minutes; and finally 1 cycle of 72°C for 5 minutes. The PCR products were analyzed on 1.5% agarose gel stained with GelRed® in 1X TAE buffer run at 100V for 30 minutes. A 100-bp ladder (Vivantis®) was used as a molecular weight marker, and the gel was visualized under a UV transilluminator (Clinx Science®).

Sequence data analysis

PCR products were sent to the Philippine Genome Center, Philippines, for PCR purification and sequencing. The partial 16S rDNA sequences were imported to BioEdit Sequence Alignment Editor version 7.0.5 to create a consensus sequence. Sequences were matched with the sequences of reference strains in GenBank database through BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov). The nucleotide similarity, e-value, nucleotide coverage, and maximum score were the bases for the putative identification of isolates.

RESULTS AND DISCUSSION

Initial screening for laminarinase activity

A total of 249 microbial isolates were qualitatively screened for laminarinase activity. Among these isolates, 141 were yeasts, while 108 were bacteria. Table 1 shows the plant associations of the isolates. Of the bacterial strains, 47 were isolated from different forest species, 31 were from annuals, and 30 from rhizosphere. Among the yeasts, 100 were endophytes from Narra and 16 were endophytes from epiphytes of Narra, while 25 were from forest soil. Initial screening of the isolates involved the determination of growth in a medium containing laminarin as sole carbon source (Kim et al. 2014). Growth indicated the ability of the isolate to utilize the carbon source through the production of the enzyme laminarinase that can catalyze the degradation of the storage glucan into glucose (Qin et al. 2017; Salyers et al. 1977). Results showed that all yeasts were unable to utilize laminarin for growth. However, 15

bacterial isolates exhibited growth on laminarin. These isolates are N1L1, N1L2, N1L3, N1L5, N2L2, N2L3, N1B6, N2B2, NF3,

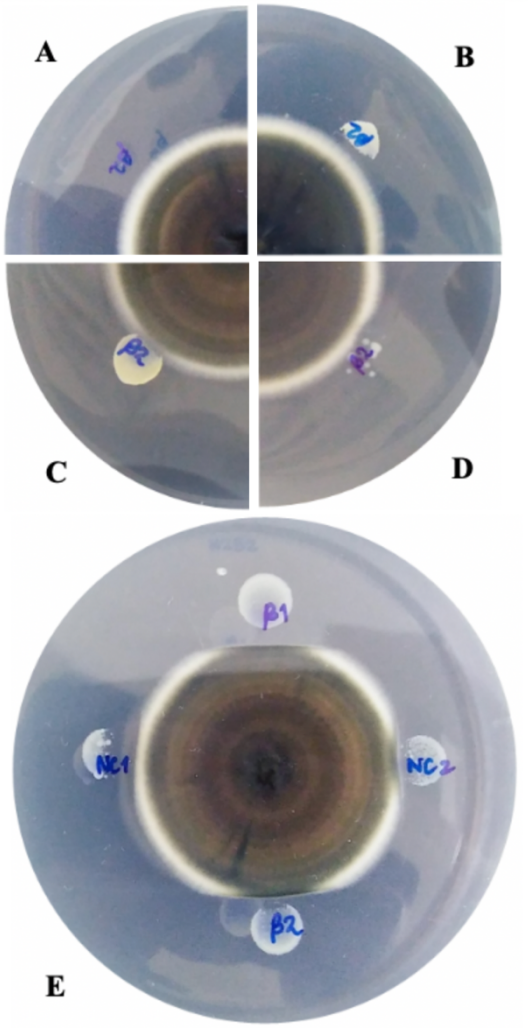

Figure 3: Preliminary antagonism assay against *Cladosporium* species. Isolates Be3 (A), NIL5 (B), UL6 (C), NUL4 (D), and N2B2 (E) against the phytopathogenic fungi *Cladosporium* species. "β" is the broth supplemented with laminarin, "NC" is the control without laminarin.

NL2, NUL4, UR2, UL6, UL8, and Be3. Note that of the 15 isolates, 11 were Narra endophytes, 3 were associated with *Chrystella* sp., while strain Be3 was isolated from *Ficus* sp. Positive isolates were further screened by using Gram's Iodine Plate with laminarin. Among the 15, only 5 isolates— N1L5, N2B2, NUL4, Be3, and UL6— produced the largest zones of clearing relative to the other isolates after two to three days of incubation (fig. 1).

Growth curve of isolates on a Minimal Salts Medium containing laminarin

Growth curves of the top five isolates were determined in Minimal Salts Medium (MSM) (fig. 2) with laminarin as sole carbon source. N1L5 had a steep log phase and attained its highest cell density at 12 hours but declined considerably after that. N2B2 exhibited a log phase that was not as steep and reached a stationary phase after 24 hours. NUL4 exhibited poorer growth relative to N1L5 and N2B2, while isolates Be3 and UL6 were barely able to grow in the liquid culture. Concurrent with the growth curve, the ability of the isolates to effect antagonistic activity against the phytopathogenic fungi *Cladosporium* sp. was determined. Aliquots obtained from each time point of the 48-hour growth period were used for determining antagonistic activity. As shown in figure 3, plates inoculated with 48-hour-old cultures of the five isolates showed

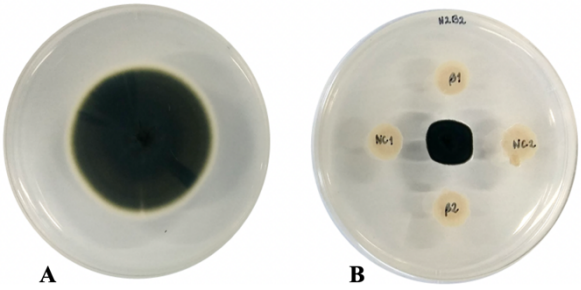

Figure 4: Antagonistic property of N2B2 grown in media with and without laminarin. Antifungal activity of N2B2 (B) against phytopathogenic fungi grown in media with laminarin ("β") and without ("NC") compared to negative control containing no bacterial cells (A).

that only N2B2 exhibited inhibition of mycelial growth. The other isolates did not have any effect on the fungal pathogen (fig. 3). This experiment showed that despite the ability of N1L5 and the other isolates to utilize laminarin as a carbon source by producing laminarinase they were not able to exhibit antifungal activity similar to N2B2. Surprisingly, spot plating of the N2B2 cells growing on MSM without laminarin also showed inhibition of *Cladosporium* sp., suggesting that antimicrobial activity of the isolate was not confined to laminarinase production and could be due to production of other compounds (fig. 4).

To determine whether the bioactive compound was secreted extracellularly, cell-free extracts of the culture broth of N2B2 were used for antagonism assay (Zouari et al. 2016; Silva et al. 2015; Berg et al. 2005). Cells were also subjected to mechanical disruption through sonication or bead beating to determine if the antifungal compound was intracellularly located. All results showed that the antagonistic activity against *Cladosporium* sp. was lost when cell-free extracts of the isolate were used (data not shown) and that antagonism was displayed only by live, growing cells.

Most secondary metabolites are secreted extracellularly (Karlovsky 2008; Martín et al. 2005), hence their role in controlling growth of other microorganisms. If N2B2 produced extracellular compounds, then the culture supernatant or even the sonicated or ruptured cells should have exhibited antimicrobial activity. Because only live cells were able to elicit a negative response from the fungus, then the antimicrobial compound appears to be produced by live cells. Other possible mechanisms of antifungal activity might be the production of lipopeptides (Zouari et al. 2016; Ongena et al. 2005), volatile organic compounds (Gotor-Vila et al. 2017; Raza et al. 2016), and polyketides (Chen et al. 2007). However, further study is needed to pinpoint the exact mechanism of antifungal activity for N2B2.

Growth of N2B2 on a medium without laminarin

N2B2 was grown in nutrient broth to determine if activity was maintained when the isolate was grown in an undefined medium, i.e., Nutrient Broth without laminarin. Results showed that the antagonistic activity was sustained whether the media contained laminarin or not (fig. 5). Biofungicide activity can be diverse and determined by production of laminarinases, antifungal volatiles, or growth-inhibiting metabolites, by inhibition of spore germination and decreased germinal tube length, and by competition for carbon sources and/or iron (siderophores) (Nally et al. 2015). The results of this study seem to reinforce this observation since antimicrobial activity is not limited to laminarinase production.

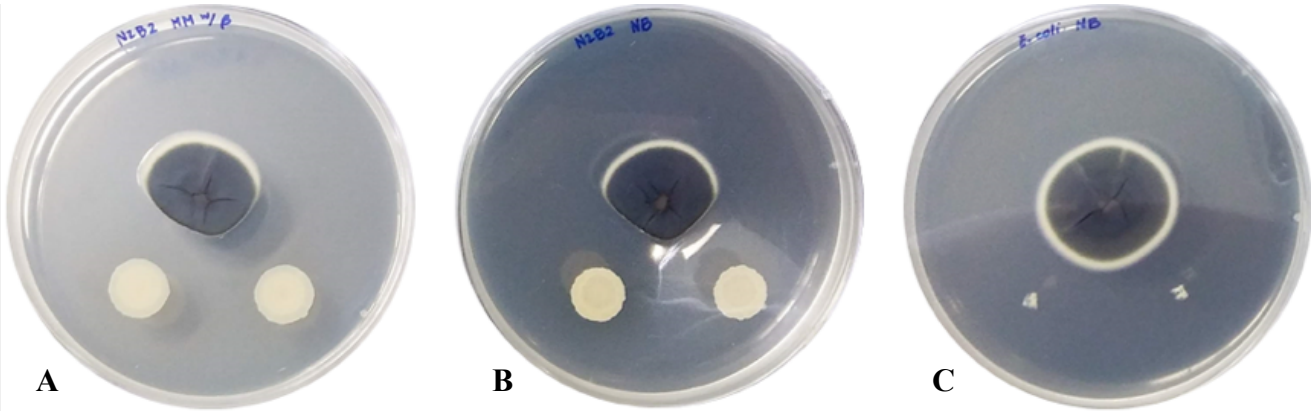

Figure 5: Antagonistic property of N2B2 grown in nutrient broth without laminarin. Culture broth of N2B2 enriched in minimal salts medium with laminarin (A), in nutrient broth (B), and *Escherichia coli* in nutrient broth as negative control (C).

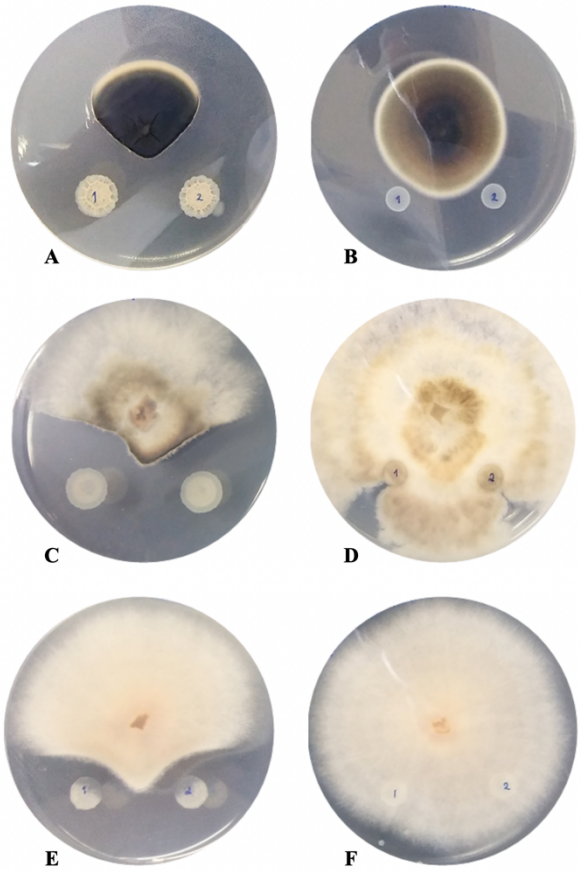

Figure 6: Antagonistic activity of N2B2 against phytopathogenic fungi. Antifungal activity of isolate N2B2 (left column) against known phytopathogenic fungi, *Cladosporium* sp. (A), *Diaporthe* sp. (C), and *Fusarium oxysporum* (E). Control = *E. coli* (right column). Incubated at 28°C for three to seven days.

Antagonistic activity against phytopathogenic fungi

To determine the specificity of the antifungal activity produced by N2B2, additional antagonism assays were performed against fungal samples belonging to genus *Diaporthe* and *Fusarium oxysporum* (Guarnaccia and Crous 2017; Balajee et al. 2009). Results showed that N2B2 enriched in nutrient broth exhibited antagonistic activity against both *Diaporthe* sp. and *Fusarium oxysporum* (fig. 6) indicating that the activity of N2B2 was not specific to *Cladosporium* sp. alone

Identification of N2B2 and its implications on biocontrol

N2B2 was isolated from Narra, an indigenous tree species. Sequence analysis of N2B2 (maximum score: 2599, total score: 2599, query cover: 100%, e-value: 0.0, percent identity: 99.58%) has been identified as *Bacillus amyloliquefaciens.* This species of soil bacteria is usually found in plant rhizospheres. Evidence has shown that this species can stimulate plant growth by releasing volatile organic compounds (Lugtenberg and Kamilova 2009), can produce phytohormonelike compounds (Idris et al. 2007), and has the potential to suppress plant-pathogenic bacteria and fungi through the production of cyclic lipopeptides (Arguelles-Arias et al. 2009; Caldeira et al. 2006). A genome analysis of *B. amyloliquefaciens* revealed a large number of gene clusters implicated in the production of antibiotics and secondary metabolites (Belbahri et al. 2017). The presence of antimicrobial compounds such as surfactin, fengycin, iturin A, macrolactin, difficidin, bacillaene, bacilysin, bacillibactin was also revealed (Arguelles-Arias et al. 2009). This study corroborates other researches that demonstrate the ability of the bacterium to produce antifungal compounds. However, further research is needed to pinpoint the actual mechanism of antifungal activity of N2B2. This is the first mention in the literature that *B. amyloliquefaciens* was isolated from a forest species, Narra (*Pterocarpus indicus*), indigenous to the Philippines.

CONCLUSION

The continuous search for alternative ways to address the problems in crop production while inflicting minimum harm to the farmer and the environment has led to the discovery of different biocontrol organisms that are potent and capable of targeting a broad spectrum of phytopathogens. Researches have shown that *Bacillus amyloliquefaciens* is capable of producing myriad of metabolites and organic compounds with antifungal activity. The bioactivity may be attributed to the production of laminarinase and other antimicrobial compound(s). This study has provided preliminary data on antifungal activity of N2B2 against *Cladosporium* sp., *Diaporthe* sp., and *Fusarium oxyporum*, highlighting the possible application of N2B2 as a biofungicide in agriculture. Further research is needed to elucidate the mechanism of antifungal activity. Despite the mounting researches on endophytes, the microbial communities of indigenous forest species are largely uncharacterized and untapped. This is the first mention in the literature of the isolation of *B. amyloliquefaciens* from an indigenous forest species, illustrating the vast potential of endophytic communities for novel applications.

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