



Aspergilli screened for antagonistic effects on fungal plant pathogens and for endoglucanase and xylanase activities

Tang K^{1,2,3}, Srihom C^{†,4}, Nuankaew S^{†,2,3}, Chuaseeharonnachai C^{2,3}, Kwantong P^{2,3}, Somrithipol S^{2,3}, Cheewangkoon R^{5,6}, Piasai O⁷ and Boonyuen N^{2,3*}

¹Diploma of Biomedical Sciences, Applied Science School, Temasek Polytechnic, 21 Tampines Avenue 1, 529757, Singapore

²Plant Microbe Interaction Research Team (APMT), Integrative Crop Biotechnology and Management Research Group (ACBG), National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Thanon Phahonyothin, Tambon Khlong Nueng, Amphoe Khlong Luang, Pathum Thani 12120, Thailand

³BIOTEC, National Science and Technology Development Agency (NSTDA), 113 Thailand Science Park, Thanon Phahonyothin, Tambon Khlong Nueng, Amphoe Khlong Luang, Pathum Thani 12120, Thailand

⁴National Biobank of Thailand (NBT), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Thanon Phahonyothin, Tambon Khlong Nueng, Amphoe Khlong Luang, Pathum Thani 12120, Thailand

⁵Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand

⁶Research Center of Microbial Diversity and Sustainable Utilization, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

⁷Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand

Tang K, Srihom C, Nuankaew S, Chuaseeharonnachai C, Kwantong P, Somrithipol S, Cheewangkoon R, Piasai O, Boonyuen N 2021 – Aspergilli screened for antagonistic effect on fungal plant pathogens and for endoglucanase and xylanase activities. *Current Research in Environmental & Applied Mycology (Journal of Fungal Biology)* 11(1), 274–283, Doi 10.5943/cream/11/1/20

Abstract

Twenty-six selected *Aspergilli* isolates were quantitatively pre-screened for endoglucanase and xylanase activities and for antagonistic effect on growth of plant pathogens using a dual plug inoculation method on agar plates. The *Aspergilli* were identified based on β -tubulin and calmodulin gene sequencing data using GenBank-based BLASTn analysis (NCBI). Antagonistic activity of the *Aspergilli* against fungal plant pathogens (i.e., *Curvularia eragrostidis*, *Colletotrichum gloeosporioides*, *Neopestalotiopsis clavispora*, *Fusarium incanatum* and *Sclerotium rolfsii*) was measured *in vitro* after seven days of incubation at room temperature. The highest antagonistic *Aspergilli* against *Curvularia eragrostidis*, *Colletotrichum gloeosporioides*, *Neopestalotiopsis clavispora*, *Fusarium incanatum* and *Sclerotium rolfsii* were identified as *Aspergillus carbonarius* WP 2, *A. parasiticus* JNF 222, *A. carbonarius* WP 2, *A. carbonarius* TBRC 402 and *A. giganteus* TBRC 400, respectively. The highest enzymatic index (EI) of endoglucanase and xylanase activity found in *Aspergillus puniceus* TBRC 2711 and *A. candidus* TBRC 2770 were 2.92 and 2.88, respectively. In this study, the preliminary screening reveals the potential of *Aspergilli* isolates as sources of endoglucanase and xylanase activity and as effective biocontrol agents against plant pathogenic fungi.

Keywords – *Aspergillus* – BLAST algorithm – fungal antagonist – micro fungi – Thailand

Introduction

Aspergilli are filamentous fungi found in various environments. They have economic importance for industrial production of enzymes and bioactive metabolites such as antibiotics, organic acids and medications (Frisvad & Larsen 2015, He et al. 2018, Li et al. 2020, Ntana et al. 2020). The chemical structures of *Aspergillus* metabolites are diverse and novel and include polysaccharides, alkaloids, polyketones, diketopyrazines and terpenes (Sanchez et al. 2012, Kjærboelling et al. 2018). In addition to antibacterial activities, some have anticancer and antiviral activities (Ma et al. 2014, Monggoot et al. 2018, Yodsing et al. 2018). Some such as *A. oryzae* are widely used in food fermentation because of their high exoenzyme activities (Samson et al. 2014). Cellulases and xylanases are of particular interest for enzymatic hydrolysis of biomass into sugars that can then be fermented to produce ethanol in biorefineries (Dashtban et al. 2010, Abdella et al. 2020, Iram et al. 2020).

In agriculture, fungal plant pathogens cause severe losses to crops worldwide and reduce the quality and quantity of agricultural commodities (Hariharan & Prasannath 2021). Examples include *Curvularia eragostidis*, *Neopestalotiopsis clavispora*, *Fusarium incarnatum* and *Sclerotium rolfsii* (Hyde et al. 2014, Kelly et al. 2017, Cacciola & Gullino 2019, Gurung et al. 2020, Wang & Wang 2021). World tendencies are shifting away from use of chemically synthesized pesticides towards biocontrol approaches and strategies for plant disease management (Zhang et al. 2020). Fungal antagonists play a significant role in controlling plant pathogens and diseases and they are increasingly being used as biocontrol agents (Shtienberg & Elad 2002, Latz et al. 2018, Thambugala et al. 2020). Previously, few *Aspergillus* species were described and proposed as potential biocontrol agents against plant fungal pathogens such as *Sclerotinia sclerotiorum* (Atallah & Yassin 2020), *Pythium aphanidermatum* (Al-Shibli et al. 2019), *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Pestalotiopsis mangiferae* and *Rhizoctonia solani* (Sanjay et al. 2018). The aim of this study was to carry out a preliminary screen of 26 Aspergilli isolates for their dual potential to serve as endocellulase (EDG) and xylanase (XLN) producers for enzymatic hydrolysis of biomass into sugars and as antagonistic agents against fungal plant pathogens. The effect of Aspergilli to control 5 selected fungal plant pathogens was performed *in vitro* by the dual culture technique in agar plates.

Materials & Methods

Fungal sources of Aspergilli isolates and fungal plant pathogens

Twenty-six *Aspergillus* isolates were selected from the BIOTEC Culture Collection (BCC; <http://www.tbrcnetwork.org>) at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani (<http://www.biotec.or.th>), Thailand. Most isolates had been tentatively identified as *Aspergillus* species based on their morphological characteristics and internal transcribed spacer (ITS) sequence data (data not shown). All isolates were checked the contamination by inoculation onto 9-cm petri dishes containing Difco™ Potato Dextrose Agar (PDA) medium and incubated at room temperature for 7 days. Seven-day old fungal plant pathogens on PDA viz *C. eragostidis* DH 052, *Co. gloeosporioides* LT 020, *N. clavispora* PP 026, *F. incarnatum* F 019 and *S. rolfsii* PP 55 isolated from leaf spot (*Musa sapientum* L.), Anthracnose (*Mangifera indica* L.), fruit rot (*Garcinia mangostana* L.), wilt (*Musa sapientum* L.) and wilt (*Solanum tuberosum* L.), respectively were provided by Kasetsart University Fungal Culture Collection (KUFC), Department of Plant Pathology, Faculty of Agriculture, Thailand.

Pre-screening for the qualitative presence of hydrolytic xylanase and endoglucanase

To screen for extracellular xylanase (XLN: endo- β -1,4-xylanase) activity, Czapek's medium containing modified xylan (CZAX) was used. The CZAX agar was inoculated with a single 1 μ l spore suspension spot and allowed to incubate in the dark for 3-4 days at 26°C. Plates were subsequently flooded with Gram's iodine, and hemicellulose-degrading ability was determined by the size of clearing zone around colonies. To screen for endoglucanase (EDN: endo- β -1,4-

glucanase) activity, carboxymethylcellulose (CMC) selective agar containing 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.05% KCl, 0.2% CMC sodium salt, 0.02% peptone and 1.7% Difco™ agar was used. The CMC agar was inoculated with a single spot of 1 µl spore suspension and allowed to incubate in the dark for 3 days at 26°C. Subsequently, fungal plates were flooded with Gram's iodine, and cellulose-degrading ability was determined by the size of clearing zone around colonies. Based on the diameter of the clear zones found at the top and bottom of the petri plates, visual qualitative screening of XLN and EDN activities were conducted as modified from the method of Boonyuen et al. (2014). An Enzymatic Index (EI) of XLN and EDN activity was calculated based on the formula: R/r, where R was the diameter of the entire clear zone, and r was the diameter of the fungal colony.

***In vitro* pre-screening of antagonistic fungi by dual plug assays**

Screening of the *Aspergilli* isolates for antagonistic activity was carried out by inoculating each test isolate in separate dual cultures with 5 selected plant pathogens (*C. eragrostidis* DH052, *Co. gloeosporioides* LT 020, *N. clavispora* PP 026, *F. incanatum* F 019 and *S. rolfsii* PP 55) in Petri dishes containing PDA followed by incubation for 7 days as previously described by Adebola & Amadi (2010). The young mycelia from the colony margin of each antagonistic *Aspergilli* isolates and the specific plant pathogenic fungus were cut using a sterile cork borer (0.5 cm diameter) and placed 2 cm away from the edge of the petri plate. Mycelium plug of the *Aspergillus* isolate was inoculated for 2 days before the fungal pathogen inoculated. For the negative fungal control, a single plug of the pathogen was inoculated onto a 9 cm PDA petri dish. Inoculum mycelium plugs were obtained from the margins of actively growing 7-day old cultures. Plates were incubated in the darkness at room temperature for 25 days, and measurements of radial growth of the plant fungal pathogens were taken after incubation for 7 days. Percent inhibition of average radial growth (I) was calculated in relation to the growth of the negative control as follows: $I = (R1-R2)/R1 \times 100\%$. Where I = the percent inhibition of radial growth (%), R1 = average of colony radius of the pathogen in the control plate (mm) and R2 = average of the colony radius of the fungal pathogen (mm) in the presence of *Aspergillus* isolates (Khleekorn & Wongrueng 2014). Dual cultures and controls were done in triplicate to ensure reproducibility. All fungal isolates were analyzed by one-way analysis of variance (ANOVA) using SPSS version 22 software, and a Duncan's test at $p \leq 0.05$ was used to determine significant differences among the means.

Identification of *Aspergilli* using sequence analysis

Genomic DNA was extracted from each isolate using the modified CTAB method as described by Boonyuen et al. (2011). The isolates were identified using partial β -tubulin (*benA*) and Calmodulin (*CaM*) gene targets. β -tubulin and *CaM* targets were amplified using the primer pairs Bt2a/Bt2b (Glass & Donaldson 1995) and CMD5/CMD6 (Hong et al. 2005, Raja et al. 2017), respectively. PCR was performed in 25 µl reaction mixtures containing 20.4 µl Nanopure H₂O, 2.5 µl DreamTaq buffer, 0.5 µl 10mM dNTP mix, 0.5 µl of each primer, 0.1 µl Dream Taq DNA polymerase (ThermoFisher, USA) & 0.5 µl of fungal DNA template. Amplifications were carried out using a T100™ Thermal Cycler (BIO-RAD) with the following protocols. For *benA*, there was initial denaturation of 94°C for 2 min followed by 30 cycles of 94°C for 2 min, 54°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. For *CaM*, there was initial denaturation of 94°C for 10 min followed by 35 cycles of 94°C for 50 s, 55°C for 50 s, 72°C for 1 min and a final extension at 72°C for 7 min. The PCR amplicons were verified by 0.8% agarose gel electrophoresis and visualized by UV before being sent to Macrogen Inc. (South Korea) for sequencing. Consensus sequences of *benA* and *CaM* were assembled using BioEdit sequence alignment editor v.7.2 (Hall 2005). The sequences were compared with sequence references in the GenBank database using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Identity percentages were used to compare sequences within genotypic groups. In this study, the preferential cut-off for fungal species inclusion was 98% identity.

Results

Activities of Xylanase and endoglucanase and identity of the Aspergilli

As shown in Table 1, the twenty-six Aspergilli isolates exhibited varying levels of xylanase (XLN) and endoglucanase (EDN) activity. One isolate (TBRC 2770) exhibited the highest activity of XLN (EI of 2.88 mm) while TBRC 2711 exhibited the highest EDN activity (EI = 2.92). For XLN, 4 isolates (15.38%) showed no qualitative activity (TBRC 2495, TBRC 301, WP 5 and TBRC 2711) while 13 isolates (50%) failed to exhibit any EDN activity. In this study, none of the isolates exhibited ligninolytic activity (data not shown)

The BLAST results on % identity are summarized in Table 1. Of the 26 isolates studied, 24 were identified as species in the genus *Aspergillus* (11 sections, including Candidi, Flavipedes, Nigri, Clavati, Cremei, Flavi, Fumigati, Nidulantes, Usti, Vesicolores and Terrei). For example, based on sequencing and morphology TBRC 2770 showed 99.62% identity (*benA*) and 98.88% (*CaM*) to *A. candidus* TBRC 2711 gave homology greater than 99% to *A. puniceus* (Table 1). Isolates WP 2, JNF 222, ATCC 56067 were identified as *A. carbonarius*, *A. parasiticus* and *A. foetidus*, respectively, using both *benA* and *CaM* sequences. Based on *CaM* data only, three isolates (KPFCN 20, TBRC 2495 and ASP 00821) were identified into *A. dimorphicus*, *A. fumigatus* and *A. rugolus*, respectively. One isolate (TBRC 400) was identified as an *A. giganteus* isolate using only Btub (Table 1). For isolate WP 1, the information from *benA* and *CaM* sequences was insufficient for identification to the species level.

Table 1 Taxonomic classification, enzymatic screening and antagonistic activity of *Aspergillus* isolates

Original Strains number	Sequence based identification		Enzymatic Index (EI)		Percent inhibition of average radial growth of fungal plant pathogens after incubation for 7 days ^{1/}				
	Closely related species (closest fungal match by BLAST hit)	% Identity (<i>benA</i> , <i>CaM</i>)	XLN	EDG	<i>Curvularia eragrostidis</i> DH 052	<i>Colletotrichum gloeosporioides</i> LT 020	<i>Neopestalotiopsis clavisporea</i> PP 026	<i>Fusarium incanatum</i> F 019	<i>Sclerotium rolfsii</i> PP 55
TBRC 2770	<i>A. candidus</i>	99.62, 98.88	2.88¹	2.85	57.89±5.58^{ab} cd	0.00±0.00 ^j	37.7±3.93 ^{cf}	0.00±0.00 ^k	49.05±5.75 ^a
TBRC 4217	<i>A. polyporicola</i>	99, 98	1.16	2.31	48.25±5.41 ^{bc} de	17.46±4.49 ^{hi}	23.81±5.83 ^{hi}	52.38±2.69^{hi} j	0.00±0.00 ^e
WP 2	<i>A. carbonarius</i>	99.8, 99	1.08	-	68.42±0.00^a 3	50.79±8.09^{ab}	61.90±0.00^{a,5}	73.81±4.45^b c	18.57±3.09 ^b c
KPFCS 3	<i>A. polyporicola</i>	98.42, 98.09	1.12	2.32	48.25±4.47 ^{bc} de	19.84±2.24 ^{hi}	31.67±4.98 ^{fg}	55.24±5.51 ^g hi	0.00±0.00 ^e
TBRC 400	<i>A. giganteus</i>	100, NA	1.20	2.46	49.12±8.68 ^{bc} d	32.54±2.24 ^{cdefgh}	43.65±2.24 ^{cde}	67.62±1.78^c def	52.86±0.00^a .7
KPFCN 20	<i>A. dimorphicus</i>	NA, 99.81	1.68	-	45.61±2.48 ^{cd} e	16.67±0.00 ^{hi}	0.00±0.00 ^k	54.29±0.00^{hi}	0.00±0.00 ^e
TBRC 2497	<i>A. nomius</i>	100, 100	1.14	-	54.39±1.24^{ab} cd	19.84±12.5 ^{hi}	13.49±2.97 ^j	51.43±5.08^{hi} j	19.05±14.2 5 ^{bc}
TBRC 309	<i>A. flavus</i>	100, 100	1.43	2.50	46.49±8.13 ^{bc} de	0.00±0.00 ^j	0.00±0.00 ^k	64.29±0.00^d ef	0.00±0.00 ^e

Table 1 Continued.

Original Strains number	Sequence based identification		Enzymatic Index (EI)		Percent inhibition of average radial growth of fungal plant pathogens after incubation for 7 days ^{1/}				
	Closely related species (closest fungal match by BLAST hit)	% Identity (<i>benA</i> , <i>CaM</i>)	XLN	EDG	<i>Curvularia eragrostidis</i> DH 052	<i>Colletotrichum gloeosporioides</i> LT 020	<i>Neopestalotiopsis clavisporea</i> PP 026	<i>Fusarium incanatum</i> F 019	<i>Sclerotium rolfsii</i> PP 55
JNF 222	<i>A. parasiticus</i>	99.81, 100	1.22	-	47.37±0.00 ^{bcde}	53.97±4.49^{a,4}	55.56±2.24^b	68.10±0.67^{cdef}	21.43±0.00 ^b
RIF 0001	<i>A. tamaritii</i>	99.8, 99.78	1.26	-	54.39±5.41^{abcd}	38.10±0.00 ^{abcdefg}	50.00±0.00^{bc}	61.43±4.04^{fg}	13.33±5.99 ^{bcd}
MD 00136	<i>A. frequens</i>	100, 99.83	1.18	1.58	42.98±1.24 ^{cd}	22.22±14.72 ^{ghi}	18.25±2.97 ^{ij}	55.24±3.75^{ghi}	0.48±0.67 ^e
TBRC 2495	<i>A. fumigatus</i>	NA, 100	-	1.73	63.16±5.68^{ab}	37.30±5.94 ^{bcdefg}	42.06±1.12 ^{dc}	63.81±2.43^{ef}	13.33±9.43 ^{bcd}
TBRC 301	<i>A. fumisynnematus</i>	100, 99.63	-	-	66.67±2.48^a	40.48±3.89 ^{abdef}	38.10±1.94 ^{cf}	64.29±4.21^{ef}	12.38±17.5 ^{1bcde}
WP 5	<i>A. fischeri</i>	100, 100	-	1.92	57.89±3.72^{abcd}	47.62±6.73 ^{abc}	46.83±1.12 ^{cd}	67.62±1.35^{cef}	0.00±0.00 ^e
ASP 00821	<i>A. rugulosus</i>	NA, 100	1.20	-	44.74±2.15 ^{cd}	30.95±1.94 ^{defgh}	23.02±1.12 ^{hi}	50.95±3.37^{hij}	7.62±9.78 ^{cd}
KUFA 0012	<i>A. fennelliae</i>	98.04, 97.28	1.23	N/A	58.77±11.03^{abcd}	45.24±0.00 ^{abcde}	23.02±1.12 ^{hi}	53.81±0.67^{hi}	2.86±2.02 ^e
TBRC 744	<i>A. quadrilineatus</i>	100, 99	1.08	1.68	31.58±22.43 ^e	28.57±1.94 ^{fgh}	27.78±2.97 ^{gh}	55.71±1.17^{ghi}	2.86±2.02 ^e
TBRC 1412	<i>A. amoenus</i>	100, 100	1.61	-	42.11±2.15 ^{de}	19.05±19.15 ^{hi}	1.59±2.24 ^k	46.19±5.87 ^j	0.00±0.00 ^e
TBRC 402	<i>A. carbonarius</i>	100, 100	1.10	-	63.16±11.96^{ab}	0.00±0.00 ^j	48.41±2.24 ^{cd}	81.9±3.37^{a,6}	0.00±0.00 ^e
ATCC 56067	<i>A. foetidus</i>	100, 100	1.39	-	67.54±1.24^a	46.03±2.24 ^{abcd}	48.41±4.05 ^{cd}	75.24±1.78^b	0.00±0.00 ^e
WP 1	<i>A. luchuensis</i>	99.82, 99.82	1.06	-	59.65±3.28^{abc}	45.24±0.00 ^{abcde}	42.86±0.00 ^{dc}	71.43±2.02^{bcd}	8.57±1.17 ^{bcde}
TBRC 2294	<i>A. sydowii</i>	99.76, 100	2.03	-	44.74±2.15 ^{cd}	7.94±6.25 ^{ij}	34.13±4.49 ^{fg}	0.00±0.00 ^k	0.00±0.00 ^e
TBRC 5169	<i>A. terreus</i>	99.82, 99	1.32	1.67	45.61±3.28 ^{cd}	29.37±2.97 ^{efgh}	38.1±3.89 ^{cf}	64.76±2.94^e	4.29±0.00 ^{de}
KPFCN 8	<i>A. allahabadii</i>	99.24, 99.46	1.53	2.12	47.37±4.30 ^{bcde}	19.05±14.02 ^{hi}	34.13±1.12 ^{fg}	49.52±1.78 ^{ij}	16.67±6.73 ^{bcd}
TBRC 2787	<i>A. calidoustus</i>	100, 99.82	1.20	2.38	57.02±12.41^{abcd}	32.54±4.49 ^{defgh}	29.37±5.61 ^{gh}	57.14±1.17^{gh}	0.00±0.00 ^e
TBRC 2711	<i>A. puniceus</i>	99.34, 99.25	-	2.92²	54.39±4.47^{abcd}	37.30±4.49 ^{bcdefg}	43.65±2.24 ^{cdc}	68.57±4.04^{cde}	0.00±0.00 ^e

Remark: ^{1,2} = the best enzyme producers in xylanase (XLN) and endoglucanase (EDN) are shown in bold text and grey background

^{3,4,5,6,7} = the best antagonistic *Aspergilli* against *C. eragrostidis*, *Co. gloeosporioides*, *N. clavisporea*, *F. incanatum* and *S. rolfisii*, respectively after incubation for 7 days in room temperature are bolded. (NA) No data available data. ^{1/} Mean followed by the same letter are not significantly different by Duncan's new multiple range test (P<0.05)

Antagonism of *Aspergilli* on the growth of fungal plant pathogens in co-culture

Tested *Aspergillus* isolates exhibited variable antagonism against the 5 fungal plant pathogens ranging from 0 to 89% inhibition (Table 1). In the control treatment, *C. eragrostidis*, *Co. gloeosporioides*, *N. clavisporea*, *F. incanatum* and *S. rolfisii* (i.e., Fig. 1B, 1D, 1F) colonized entire plates after incubation at room temperature for 7 days. None of the *Aspergilli* tested were able to completely inhibit mycelial growth of 5 selected fungal plant pathogens. However, one isolate (*A. carbonarius*) was able to inhibit 50% or more growth (grey background in Table 1) of all for pathogens except *S. rolfisii*, and 2 isolates (*A. parasiticus* and *A. tamarii*) were able to inhibit 50% or more of the growth of 3 pathogens. At the same time, 11 isolates were able to inhibit 50% or more of the growth of 2 pathogens. *Fusarium incanatum* was the most sensitive to the *Aspergilli* (50% inhibition or more for 22 of the 26 *Aspergilli* tested and ranging from 51 to 82% inhibition). *Curvularia eragrostidis* was the next most sensitive (50% inhibition or more for 13 of the 26 *Aspergilli* tested and ranging from 51 to 69% inhibition). The most unaffected pathogen was *S. rolfisii* that showed 53% inhibition with *A. giganteus* only.

Table 1 reveals that *A. carbonarius* WP 2 gave the highest percent inhibition of growth against to *C. eragrostidis* (68.42%) and *N. clavisspora* (61.9%). This inhibition was clearly discerned by limited growth of a fungal plant pathogen mycelium in the zone surrounding the colony of *A. carbonarius* WP 2. In Table 1, it can be seen that *A. carbonarius* TBRC 402 and *A. giganteus* TBRC 400 showed the highest antagonistic activity to *F. incanatum* and *S. rolfisii* with 81.9% and 52.86%, respectively. Additionally, *A. carbonarius* WP 2 showed the strongest inhibitory effect on both *Co. gloeosporioides* and *N. clavisporea*. Interestingly, *A. fischeri* WP 5 inhibited the growth of *S. rolfisii* when its mycelium contacted that of *A. fischeri* WP 5 and this also resulted in sclerotia production by *S. rolfisii* (Fig. 1E). In addition, *A. parasiticus* JNF 222 showed antagonistic activity against *N. clavisporea* PP 026, while *A. foetidus* ATCC 56067 exhibited antagonistic effect to *F. incanatum* F 019 as shown in Fig. 1A, 1C.

Discussion

Our results showed that 22 of the *Aspergilli* isolates we studied produced XLN while only 13 produced EDG. This is in line with a previous report indicating that species of *Aspergilli* often produce xylanolytic enzymes (Chukwuma et al. 2020) and cellulolytic enzymes (i.e., Arnthong et al. 2020, Namnuch et al. 2020). We also showed that many of the *Aspergilli* exhibited antagonistic activity against 5 plant pathogens in the form of inhibition zones in dual inoculated agar plates. These results are also in accordance with the data reported by Adebola & Amadi (2010) showing *in vitro* fungal interactions in the form of growth inhibition zones. Our findings are also consistent with those of previous studies in which several *Aspergillus* species such as *A. terreus*, *A. niger*, *A. awamori*, *A. niger* and *A. nidulans* were reported to be effective against fungal plant pathogens and fungal-like microbes (i.e., Nesha & Siddiqui 2017, Sanjay et al. 2018, Al-Shibli et al. 2019, Rezvani et al. 2020). They have also been reported to inhibit aflatoxigenic *A. flavus* in maize crops (Krishnamurthy et al. 2020). In the findings of Nigam et al. (2014), *A. flavus* was also found to be an effective antagonistic agent that helped to improve seed germination and control root rot, but with relatively low efficacy. The antagonistic activity is likely due to volatile and/or diffusible antibiotics (Boddy 2000). For example, *A. foetidus* ATCC 56067 can inhibit the growth of *F. incanatum*. This observation agrees with the reports by Boddy (2000), Boddy et al. (2007) who proposed that *Aspergillus* can probably produce antibiotics (Waksman & Geiger 1944) and antifungal metabolites (Sornakili et al. 2020). In co-cultures, *S. rolfisii* was able to overgrow *A. fischeri* WP 5 at the interaction zone (Fig. 1E). However, the mycelial density of *Sclerotium* sp. was reduced and the production of sclerotia was induced. Similarly, Boddy (2000) reported that

antagonistic interactions can cause mycelial morphology to change and mycelium density to be re-distributed in the zone of interaction.

In Fig. 1E, the formation of sclerotia by *S. rolfii* after interaction with *A. fischeri* WP 5 (Watkinson et al. 2015) is a response indicator of unfavorable growth conditions for *Sclerotium*. The dormant survival of *Sclerotium* sp. in the form of sclerotia would allow it to revive after favorable growth conditions returned (Smith 1972). Similarly, Hu et al. (2013) and Yadav et al. (2012) found that formation of sclerotia by *S. sclerotiorum* could be controlled by *A. aculeatus* and *Aspergillus* sp. under laboratory conditions, also in agreement with the findings in this study.

In our study, *A. carbonarius* WP 2 gave the highest percentage inhibition for the two fungal plant pathogens *Co. gloeosporioides* and *N. clavispora*, indicating that it may have potential as a biocontrol agent against *Co. gloeosporioides* and *N. clavispora*. Similarly, *A. giganteus* TBRC 400 was the only isolate with high antagonistic activity against the *Sclerotium* isolate we tested. Thus, it may be a good candidate for further study in the control of *Sclerotium*.

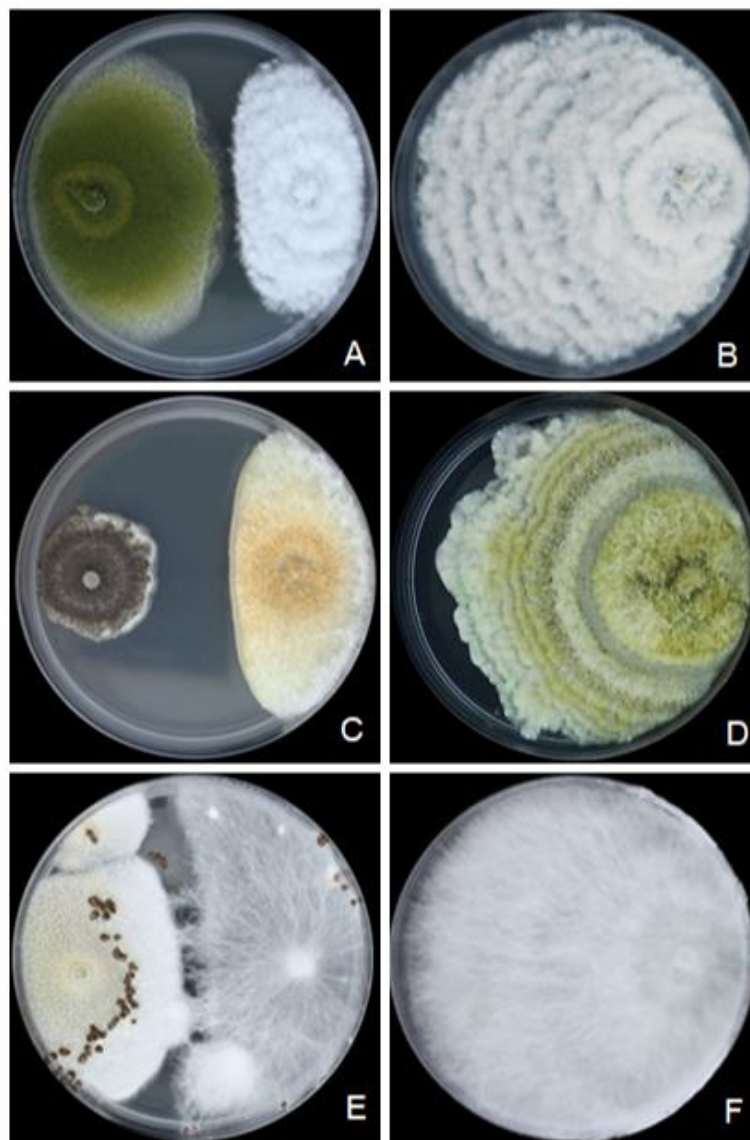


Fig. 1 – Selected photomicrographs of agar plate interactions between Aspegilli and plant pathogens. The Aspergilli isolates are on the left side of the plate and the pathogens on the right side. A *A. parasiticus* JNF 222 against *N. clavispora* PP 026. B *N. clavispora* control plate. C *A. foetidus* ATCC 56067 against *F. incanatum* F 019. D *F. incanatum* control plate. E *A. fischeri* WP 5 vs *S. rolfii* PP 55 showing contact inhibition with sclerotia formation. F *S. rolfii* control plate.

With respect to the quantification ENG and XLN in this study, we recommend that solid state fermentation be used to further optimize enzymatic activity. For antagonistic aspects, the findings of this paper revealed that some *Aspergilli* exhibited antagonistic activity when co-cultured with fungal plant pathogens and thus may have potential as biocontrol agents against selected plant fungal pathogens. Additional studies are still required to determine whether there will be any practical utility of the *Aspergilli* for biocontrol of plant fungal pathogens. It is vital to test the *Aspergilli* isolates for antagonistic activity in crops under field conditions and for their ability to colonize any intended host plants without negative effects. Alternatively, the *Aspergilli* may be cultured, and promising active culture ingredients may be extracted for use as agrochemicals. It is also important to realize that the efficacy of biological control agents can be variable and is dependent on pathogen inoculum level and environmental conditions.

Acknowledgements

This work was supported by National Center for Genetic Engineering and Biotechnology (BIOTEC). This paper partially fulfilled the requirement for a diploma of Biomedical Sciences, Applied Science School, Temasek Polytechnic, Singapore by the first author who did the research at BIOTEC under the Students' Internship Program in 2019. The last author wishes to thank Drs. Jennifer Luangsa-ard, Theerayut Toojinda and Wonnop Visessanguan for their support with fungal research at BIOTEC. BIOTEC International Cooperation Section (ICS) is thanked for their assistance in coordinating the program for the first author. Chen Yang and Bryan Teo from Temasek Polytechnic, Singapore are thanked for their help in antagonistic screening at BIOTEC. Prof. Dr. Timothy W. Flegel (Professional Authorship Center; PAC) is thanked for his critical review and English editing of the paper before submission to the journal. The reviewers and editor are greatly thanked for their valuable suggestions.

References

- Abdella A, Segato F, Wilkins MR. 2020 – Optimization of process parameters and fermentation strategy for xylanase production in a stirred tank reactor using a mutant *Aspergillus nidulans* strain. *Biotechnology Reports*, 26.
- Adebola M, Amadi J. 2010 – Screening three *Aspergillus* species for antagonistic activities against the cocoa black pod organism (*Phytophthora palmivora*). *Agriculture and Biology Journal of North America*, 362–365.
- Al-Shibli H, Dobretsov S, Al-Nabhani A, Maharachchikumbura SSN et al. 2019 – *Aspergillus terreus* obtained from mangrove exhibits antagonistic activities against *Pythium aphanidermatum*-induced damping-off of cucumber. *Peer J*, 10.
- Arnthong J, Siamphan C, Chuaseharonnachai C, Boonyuen N, Suwannarangsee S. 2020 – Towards a miniaturized culture screening for cellulolytic fungi and their agricultural lignocellulosic degradation. *Journal of Microbiology and Biotechnology*, 30(11), 1670–1679.
- Atallah O, Yassin S. 2020 – *Aspergillus* spp. eliminate *Sclerotinia sclerotiorum* by imbalancing the ambient oxalic acid concentration and parasitizing its sclerotia. *Environmental microbiology*, 22 (12), 5265–5279.
- Boddy L. 2000 – Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbiology Ecology*, 31(3), 185–194.
- Boddy L, Frankland J, West P van. 2007 – *Ecology of saprotrophic basidiomycetes*. Elsevier.
- Boonyuen N, Chuaseharonnachai C, Suetrong S, Veera VS et al. 2011 – Savoryellales (Hypocreomycetidae, Sordariomycetes): A novel lineage of aquatic ascomycetes inferred from multiple-gene phylogenies of the genera *Ascotaiwania*, *Ascothailandia*, and *Savoryella*. *Mycologia*, 103(6), 1351–1371.
- Boonyuen N, Manoch L, Luangsa-ard JJ, Piasai O et al. 2014 – Decomposition of sugarcane bagasse with lignocellulose-derived thermotolerant and thermoresistant *Penicillia* and *Aspergilli*. *International Biodeterioration & Biodegradation*, 92, 86–100.

- Cacciola SO, Gullino ML. 2019 – Emerging and re-emerging fungus and oomycete soil-borne plant diseases in Italy. *Phytopathologia Mediterranea*, 58(3), 451–472.
- Chukwuma OB, Rafatullah M, Tajarudin HA, Ismail N. 2020 – Lignocellulolytic enzymes in biotechnological and industrial processes: A review. *Sustainability*, 12(18): 1–31.
- Dashtban M, Schraft H, Syed TA, Wensheng Q. 2010 – Fungal biodegradation and enzymatic modification of lignin. *International Journal of Biochemistry and Molecular Biology*, 1 (1), 36–50.
- Frisvad JC, Larsen TO. 2015 – Chemodiversity in the genus *Aspergillus*. *Applied Microbiology and Biotechnology*, 99(19), 7859–7877.
- Glass NL, Donaldson GC. 1995 – Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology*, 61(4), 1323–1330.
- Gurung K, Dasila K, Pandey A, Bag N. 2020 – *Curvularia eragrostidis*, a new threat to large cardamom (*Amomum subulatum* Roxb.) causing leaf blight in Sikkim. *Journal of Biosciences*, 45(1).
- Hall T. 2005 – BioEdit Sequence Alignment Editor for Windows 95/98/NT/XP.
- Hariharan G, Prasannath K. 2021 – Recent Advances in Molecular Diagnostics of Fungal Plant Pathogens: A Mini Review. *Frontiers in Cellular and Infection Microbiology*, 10.
- He Y, Wang B, Chen W, Cox RJ et al. 2018 – Recent advances in reconstructing microbial secondary metabolites biosynthesis in *Aspergillus* spp. *Biotechnology Advances*, 36(3), 739–783.
- Hong S-B, Go S-J, Shin H-D, Frisvad JC, Samson RA. 2005 – Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia*, 97(6), 1316–1329.
- Hu X, Webster G, Xie L, Yu C et al. 2013 – A new mycoparasite, *Aspergillus* sp. ASP-4, parasitizes the sclerotia of *Sclerotinia sclerotiorum*. *Crop Protection*, 54, 15–22.
- Hyde KD, Nilsson RH, Alias SA, Ariyawansa HA et al. 2014 – One stop shop: backbones trees for important phytopathogenic genera: I, *Fungal Diversity*, 67(1), 21–125.
- Iram A, Cekmecelioglu D, Demirci A. 2020 – Screening of bacterial and fungal strains for cellulase and xylanase production using distillers' dried grains with solubles (DDGS) as the main feedstock. *Biomass Convers Biorefinery*.
- Kelly LA, Tan YP, Ryley MJ, Aitken EAB. 2017 – *Fusarium* species associated with stalk rot and head blight of grain sorghum in Queensland and New South Wales, Australia. *Plant Pathology*, 66(9), 1413–1423.
- Khleekorn S, Wongrueng S. 2014 – Evaluation of antagonistic bacteria inhibitory to *Colletotrichum musae* on banana. *Journal of Agricultural Technology*, 10(2), 383–390.
- Kjærboelling I, Vesth TC, Frisvad JC, Nybo JL et al. 2018 – Linking secondary metabolites to gene clusters through genome sequencing of six diverse *Aspergillus* species. *Proceedings of the National Academy of Sciences of the United States of America*, 115(4), 753–761.
- Krishnamurthy R, Padma PR, Dhandapani K. 2020 – Antagonistic efficiency of *Aspergillus giganteus* as a biocontrol agent against aflatoxigenic *Aspergillus flavus* infecting maize. *Journal of Pure and Applied Microbiology*, 14(1), 527–539.
- Latz MAC, Jensen B, Collinge DB, Jørgensen HJL. 2018 – Endophytic fungi as biocontrol agents: elucidating mechanisms in disease suppression. *Plant Ecology and Diversity*, 11(5-6), 555–567.
- Li C, Zhou J, Du G, Chen J et al. 2020 – Developing *Aspergillus niger* as a cell factory for food enzyme production. *Biotechnology Advances*, 44.
- Ma X, Zhu T, Gu Q, Xi R et al. 2014 – Structures and antiviral activities of butyrolactone derivatives isolated from *Aspergillus terreus* MXH-23. *Journal of Ocean University of China*, 13(6), 1067–1070.
- Monggoot S, Pichaitam T, Tanapichatsakul C, Pripdeevech P. 2018 – Antibacterial potential of secondary metabolites produced by *Aspergillus* sp., an endophyte of *Mitrephora wangii*. *Archives of Microbiology*, 200(6), 951–959.

- Namnuch N, Thammasittirong A, Thammasittirong SN. 2020 – Lignocellulose hydrolytic enzymes production by *Aspergillus flavus* KUB2 using submerged fermentation of sugarcane bagasse waste. *Mycology*.
- Nesha R, Siddiqui ZA. 2017 – Effects of *Paecilomyces lilacinus* and *Aspergillus niger* alone and in combination on the growth, chlorophyll contents and soft rot disease complex of carrot. *Scientia Horticulturae*, 218, 258–264.
- Nigam R, Saxena P, Singh DP. 2014 – Study on efficacy of biocontrol agents against root rot pathogens of cowpea. *Biochemical and Cellular Archives*, 14(2), 413–419.
- Ntana F, Mortensen UH, Sarazin C, Figge R. 2020 – *Aspergillus*: A powerful protein production platform. *Catalysts*, 10(9), 1–29.
- Raja HA, Miller AN, Pearce CJ, Oberlies NH. 2017 – Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *Journal of Natural Products*, 80(3), 756–770.
- Rezvani V, Taheri P, Pourianfar HR, Drakhshan A. 2020 – Biocontrol and plant growth promotion activities of endophytic and rhizospheric fungi from almond trees (*Prunus dulcis*) indigenous in the Northeast of Iran. *Current Research in Environmental & Applied Mycology (Journal of Fungal Biology)*, 10(1), 50–62.
- Samson RA, Visagie CM, Houbraken J, Hong S et al. 2014 – Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Studies in Mycology*, 78, 141–173.
- Sanchez JF, Somoza AD, Keller NP, Wang CCC. 2012 – Advances in *Aspergillus* secondary metabolite research in the post-genomic era. *Natural Product Reports*, 29(3), 351–37.
- Sanjay R, Esakkiraja E, Duraipandi A. 2018 – Biocontrol and growth promotion activity of endophytic *Aspergillus* spp. from Mango (*Mangifera Indica* L.). *Asian Journal of Microbiology, Biotechnology & Environmental Sciences*, 20(2), 513–517.
- Shtienberg D, Elad Y. 2002 – Is it possible to cope with the variability of biocontrol? *Bulletin IOBC/WPRS* 25, 1–4.
- Smith AM. 1972 – Biological control of fungal sclerotia in soil. *Soil Biology & Biochemistry*, 4(2):131–132.
- Sornakili A, Thankappan S, Sridharan AP, Nithya P, Uthandi S. 2020 – Antagonistic fungal endophytes and their metabolite-mediated interactions against phytopathogens in rice. *Physiological and Molecular Plant Pathology*, 112.
- Thambugala KM, Daranagama DA, Phillips AJL, Kannangara SD, Promputtha I. 2020 – Fungi vs. Fungi in Biocontrol: An Overview of Fungal Antagonists Applied Against Fungal Plant Pathogens. *Frontiers in Cellular and Infection Microbiology*, 10.
- Waksman SA, Geiger WB. 1944 – The Nature of the Antibiotic Substances Produced by *Aspergillus fumigatus* 12, *Journal of Bacteriology*, 47(4), 391–397.
- Wang M, Wang H. 2021 – First report of leaf anthracnose caused by *Colletotrichum liriopes* on *Ophiopogon japonicus* in China. *Crop Protection*, 140.
- Watkinson S, Boddy L, Money NP. 2015 – *The Fungi: Third Edition*.
- Yadav S, Srivastava AK, Singh DP, Arora DK. 2012 – Isolation of oxalic acid tolerating fungi and decipherization of its potential to control *Sclerotinia sclerotiorum* through oxalate oxidase like protein. *World Journal of Microbiology and Biotechnology*, 28(11), 3197– 3206.
- Yodsing N, Lekphrom R, Sangsopha W, Aimi T, Boonlue S. 2018 – Secondary Metabolites and their Biological Activity from *Aspergillus aculeatus* KKU-CT2. *Current Microbiology*, 75(5), 513–518.
- Zhang H, Godana EA, Sui Y, Yang Q et al. 2020 – Biological control as an alternative to synthetic fungicides for the management of grey and blue mould diseases of table grapes: a review. *Critical Reviews in Microbiology*, 46(4), 450–462.