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ENDOPHYTIC *Aspergillus* spp. ASSOCIATED WITH *Plectranthus amboinicus* LEAVES AS POTENTIAL BIOCONTROL AGENTS AGAINST *Fusarium verticillioides*

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18 **ENDOPHYTIC *Aspergillus* spp. ASSOCIATED WITH *Plectranthus amboinicus* LEAVES**
19 **AS POTENTIAL BIOCONTROL AGENTS AGAINST *Fusarium verticillioides***

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20

21 **ABSTRACT**

22 *Fusarium verticillioides* is the causal agent of ear, stalk and root rot of maize that results in
23 the severe reduction in yields and quality of infected products. Endophytic fungi have been purported
24 as potential candidates in controlling pathogens since they are considered strong plant mutualists that
25 confer disease resilience to their host. The present study was carried out to determine the *in vitro*
26 antagonistic activity and biocontrol potential of endophytic *Aspergillus* spp. associated with *P.*
27 *amboinicus* leaves against *F. verticillioides*. Three fungal endophytes from the genus *Aspergillus*
28 were isolated and identified from the leaves of *P. amboinicus*, namely *A. flavus*, *A. terreus* and *A.*
29 *niger*. The fungal isolates were tested for antagonism against *F. verticillioides* in dual culture plates.
30 Results indicate that the *Aspergillus* endophytes can restrict growth of *F. verticillioides* and
31 employ varying mechanisms of antagonism. *A. niger* inhibited *F. verticillioides* by 47.37%,
32 followed by *A. flavus* (41.02%) and *A. terreus* (27.91%) respectively. Observations of dual
33 culture plates revealed that *A. flavus* and *A. niger* antagonized the pathogen via overgrowth
34 mechanism while *A. terreus* employed antibiosis to restrict the growth of *F. verticillioides*. The
35 varying degrees of antagonism exhibited by the *Aspergillus* endophytes show their potential as
36 biocontrol agents and source of bioactive compounds.

37

38 **Keywords**: *Aspergillus*, biocontrol, endophytic fungi, *Fusarium*, rot

39

40 **INTRODUCTION**

41 *Fusarium verticillioides* is one of the most commonly reported soil-borne fungal pathogens
42 infecting maize (Abbas et al., 1998; Bacon and Hinton, 1996). It is the causal agent of ear, stalk and
43 root rot of maize. This fungus also produces secondary metabolites such as fumonisins that
44 accumulate in maize kernels, consequently causing severe reductions in yields and quality of products
45 (Leyva-Madrigal et al., 2017; Chu and Li, 1994). Plant pathogens demands to be managed and
46 controlled in order to maintain the abundance and quality of agricultural products around the world
47 (Pal and Gardener, 2006). Currently, chemical fungicides are the most effective agents in preventing
48 the infection of *F. verticillioides*. However, efforts to control postharvest diseases employing
49 synthetic chemical control agents pose danger to the environment and may affect the diversity of soil
50 microorganisms (Cardoso et al., 2010).

51 Endophytic fungi are microorganisms that inhabit internal plant tissues without causing any
52 apparent symptoms and this relationship usually involves metabolic interactions (Bacon and White,
53 2000; Petrini, 1991; Wilson, 1995). Studies and growing evidences support and indicate that

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54 endophytes are found in all plants and are extremely abundant and diverse (Arnold et al., 2000).
55 Endophytic fungi are believed to be strong plant mutualists and can confer increased resilience against
56 pests and plant pathogens (Carroll, 1988). Since these microorganisms are systemically distributed
57 throughout the host via metabolic translocation, they are interesting candidates for the biological
58 control (Rai et al. 2007). Many endophytic fungi have been evaluated for their biocontrol potential
59 against different plant pathogens. The elaboration on the mechanisms and action of these fungi as
60 antagonists is necessary to determine their efficacy as biocontrol agents because they can act against
61 pathogens in several ways (Rahman et al., 2009). Furthermore, biocontrol using fungi has proven to
62 be long-lasting on its effect and has the advantage of not requiring repeated periodic application as in
63 the case of chemical fungicides (Okigbo and Ikediugwu, 2000).

64 Numerous reports have been available on the potential use of *Aspergillus* species as
65 biocontrol agents (Gomathi and Ambikapathy, 2011). Studies suggests that *Aspergillus* species
66 used as biological control agents against fungal pathogens such as *Fusarium oxysporum*,
67 *Pythium* spp. and *Sclerotinia sclerotiorum* act through competition, mycelial lysis,
68 mycoparasitism and antibiosis via the synthesis of volatile and/or non-volatile metabolites
69 (Daami-Remadi *et al*, 2006; Bhattacharyya and Jha, 2011).

70 The finding that endophytic fungi could be utilized as biological control agents encourage
71 more studies and researches involving these microorganisms. Therefore, the present study was carried
72 out to determine the *in vitro* antagonistic activity and biocontrol potential of endophytic *Aspergillus*
73 spp. associated with *P. amboinicus* against *F. verticillioides*.

74

75 ## MATERIALS AND METHODS

76 ### Collection of Plant Material

77 Mature and healthy leaf samples of *P. amboinicus* were collected during May 2018 from
78 Echague, Isabela (16.6701° N, 121.7171° E). The authentication of plant materials was done on the
79 basis of taxonomic characters through the assistance of an experienced botanist. Samples were
80 transported in sterile polypropylene bags and processed within 6 hours of collection.

81

82 ### Isolation of Endophytic Fungi

83 Surface sterilization of plant samples and isolation of endophytic fungi was done using the
84 method described by Kusari et al. (2009) with minor modifications. Leaves of *P. amboinicus* were
85 washed and rinsed with running tap water and cut into 10 mm (length) by 5 mm (width) segments.
86 Then, each segment was surface sterilized by sequential immersion in 75% ethanol for 2 minutes, 1%
87 Sodium hypochlorite (NaOCl) for 3 minutes, and then once again in 75% ethanol for 1 minute. The
88 leaf segments were finally rinsed three times in sterile distilled water to remove excess sterilant and

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89 blot dried in sterile filter paper. Afterwards, the leaf segments of *P. amboinicus* were inoculated onto
90 Potato Dextrose Agar (PDA) plates supplemented with streptomycin (1 ml/L) to suppress bacterial
91 growth. Four (4) leaf segments were equidistantly placed on each amended PDA plate. The plates
92 were then sealed with parafilm and incubated at 28°C until the growth of endophytic fungi was
93 detected. The hyphal tip of each endophytic fungi growing out from the leaf segments were separately
94 transferred into new amended PDA plates and routinely maintained.

95

96 **Identification of Endophytic Fungi**

97 Endophytic fungi were identified according to their macroscopic and microscopic
98 characteristics such as the morphology of fruiting structures and spore morphology. Colony
99 morphology of the endophytes was observed on Coconut Water Agar (CWA), Potato Dextrose Agar
100 (PDA) and Malt Extract Agar (MEA). To ascertain identification of species, microscopic examination
101 of morphological structures was conducted using the agar block technique. The identification of fungi
102 was done using the keys and descriptions provided by Quimio and Hanlin (1999) and Samson et al.
103 (2014).

104

105 **Source of Test Fungus**

106 Pure cultures of the pathogenic fungus *F. verticillioides* were obtained from the maintained
107 cultures of Mycology Laboratory, College of Arts and Sciences, ISU– Echague, Isabela. The cultures
108 were transferred into sterilized Potato Dextrose Agar (PDA) plates and incubated at ±30 °C to allow
109 growth of the mycelia for seven (7) days.

110

111 ***In vitro* Antagonistic Activity**

112 The *in vitro* biocontrol potential and antagonistic activity of the endophytic fungi was tested
113 against the pathogenic fungus *F. verticillioides* using the dual culture method described by Matroudi
114 et al. (2009) and John et al. (2010). Agar plugs of each endophytic fungi and pathogen were obtained
115 from the edge of 7-day-old pure cultures using sterile cork borer. The plugs were aseptically
116 transferred 20 mm apart respectively on the center of 90 mm MEA plates. Control plates were
117 inoculated with the pathogenic fungi alone. Plates were incubated at 37°C for 15 days. The
118 interactions exhibited by the co-cultures were monitored daily and the diameter of growth of both
119 endophyte and pathogen were recorded at 5, 10 and 15 days, respectively. All control and test plates
120 were conducted in triplicates. Percentage inhibition was calculated as compared to control (Gaspar et
121 al., 2004). The growth inhibition was calculated by using the formula:

122 $$\text{Percent Inhibition (\%)} = \frac{\text{Pathogen growth in control} - \text{Pathogen growth in test}}{\text{Pathogen growth in control}} \times 100$$

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124

125 **Statistical Analysis**

126 Each of the tests was carried out using Completely Randomized Design (CRD) with three
127 replicates for each treatment. All means were treated statistically using one-way Analysis of Variance
128 (ANOVA) and compared by Tukey's Honest Significant Difference test at $p < 0.05$ using IBM™
129 SPSS v25.

130

131 **RESULTS AND DISCUSSION**

132 **Identification and Characterization of Endophytic Fungi**

133 Eight endophytic fungi were isolated from the healthy and asymptomatic leaves of *P.*
134 *amboinicus*. Three fungal isolates belonging to *Aspergillus* species were selected for the
135 determination of biocontrol potential. The morphology and growth characteristics of the *Aspergillus*
136 endophytes on CWA, PDA and MEA were recorded (Table 1).

137

138 Table 1 Macroscopic and microscopic characteristics of the *Aspergillus* endophytes.

Endophytic Fungi	Culture Media	Macroscopic Characteristics			Microscopic Characteristics		
		Colony Color	Reverse Color	Colony Density	Shape of Vesicle	Texture of Conidia	Seriation
A. flavus	CWA	Brown-green	White	Abundant	Sub-globose	Smooth/Finely roughened	Biseriate
	PDA	Parrot green	White	Luxuriant			
	MEA	Yellow	Light yellow	Abundant			
A. terreus	CWA	Beige	Tan	Sparse	Pyriform	Smooth	Biseriate
	PDA	Cinnamon	Brown	Sparse			
	MEA	Cream yellow	Yellow orange	Abundant			
A. niger	CWA	Grey	White	Luxuriant	Globose	Echinulate	Biseriate
	PDA	Black	Cream	Abundant			
	MEA	Brown-black	Black	Luxuriant			

139

140 The colony color of *A. flavus* endophytes ranged from yellow to olive green which turned
141 dark green with age (Fig. 1A). The margin and form are both filamentous and elevation is slightly
142 raised. Production of colorless exudates and brown-black sclerotia was observed in various plates
143 after extended incubation. On the other hand, colonies of *A. terreus* showed cinnamon-brown color

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with floccose white mycelia which eventually turns brown to yellow-brown consisting of a dense felt of conidiophores (Fig. 1B). Reverse morphology was brownish to orange in color, indicating the secretion of metabolites into the medium. Sclerotia was absent. Meanwhile, the colony morphology of *A. niger* has a distinct black-brown colony (Fig. 1C). It shows both filamentous on margin and form and umbonate elevation. The colonies initially grow with felt-like yellow to white hyphae, turning black with the formation of conidia. Formation of black sclerotia and black exudate beads was observed

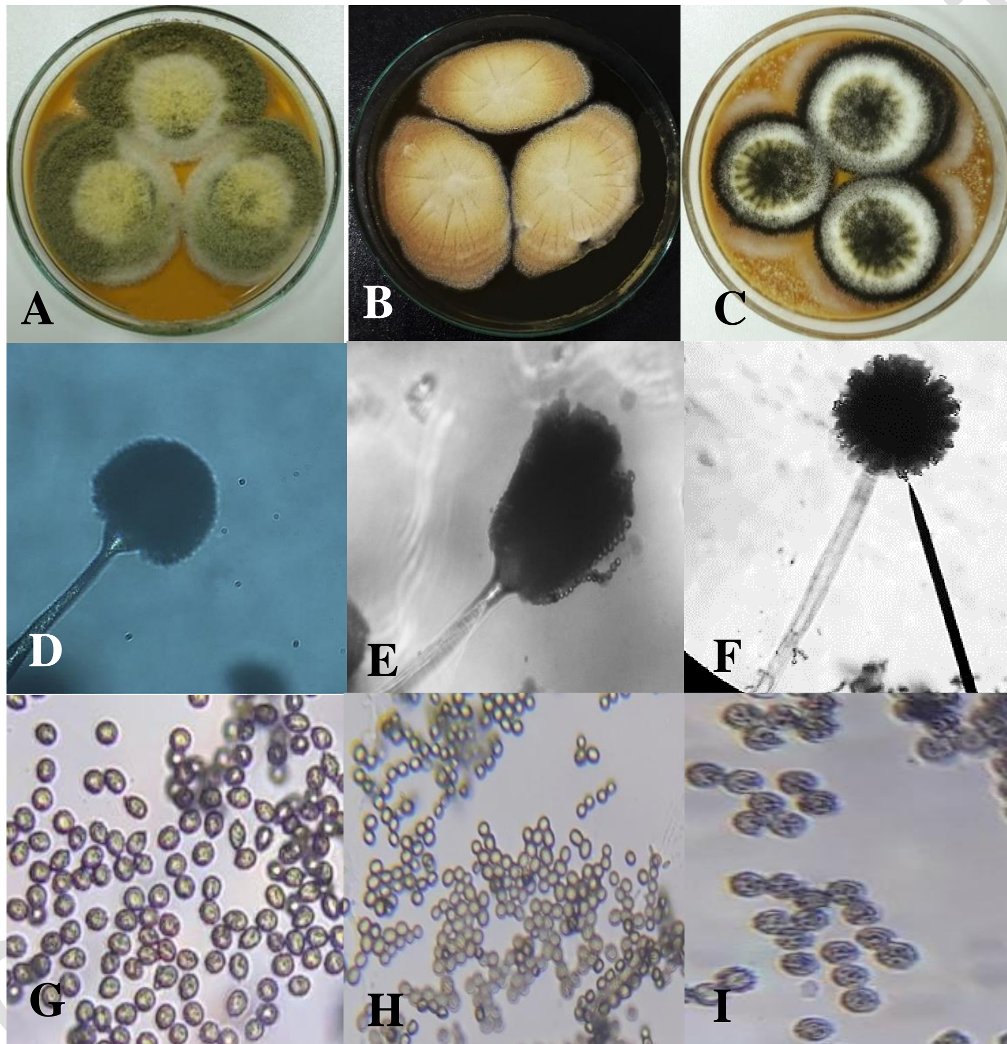

Figure 1 Colony morphology of (A) *A. flavus,* (B) *A. terreus* and (C) *A. niger;* Conidiophore of (D) *A. flavus,* (E) *A. terreus* and (F) *A. niger*; Conidia of (G) *A. flavus,* (H) *A. terreus* and (I) *A. niger*

The microscopic structures of the endophytes were observed in a microscope through the agar block method. The vesicles of *A. flavus* have sub-globose shape, while *A. terreus* and *A. niger* have pyriform and globose shaped vesicles respectively (Fig. 1D-1F). The asexual conidia of *A. terreus* are smooth and hyaline, while *A. flavus* conidia are finely roughened compared to the conidia of *A. niger* which are echinulate (Fig. 1G-1I). The endophytic fungi also exhibited some common

188 morphological structures such as biseriate conidial heads and septate hyaline hyphae. The obtained
189 microscopic descriptions of the *Aspergillus* endophytes coincide with the keys and descriptions
190 provided by Samson et al. (2002).
191
192 ***In vitro* Antagonistic Activity of Endophytic Fungi**
193 The radial mycelial growth and percentage inhibition in colony growth of *F. verticillioides*
194 by endophytic strains of *Aspergillus* is presented in Table 2. The results show that the endophytes
195 can varyingly restrict the growth of *F. verticillioides*. Among the three fungi, *A. niger* produced
196 the largest inhibition on the mycelial growth of *F. verticillioides* by 47.37%, followed by *A. flavus*
197 (41.02%) and *A. terreus* (27.91%). *F. verticillioides* recorded a mean radial growth diameter of
198 69.91 mm on the control plate. In comparison, the pathogenic fungi produced radial growth of
199 49.85mm, 60.92 mm and 44.48 mm in the dual culture with *A. flavus*, *A. terreus* and *A. niger*
200 respectively. The smaller radial growth on the dual culture plates indicated the presence of
201 antagonism between the pathogen and the endophytes.
202
203 Table 2 Radial mycelial growth of *F. verticillioides* and percent inhibition of the pathogen by three
204 endophytic strains of *Aspergillus* after 14 days of incubation.

Fungal Antagonists	***F. verticillioides***	
	Radial Mycelial Growth (mm)	**Percent Inhibition (%)**
A. flavus	49.85±10.64[a]	28.69%
A. terreus	60.92±11.51[b]	12.86%
A. niger	44.48±4.39[a]	36.38%
Control	69.91±16.31[b]	-

206 **Note**: Values are means of three replications. Means in the same column not sharing the same superscript are
207 significantly different at 5% significance level.
208

209 All the fungal antagonists were determined to inhibit the mycelial growth of F.
210 verticillioides involving different antagonistic mechanisms (Figure 9). Observations of dual
211 culture plates of *A. flavus* and *F. verticillioides* indicate that the endophyte antagonized the
212 pathogen through overgrowth mechanism. The overgrowth is achieved when a fungus exhibits
213 higher growth rate, tolerance against metabolites produced and higher capacity of antibiotic
214 production (Mathiavanan et al., 2000). A noticeable change in the morphology of *A. flavus* in all
215 co-culture plates can also be observed wherein the isolates became highly floccose and conidia
216 are rarely present (Fig. 9A). The variation suggests that the *A. flavus* isolates are adapting and

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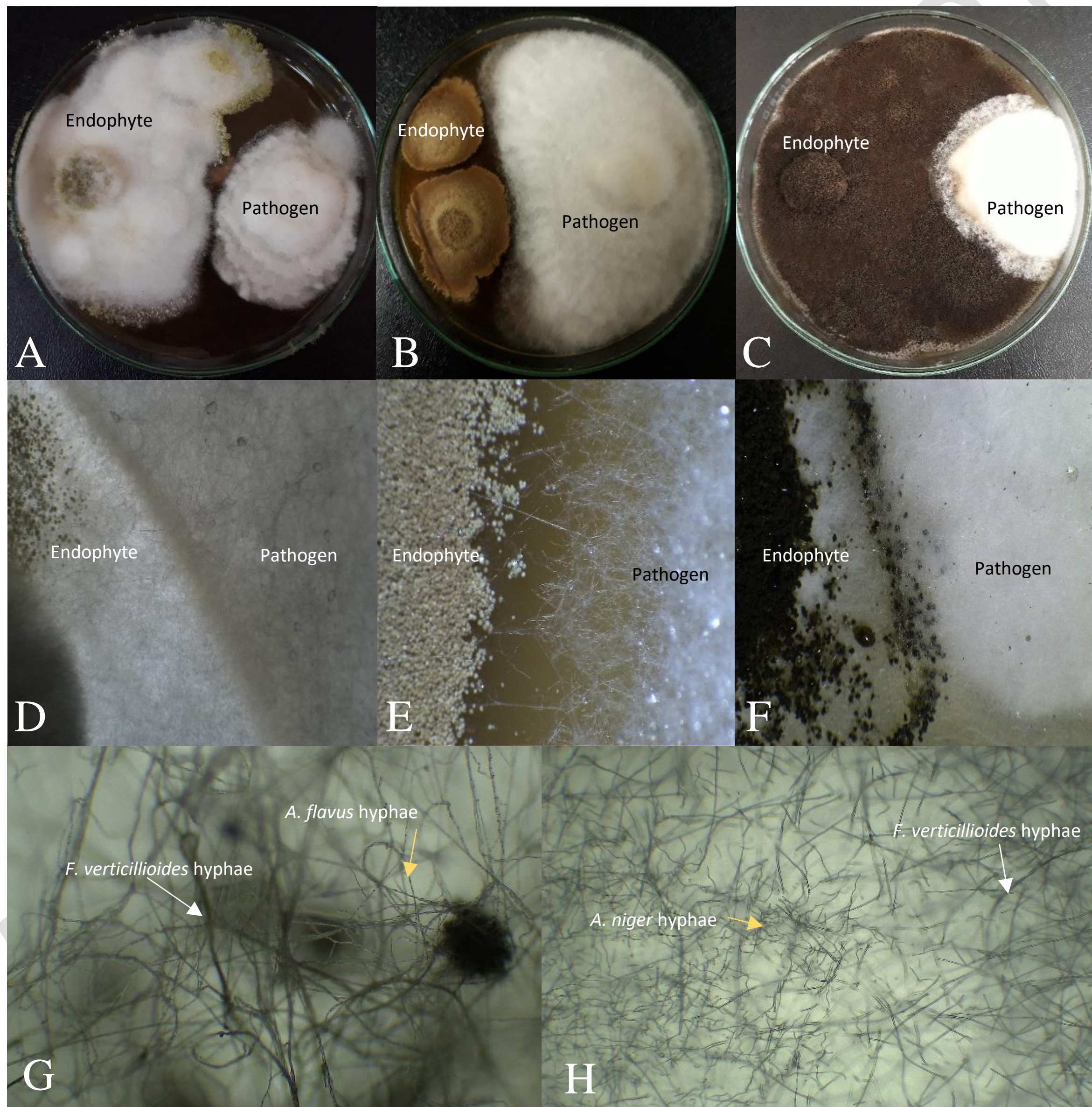
217 responding to the presence of *F. verticillioides*. *Aspergillus* species are known to grow via the
218 formation of a floccose mycelium, producing aerial hyphae that are capable of enhanced oxygen
219 absorption and increased rates of respiration (Rahardjo et al., 2005).

220 Isolates of *A. niger* outgrew those of *F. verticillioides*, which implies that the antagonism
221 involved is overgrowth mechanism. Macroscopic observation also revealed that the *A. niger* have
222 mutually intermingled growth with *F. verticillioides* without any zone of inhibition, indicating the
223 failure of the production of antibiotics either by the pathogen or by the antagonist.
224



225 Figure 9 Antagonism of *F. verticillioides* with (A, D) *A. flavus,* (B, E) *A. terreus* and (C, F) *A.*
226 *niger*; Microscopic hyphal interactions of (G) *A. flavus* and *F. verticillioides* and (H) *A.*
227 *niger* with *F. verticillioides*.
228

229 In the case of *A. terreus*, zone of inhibition was clearly observed in which there was a
230 conspicuous space between the antagonist and the test fungus (Fig. 9E). The inhibition zone is

observed in all co-culture plates of *A. terreus* and *F. verticillioides* and mycelial growth of both fungi is either stunted or severely decreased in the region. The formation of a zone of inhibition is an indication of the production of antibiotic substances either by the pathogen against antagonistic fungi or vice versa (Gomathi and Ambikapathy, 2011).

The capacity of the *Aspergillus* species to inhibit *Fusarium* isolates has been reported by several authors. Patibandn and Sen (2007) and Dwivedi and Enespa (2013) concluded that *A. niger* initiated lysis of *F. oxysporum* mycelium through antibiosis. Abdallah et al. (2015) also found out that several *Aspergillus* species including *A. niger*, *A. flavus* and *A. terreus* are successful biocontrol agents against *Fusarium sambucinum* and *Phytophthora erythroseptica*. Moreover, *A. niger* was also reported as one of the best antagonists for growth inhibition of several soil-borne, seed-borne and foliar plant pathogens (Kamil et al., 2009; Ahmed and Upadhyay, 2009). Additionally, certain atoxigenic strains of *A. flavus* have been reported to competitively exclude aflatoxin-producing strains during crop infection and thereby reduce aflatoxin contamination. One of these, AF36, has been registered as a biological control for the competitive exclusion of aflatoxin producing fungi from cottonseed (Cotty, 2018).

According to Abdallah et al. (2015), *Aspergillus* species have diverse adaptations and responses to cellular stress which allows them to be resilient in the presence of other organisms. These include the deployment of biophysically diverse compatible solutes and functionally diverse protein-stabilization proteins; hyperaccumulation of melanin in the cell wall; oxidative stress responses; ability to resist high temperatures; the production of extracellular polymeric substances (EPS) and formation of biofilms; and the ability to compete with other microbes.

CONCLUSION

Three *Aspergillus* endophytes isolated from the foliar segments of *P. amboinicus* were determined to restrict the growth of *F. verticillioides* and the mechanisms involved are overgrowth and antibiosis. The capacity of these endophytes to restrict the growth of *F. verticillioides* means that these organisms can be exploited as possible alternatives to chemical control agents.

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