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BIOCONTROL POTENTIAL OF ENDOPHYTIC *Aspergillus* spp. AGAINST *Fusarium verticillioides*

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ABSTRACT

The soil-borne fungus *Fusarium verticillioides* is the causal agent of ear, stalk and root rot of maize resulting in severe reduction in yields and quality of infected products. Endophytic fungi have been reported as potential candidates in controlling pathogens since they are considered strong plant mutualists that confer disease resilience to their host. The present study was carried out to determine the *in vitro* antagonistic activity and biocontrol potential of endophytic *Aspergillus* spp. associated with *Plectranthus amboinicus* leaves against *F. verticillioides*. Three fungal endophytes from the genus *Aspergillus*, namely; *A. tamarii*, *A. terreus* and *A. niger* were isolated and identified from the leaves of *P. amboinicus*,. The fungal isolates were tested for their antagonism against *F. verticillioides* in dual culture plates. Results indicated that the *Aspergillus* endophytes can restrict the growth of *F. verticillioides* through varying mechanisms of antagonism. *A. niger* inhibited *F. verticillioides* by 47.37%, followed by *A. tamarii* (41.02%) and *A. terreus* (27.91%). Dual culture observations revealed that *A. tamarii* and *A. niger* antagonized the growth of *F. verticillioides* via overgrowth mechanism while *A. terreus* employed antibiosis to restrict the pathogen. These varying degrees of antagonism by the *Aspergillus* endophytes exhibited their potential as biocontrol agents and source of bioactive compounds.

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

INTRODUCTION

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

Endophytic fungi are microorganisms that inhabit internal plant tissues, usually involving metabolic interactions without apparent symptoms (Bacon & White 2000; Petrini 1991; Wilson 1995). Growing evidences from several studies indicate that endophytes are found in all plants and are extremely abundant and diverse (Arnold *et al.* 2000). Endophytic fungi are believed to be strong plant mutualists which can produce increased resilience against pests and plant pathogens (Carroll 1988). Since these microorganisms are systemically distributed throughout the host via metabolic translocation, they are noteworthy candidates for biological control (Rai *et al.* 2007). Studies have been done on the antagonistic mechanisms and actions, particularly the efficacy, of many endophytic fungi for their biocontrol potential against different plant pathogens (Rahman *et al.* 2009). Furthermore, considering their long-lasting effects and not requiring repeated periodic

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application, the use of fungi for biocontrol has more advantages than chemical fungicides (Okigbo & Ikediugwu 2000).

Several studies recorded that *Aspergillus* species acted as biological control agents against fungal pathogens (*Fusarium oxysporum*, *Pythium* spp. and *Sclerotinia sclerotiorum*) through competition, mycelial lysis, mycoparasitism and antibiosis via the synthesis of volatile and/or non-volatile metabolites (Gomathi & Ambikapathy 2011; Daami-Remadi *et al.* 2006; Bhattacharyya & Jha 2011).

Hence, the present study was carried out to determine the *in vitro* antagonistic activity and biocontrol potential of endophytic *Aspergillus* spp. associated with *Plectranthus. amboinicus* against *F. verticillioides*.

MATERIALS AND METHODS

Collection of Plant Material

Mature and healthy leaf samples of *Plectranthus amboinicus* were collected in May 2018 from Echague, Isabela (16.6701° N, 121.7171° E). The plant materials were authenticated based on taxonomic characters through the assistance of an experienced botanist. Samples were transported in sterile polypropylene bags and processed within 6 hours of collection.

Isolation of Endophytic Fungi

Plant samples were surface sterilized and the endophytic fungi were isolated using the method described by Kusari *et al.* (2009) with minor modifications. Leaves of *P. amboinicus* were washed and rinsed with running tap water and cut into 10 mm (length) by 5 mm (width) segments. Each segment was then surface sterilized by sequential immersion in 75% ethanol for 2 minutes, 1% Sodium hypochlorite (NaOCl) for 3 minutes, and then once again in 75% ethanol for 1 minute. The leaf segments were finally rinsed three times in sterile distilled water to remove excess sterilant and blot dried in sterile filter paper. The leaf segments were later inoculated onto Potato Dextrose Agar (PDA) plates supplemented with streptomycin (1 ml/L) to suppress bacterial growth. Four (4) leaf segments were equidistantly placed on each amended PDA plate. The plates were then sealed with parafilm and incubated at 28 °C until the growth of endophytic fungi was detected. The hyphal tip of each endophytic fungi growing out from the leaf segments were separately transferred into new amended PDA plates and routinely maintained.

Identification of Endophytic Fungi

The endophytic fungi were identified according to their macroscopic and microscopic characteristics, particulalrly the fruiting structures and spore. Colony morphology of the endophytes was observed on Coconut Water Agar (CWA), Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA). To ascertain the identification of species, a microscopic examination of morphological structures was conducted using the agar block technique. The identification of fungi was done using the dichotomous keys and descriptions provided by Quimio and Hanlin (1999) and Samson *et al.* (2014).

Source of Test Fungus

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

In vitro Antagonistic Activity

The *in vitro* biocontrol potential and antagonistic activity of the endophytic fungi were tested against the pathogenic fungus *F. verticillioides* using the dual culture method described by Matroudi *et al.* (2009) and John *et al.* (2010). Agar plugs of each endophytic fungi and pathogen were obtained from the edge of 7-day-old pure cultures using sterile cork borer. The plugs were aseptically transferred 20 mm apart respectively on the center of 90 mm MEA plates. Control plates were inoculated with the pathogenic fungi alone. Plates were incubated at 37 °C for 15 days. The interactions exhibited by the co-cultures were monitored daily and the diameter growth of both endophyte and pathogen were recorded at 5, 10 and 15 days, respectively. All control and test plates were

conducted in triplicates. Percentage inhibition was calculated as compared to control (Gaspar *et al.* 2004). The growth inhibition was calculated by using the formula:

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

Statistical Analysis

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

RESULTS AND DISCUSSION

Identification and Characterization of Endophytic Fungi

Three fungal isolates belonging to *Aspergillus* species were selected and their morphology and growth characteristics on CWA, PDA and MEA were recorded for the determination of their biocontrol potential (Table 1).

The colony color of *A. tamarii* endophytes ranged from yellow to olive green which turned dark green with age (Fig. 1A). The margin and form are both filamentous and elevation is slightly raised. Production of colorless exudates and brown-black sclerotia was observed in various plates after extended incubation. The colonies of *A. terreus* showed cinnamon-brown color 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, *A. niger* has a distinct black-brown colony (Fig. 1C). It was both filamentous on margin and form and has 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 also observed.

The microscopic structures of the endophytes were observed in a microscope through the agar block method. The vesicles of *A. tamarii* 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. tamarii* conidia are finely roughened compared to the conidia of *A. niger* which are echinulate (Fig. 1G-1I). The endophytic fungi also exhibited some common morphological structures, such as biseriate conidial heads and septate hyaline hyphae. The obtained microscopic descriptions of the *Aspergillus* endophytes coincide with the keys and descriptions provided by Samson *et al.* (2002).

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. tamarii	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			

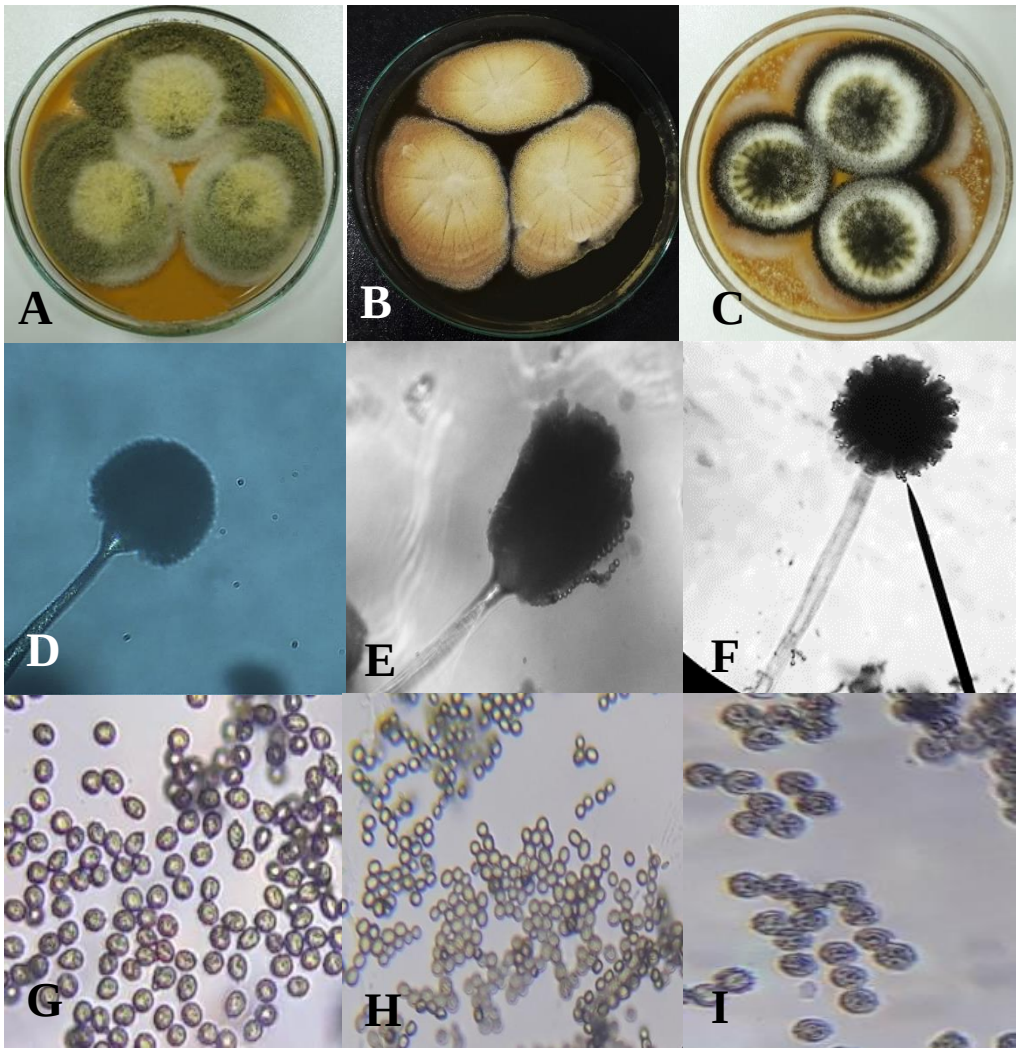

Figure 1 Colony morphology of (A) *A. tamarii;* (B) *A. terreus;* and (C) *A. niger;* Conidiophore of (D) *A. tamarii;* (E) *A. terreus;* and (F) *A. niger*; Conidia of (G) *A. tamarii;* (H) *A. terreus;* and (I) *A. niger*

In vitro Antagonistic Activity of Endophytic Fungi

Results of this study showed that the endophytes can variably restrict the growth of *F. verticillioides* (Table 2). Among the three fungi, *A. niger* produced the largest inhibition on the mycelial growth of *F. verticillioides* by 47.37%, followed by *A. tamarii* (41.02%) and *A. terreus* (27.91%). *F. verticillioides* recorded a mean radial growth diameter of 69.91 mm on the control plate. In comparison, the pathogenic fungi produced radial growth of 49.85 mm, 60.92 mm and 44.48 mm in the dual culture with *A. tamarii*, *A. terreus* and *A. niger,* respectively. The smaller radial growth on the dual culture plates indicated the presence of antagonism between the pathogen and the endophytes.

Table 2 Radial mycelial growth and percent inhibition of *F. verticillioides* by three endophytic strains of *Aspergillus* after 14 days of incubation

Fungal antagonists	*F. verticillioides*	
	Radial Mycelial Growth (mm)	Percent Inhibition (%)
A. tamarii	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]	-

Notes: Values are means of three replications. Means in the same column with different superscript indicate significant difference at $P \leq 0.05$.

All the fungal antagonists inhibited the mycelial growth of *F. verticillioides* through different antagonistic mechanisms (Fig. 2). Observations on the dual culture plates of *A. tamarii* and *F. verticillioides* indicated that the endophyte antagonized the pathogen through overgrowth mechanism. The overgrowth is achieved when a fungus exhibits a higher growth rate, tolerance against metabolites produced, and a higher capacity of antibiotic production (Mathiavanan *et al.* 2000). A noticeable change in the morphology of *A. tamarii* in all co-culture plates was also observed wherein the isolates became highly floccose and conidia were rarely present (Fig. 2A). This suggested that the *A. tamarii* isolates were adapting and responding to the presence of *F. verticillioides*. *Aspergillus* species are known to grow via the formation of a floccose mycelium, producing aerial hyphae that are capable of enhanced oxygen absorption and increased rates of respiration (Rahardjo *et al.* 2005).

Isolates of *A. niger* outgrew those of *F. verticillioides* implying that the antagonism involved is overgrowth mechanism. Macroscopic observation also revealed that the *A. niger* has a mutually intermingled growth with *F. verticillioides* without any zone of inhibition, indicating the failure of the production of antibiotics either by the pathogen or by the antagonist.

A. terreus zone of inhibition was clearly observed in which there was a conspicuous space between the antagonist and the test fungus (Fig. 2E). 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 & Ambikapathy 2011).

The capacity of *Aspergillus* species to inhibit *Fusarium* isolates has been reported by several authors. *A. niger* initiated lysis of *F. oxysporum* mycelium through antibiosis (Patibandn & Sen 2007; Dwivedi & Enespa 2013). *A. niger*, *A. tamarii* and *A. terreus* successfully controled the growth of *Fusarium sambucinum* and *Phytophthora erythroseptica* (Abdallah *et al.* 2015). *A. niger* was also reported as one of the best antagonists for several soil-borne, seed-borne and foliar plant pathogens (Kamil *et al.* 2009; Ahmed & Upadhyay 2009). Moreover, certain atoxigenic strains of *Aspergillus* 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).

Aspergillus species have diverse adaptations and responses to cellular stress which allows them to be resilient in the presence of other organisms (Abdallah *et al.* 2015). 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; production of extracellular polymeric substances (EPS) and formation of biofilms; and the ability to compete with other microbes.

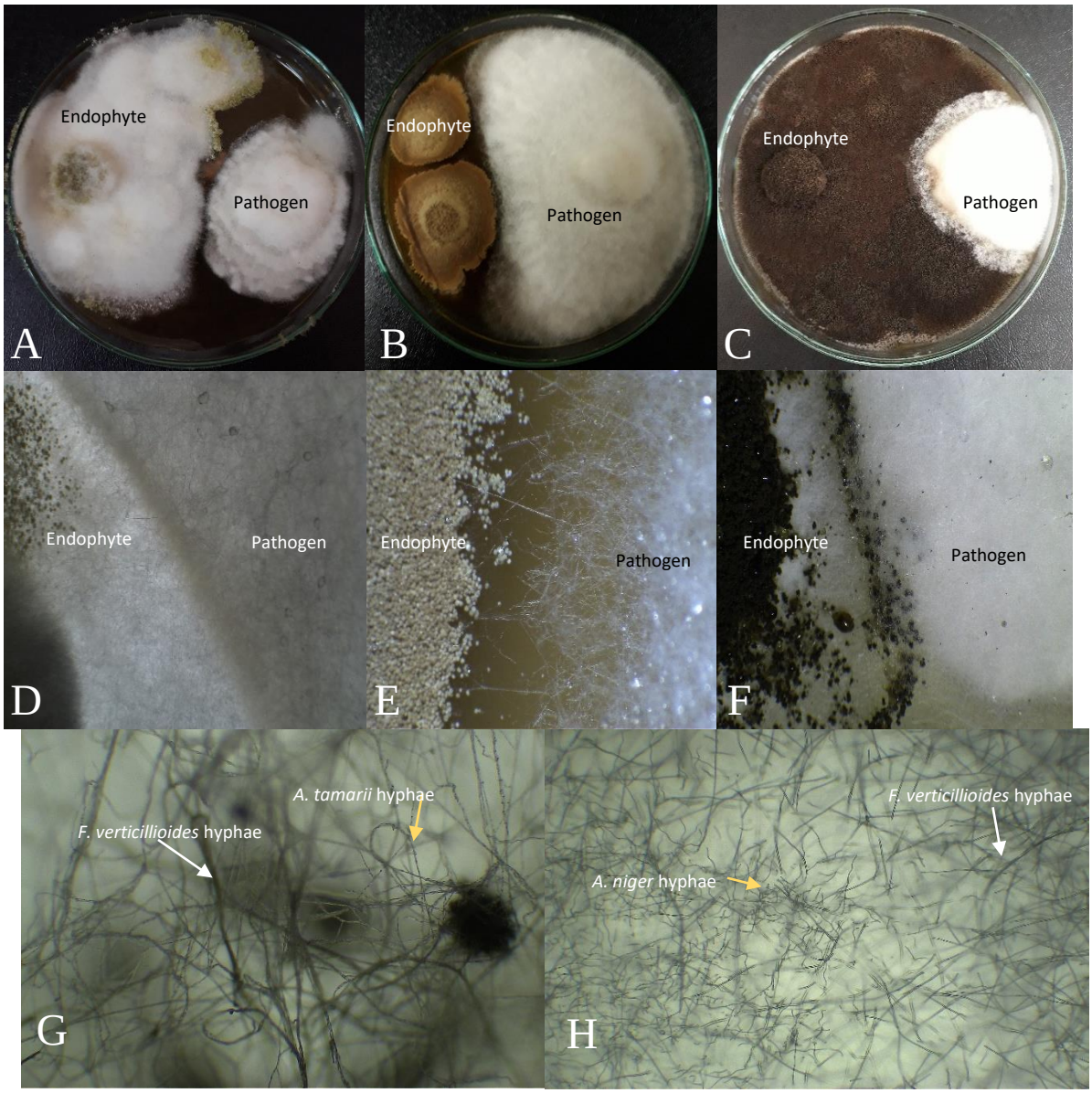

Figure 2 Antagonism of *F. verticillioides* with *A. tamarii* (A, D), *A. terreus* (B, E) and *A. niger* (C, F); Microscopic hyphal interactions of *A. tamarii* and *F. verticillioides* (G) and *A. niger* (H) with *F. verticillioides*

CONCLUSION

The three *Aspergillus* endophytes, namely *A. tamarii, A. terreus* and *A niger* isolated from the foliar segments of *Plectranthus amboinicus* restricted the growth of *Fusarium verticillioides* and the mechanisms involved overgrowth and antibiosis. The capacity of these endophytes to restrict the growth of *F. verticillioides* implied that these organisms can be exploited as possible alternatives to chemical control agents.

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