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Research

Mycotoxin Production by *Fusarium proliferatum* and *Fusarium fujikuroi* Causing Stem Rot of *Hylocereus polyrhizus* in Malaysia

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ABSTRACT

Fusarium proliferatum and *Fusarium fujikuroi* are the causative pathogens of stem rot in red-fleshed dragon fruit (*Hylocereus polyrhizus*). Both species are toxigenic fungi that produce several mycotoxins, including fumonisin B_1 (FB_1), moniliformin (MON), and beauvericin (BEA). These mycotoxins exert phytotoxic effects and are involved in pathogenesis in the host plants. In this study, we investigated the ability of *F. proliferatum* and *F. fujikuroi* to produce FB_1, MON, and BEA. Polymerase chain reaction amplification using *FUM1*-specific primers detected the gene in all 44 isolates tested, indicating that all isolates produced FB_1. Isolates of *F. proliferatum* and *F. fujikuroi* produced variable concentrations of FB_1, ranging from 11.97–236.80 µg/g. MON and BEA were also produced at 0.48–174.84 µg/g and 0.28–70.02 µg/g, respectively by isolates of *F. proliferatum* and *F. fujikuroi.* These results suggest that the three mycotoxins play roles in stem rot disease development and symptom manifestation, as all isolates tested were pathogenic and led to stem rot in *H. polyrhizus*.

Key words: Beauvericin, fumonisin B_1, *Fusarium fujikuroi*, *Fusarium proliferatum*, *Hylocereus polyrhizus,* moniliformin

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INTRODUCTION

Red-fleshed dragon fruit (*Hylocereus polyrhizus*) is grown commercially because it is a profitable fruit crop. With proper management to produce a high yield of high-quality fruits, red-fleshed dragon fruit has a rapid return. In the second year of planting, the plants bear fruit, and full production is achieved in 5 years (Hamidah & Zainuddin, 2007). One of the constraints in the production of dragon fruit is the various types of diseases caused by fungi, bacteria, and viruses that infect fruit crops (Balendres & Bengoa, 2019). In Malaysia, stem rot, which is characterized by circular, sunken lesions, has been detected in most dragon fruit plantations, and the causal pathogens have been identified as *Fusarium proliferatum* and *Fusarium fujikuroi* (Masratul Hawa *et al*., 2013; 2017).

Fusarium proliferatum and *F. fujikuroi* are mycotoxigenic fungi that produce several types of mycotoxins, including fumonisin B_1 (FB_1), moniliformin (MON), beauvericin (BEA), fusaric acid, fusaproliferin, and gibberellic acid. Both *F. proliferatum* and *F. fujikuroi* are also plant pathogens. Mycotoxins produced by *Fusarium* species are reportedly associated with pathogenesis during infection and they help the fungi to compete with other organisms (Reverberi *et al*., 2010). Logrieco *et al*. (1998) reported that higher amounts of fumonisins were found in asparagus infected with *F. proliferatum*, suggesting that fumonisins play an important role in the pathogenesis of crown rot. FB_1 has also been shown to induce phytotoxic effects in maize and tomatoes (Lamprecht *et al*., 1994). In the present study, the production of mycotoxins BEA, FB_1, and MON was analyzed because they are the major mycotoxins produced by *F. proliferatum* and *F. fujikuroi*. These mycotoxins exert phytotoxic effects on several crops.

MATERIALS AND METHODS

Fungal isolates

Information on the sampling and isolation of the fungal isolates, as well as the morphological and molecular characteristics used for identification, have been described in detail by Masratul Hawa *et al*. (2013, 2017). For mycotoxin analysis, 44 *F. proliferatum* and *F. fujikuroi* isolates were selected based on sampling locations from 10 states in Malaysia (Table 1).

Table 1. Isolates of *F. proliferatum* and *F. fujikuroi* used for mycotoxin analysis

State	Sampling location	Isolate/ code	*Fusarium* species
Penang	Juru	P4029π	*F. proliferatum*
	Juru	P4042π	*F. fujikuroi*
	Juru	P4093π	*F. proliferatum*
	Tasek Gelugor	P4671π	*F. fujikuroi*
	Tasek Gelugor	P4682π	*F. fujikuroi*
Perak	Slim River	A4694π	*F. fujikuroi*
	Slim River	A4708π	*F. fujikuroi*
	Tapah	A4697π	*F. fujikuroi*
	Tapah	A4705π	*F. proliferatum*
	Tapah	A9141π	*F. proliferatum*
Selangor	Hulu Langat	B4711π	*F. fujikuroi*
	Hulu Langat	B4727π	*F. proliferatum*
	Sabak Bernam	B4713π	*F. fujikuroi*
Negeri Sembilan	Kuala Pilah	N5354π	*F. proliferatum*
	Kuala Pilah	N5359π	*F. fujikuroi*
	Kuala Pilah	N9006π	*F. fujikuroi*
	Mantin	N9004π	*F. proliferatum*
	Mantin	N9028π	*F. proliferatum*
Melaka	Sungai Rambai	M9030π	*F. proliferatum*
	Sungai Rambai	M9046π	*F. fujikuroi*
	Naning	M9036π	*F. proliferatum*
Johor	Kluang	J9067π	*F. fujikuroi*
	Kluang	J9098π	*F. proliferatum*
	Mersing	J9094π	*F. fujikuroi*
	Mersing	J9172π	*F. proliferatum*
Kelantan	Tok Bali	D9110π	*F. fujikuroi*
	Tok Bali	D9111π	*F. proliferatum*
	Tok Bali	D9112π	*F. proliferatum*
	Kota Bahru	D9125π	*F. fujikuroi*
Sarawak	Kuching	Q9077π	*F. proliferatum*
	Kuching	Q9136π	*F. fujikuroi*
	Kuching	Q9158π	*F. fujikuroi*
	Sibu	Q9020π	*F. proliferatum*
	Sibu	Q9104π	*F. proliferatum*
Sabah	Kota Kinabalu	S9060π	*F. proliferatum*
	Kota Kinabalu	S9073π	*F. proliferatum*
	Kota Kinabalu	S9169π	*F. fujikuroi*
	Kota Kinabalu	S9181π	*F. fujikuroi*
	Kudat	S9164π	*F. fujikuroi*
	Kudat	S9178π	*F. fujikuroi*
	Gurun	K9192π	*F. proliferatum*
	Gurun	K9197π	*F. fujikuroi*
Kedah	Gurun	K9199π	*F. proliferatum*
	Yan	K9202π	*F. proliferatum*

Detection of the fumonisin gene

PCR amplification using *FUM1*-specific primers distinguished between FB_1-producing and non-producing isolates. Both FB_1-producing and non-producing *Fusarium* species have been reported to be associated with a broad range of plant hosts (Jimenez *et al*., 1997; Sreenivasa *et al*., 2006). To detect toxigenic *Fusarium* species using polymerase chain reaction (PCR) amplification, only FB_1-producing isolates were detected, because specific primers targeting BEA and MON have not yet been designed.

PCR amplification of the *FUM1* gene was carried out for 44 isolates of *F. proliferatum* and *F. fujikuroi*. The fungal isolates were subcultured on potato dextrose agar and DNA was extracted using the Qiagen DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The *FUM1* gene was detected using three different primer pairs, namely Fm1/Fm2 (Proctor *et al*., 1999), FUM1-F/FUM1-R (Bluhm *et al*., 2004), and FUM1P2-F/ FUM1P2-R (Jurado *et al*., 2010). Amplification of the *FUM1* gene using the Fm1/Fm2 primer pair was carried out in a 20 µL reaction mixture containing 2 µL of 10× PCR buffer, 0.6 mM $MgCl_2$, 0.04 mM each dNTP (Promega, Madison, WI, USA), 1.0 units of GoTaq® DNA polymerase (Promega), 1.0 µM each primer, and 20 ng of genomic DNA. The PCR cycles were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s; and a final extension at 72 °C for 5 min.

For the FUM1-F/FUM1-R primer pair, 25 µL reaction mixtures were prepared containing 2.5 µL of 10× PCR Buffer, 1.5 mM $MgCl_2$, 0.1 mM each dNTP (Promega), 0.625 units of GoTaq® DNA polymerase (Promega), 0.9 µM each primer, and 50 ng of genomic DNA. The PCR cycles were as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at 60 °C for 50 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min.

For the FUM1P2-F/FUM1P2-R primer pair, PCR was performed in a 25 µL reaction mixture containing 2.5 µL of 10× PCR Buffer, 0.5 mM $MgCl_2$, 0.04 mM dNTP (Promega), 2.5 units of GoTaq® DNA polymerase (Promega), 1.0 µM each primer, and 60 ng of genomic DNA. The PCR cycles were as follows: initial denaturation at 94 °C for 85 s; 35 cycles of denaturation at 95 °C for 35 s, annealing at 64 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min.

Amplification was performed in a Peltier thermal cycler (PTC-100®; MJ Research, Inc., Deltona, FL, USA). Electrophoresis was performed on 1.5% agarose gels for 90 min at 90 V and 400 mA for the PCR products generated from the Fm1/Fm2 and FUM1-F/FUM1-R primer pairs and on a 5% agarose gel for the PCR products generated from the FUM1P2-F/FUM1P2-R primer pair. The gels were then stained with ethidium bromide. The size of the PCR fragment was estimated based on a comparison with a 100 bp DNA ladder (GeneRulers™; Fermentas, Waltham, MA, USA) for Fm1/Fm2 and FUM1-F/FUM1-R, and an ultra-low range DNA ladder (GeneRulers™) for FUM1P2-F/FUM1P2-R.

In vitro mycotoxin production

In vitro mycotoxin production was assessed using ultra-performance liquid chromatography (UPLC) based on the methods described by Logrieco *et al*. (1998). Clean corn grit was placed in 250 mL Erlenmeyer flasks, adjusted to approximately 45% moisture with distilled water, autoclaved at 121 °C for 1 h, and allowed to stand overnight. A spore suspension (1×10^7 conidia/mL) of each isolate of *F. proliferatum* and *F. fujikuroi* was prepared from a 7-day-old culture in sterile distilled water. Each flask was evenly inoculated with 2 mL of the spore suspension and incubated in the dark at 25 ± 1 °C for 30 days. The control was treated with sterile distilled water instead of the fungal spore suspension. All treatments were performed twice. To distribute the inoculum evenly, each flask was shaken once or twice daily for 3 days. After 1 month of incubation, each inoculated flask was subjected to mycotoxin analysis.

Fumonisin B_1 analysis

The procedure described by Holcomb and Thompson (1996) was adopted for FB_1 extraction. Ten grams of the inoculated corn grit was extracted with 40 mL of acetonitrile: water (50:50, v/v). The homogenized mixture was then shaken for 1 h at 200 r.p.m using a Certomat® orbital shaker (B-Braun, Melsungen, Germany), blended for approximately 10 min, and filtered through Whatman no. 4 filter paper. The filtrate was defatted twice with 40 mL of hexane and separated using a separatory funnel. The bottom layer was allowed to evaporate to dryness at 60 °C using a rotary evaporator (Eylea Rotary Evaporator N-100 with an Eyela Digital Water Bath SB-100). The residue was completely dissolved in 1 mL of methanol for purification.

The FB_1 purification procedure was conducted according to the method described by Rottinghaus *et al*. (1992). One milliliter of the sample (FB_1 extract) was added to 2.5 mL of 1% potassium chloride (KCl) and applied to a solid-phase extraction (SPE) column (Supelco; Sigma-Aldrich, St Louis, MO, USA). The column was preconditioned with methanol (5 mL) and a 1% KCl solution (5 mL) before sample application. The column was then washed with a 1% KCl solution (3 mL), followed by 2 mL of an acetonitrile:1% KCl (10:90, v/v) mixture. FB_1 was eluted from the column with 2 mL of acetonitrile: water (50:50, v/v) for pre-column derivatisation.

The FB_1 standard was purchased from Sigma-Aldrich. A standard stock solution (1,000 µg/g) was prepared in 1 mL of acetonitrile: water (50:50, v/v) and stored at 4 °C until use. Five different concentrations (20, 40, 60, 80, & 100 µg/g) of the working standard solutions were prepared in acetonitrile:water (50:50, v/v) for pre-column derivatization.

One milliliter of methanol was added to 40 mg of ortho-phthaldialdehyde (OPA) reagent. The solution was diluted with 5 mL of 0.1 M disodium tetraborate ($Na_2B_4O_7$) and 50 µL of 2-mercaptoethanol

(Shephard *et al*., 1990). The mixture was stored and kept stable for up to 1 week at 25°C in the dark, capped, or in an amber vial. The OPA reagent (800 μL) was added to the sample (purified FB_1 extract) or standard solution (200 μL), mixed well, and filtered into a 12 × 32 mm amber glass screw neck vial (Waters, Milford, MA, USA) using a 0.2 μm polytetrafluoroethylene (PTFE) hydrophobic filter (Dismic®-13 JP; Advantec , Japan) After 3 min, 10 μL of the reaction mixture was injected into the UPLC column.

A UPLC system equipped with a Waters Acquity UPLC® binary pump connected to a Waters Acquity UPLC® fluorescence detector with emission and excitation wavelengths of 440 and 335 nm, respectively, was used to detect FB_1. Chromatographic separations were conducted on a C_{18} reversed-phase column (2.1 × 100 mm, 1.7 μm; Waters). Acetonitrile:water (50:50, v/v) was used as the mobile phase after filtration through a 0.2 μm nylon transfer membrane (Amersham, Amersham, UK) at a flow rate of 0.2 mL/min. The total run time was 4 min. FB_1 quantification was determined by peak height measurements and comparison with reference standard solutions. A calibration curve of the FB_1 standard was constructed to quantify FB_1 in the isolates of *F. proliferatum* and *F. fujikuroi*.

Moniliformin analysis

Moniliformin was extracted, as described by Munimbazi and Bullerman (1998). Approximately 15 g of inoculated corn grit was added to 50 mL of 1% tetrabutylammonium hydrogen sulfate. The mixture was shaken for 1 h at 200 rpm using a Certomat® orbital shaker, blended for approximately 10 min, and filtered through the Whatman No. 4 filter paper. The filtrate was defatted with 40 mL of n-hexane and separated using a separatory funnel. The bottom layer was collected, gently mixed with 50 mL of dichloromethane, and separated using a separatory funnel. This extraction step was repeated with 50 mL of dichloromethane. The two dichloromethane extracts were combined and evaporated to dryness at 60°C using a rotary evaporator (Eyela Rotary Evaporator N-100, Eyela Digital Water Bath SB-100). The residue was then dissolved in 1 mL of double-distilled water for clean-up. MON was purified as described by Munimbazi and Bullerman (1998). A disposable SPE column (Supelco) was fitted to the end of a 10 mL syringe or the port of a vacuum manifold. The column was conditioned successively with 1 mL of methanol, 1 ml of double-distilled water, and 1 mL of 0.1 M ortho-phosphoric acid (H_3PO_4). The extracted residue was loaded onto an SPE column. Once all of the extract had passed through the column, 1 mL of double-distilled water was added to the column for washing. MON was eluted with 1 mL of 0.05 M sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$; pH 5). The eluate was filtered through a 0.2 μm PTFE hydrophobic filter (Dismic®-13 JP) into a 12 × 32 mm glass screw neck vial (Waters) and maintained at 4 °C until UPLC analysis.

The MON standard was purchased from Sigma-Aldrich. A standard stock solution (1,000 μg/g) was prepared in 1 mL of 0.05 M $NaH_2PO_4.H_2O$ (pH 5). Using the standard stock solution, different concentrations of MON, ranging from 20 to 100 μg/g, were prepared by dilution with 0.05 M $NaH_2PO_4.H_2O$ (pH 5). The standard solution was then filtered through a 0.2 μm PTFE hydrophobic filter (Dismic®-13 JP) into a 12 × 32 mm glass screw neck vial (Waters) and stored at 4 °C until UPLC analysis.

Ten microlitres of purified MON was analyzed using a UPLC comprising a Waters Acquity UPLC® binary pump connected to a Waters Acquity UPLC® photodiodiode array detector set at 229 nm. Chromatographic separations were performed on a C_{18} reversed-phase column (2.1 × 100 mm, 1.7 μm; Waters). Acetonitrile:water (50:50, v/v) was used as the mobile phase after filtration through a 0.2 μm nylon transfer membrane (Amersham) at a flow rate of 0.2 mL/min. The total run time was 3 min. MON was quantified by comparing the peak heights of the samples with the calibration curves of the standards. A calibration curve of the MON standard was generated to quantify MON, and the retention time of MON was maintained at 1.00 min for the standards and samples of *F. proliferatum* and *F. fujikuroi.*

Beauvericin analysis

BEA was extracted according to the procedures described by Kostecki *et al*. (1999) and Waskiewicz *et al*. (2009). Fifteen grams of inoculated corn grit from each isolate was homogenized with 75 mL of acetonitrile:methanol:water (16:3:1, v/v/v) and allowed to stand overnight. The mixture was then shaken for 1 hr at 200 rpm using a Certomat® orbital shaker, before blending for approximately 10 min and filtering through Whatman no. 4 filter paper. The filtrate was defatted twice with 25 mL of heptane using a separatory funnel, and the bottom layer was evaporated to dryness at 60 °C using a rotary evaporator (Eyela Rotary Evaporator N-100 with an Eyela Digital water bath SB-100). The residue was evenly dissolved in 50 mL of methanol:water (55:45, v/v) and extracted twice with 25 mL of dichloromethane. The lower aqueous phase containing BEA was evaporated to dryness at 60 °C. The residue was then dissolved in 1 mL of methanol for clean-up.

The clean-up procedure was performed according to the method described by Krska *et al*. (1997). A Discovery® SPE column (Supelco) was used after preconditioning with 2 × 1 mL of chloroform. One milliliter of the sample (BEA extract) was passed through the column. The column was washed with 4 × 1 mL of chloroform, followed by 2 × 1 mL of chloroform:methanol (99:1, v/v). BEA was eluted with 2 mL of chloroform:methanol (98:2, v/v). The eluate was evaporated to dryness at 60 °C, re-dissolved in

1 mL of methanol, filtered through a 0.2 μm PTFE hydrophobic filter (Dismic®-13 JP) into a 12 × 32 mm glass screw neck vial (Waters), and stored at 4 °C until UPLC analysis.

The BEA standards were purchased from Sigma-Aldrich. A standard stock solution (5,000 μg/g) was prepared in 5 mL of methanol and stored at 8 °C until use. Five different concentrations (20, 40, 60, 80, & 100 μg/g) of working standard solutions were prepared in methanol and filtered through a 0.2 μm PTFE hydrophobic filter (Dismic®-13 JP) into a 12 × 32 mm glass screw neck vial (Waters) and kept at 4 °C until analyzed using UPLC.

BEA was quantified using a UPLC system consisting of a Waters Acquity UPLC® binary pump connected to a Waters Acquity UPLC® photodiode array detector (set at 205 nm). Chromatographic separations were carried out on a C_{18} reversed-phase column (2.1 × 100 mm, 1.7 μm; Waters). Acetonitrile:water (90:10, v/v) was used as the mobile phase after filtration through a 0.2 μm nylon transfer membrane (Amersham). The total run time was 4 min and the flow rate was maintained at 0.2 mL/min. Ten microlitres of purified BEA was injected for each run and BEA was detected by comparing the retention time and UV spectrum against an authentic BEA standard. A calibration curve of the BEA standard was generated and BEA was quantified in the isolates by comparing the peak height of BEA with the calibration curve. The retention time of BEA was identified at 2.00 min for the standards and samples of both *F. proliferatum* and *F. fujikuroi.*

Statistical analysis

The mean mycotoxin concentrations were compared among the tested isolates. For each mycotoxin, the mean and standard deviation were calculated using Microsoft Excel 11. The concentration range of each mycotoxin is also presented.

RESULTS AND DISCUSSION

The results showed that all isolates of *F. proliferatum* and *F. fujikuroi* produced single fragments of 800, 183, and 64 bp using the Fm1/Fm2, FUM1-F/FUM1-R, and FUM1P2-F/FUM1P2-R primer pairs, respectively. This PCR fragment was not detected in the control (Figure 1, 2 & 3).

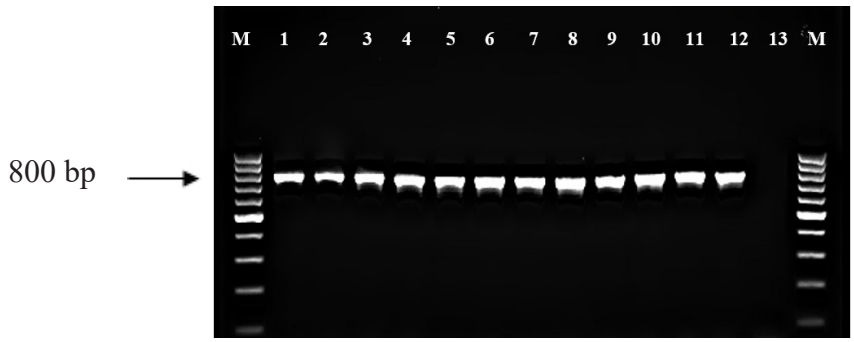

Fig. 1. PCR fragments of *FUM1* gene using Fm1/Fm2 primer of several isolates of *F. proliferatum* and *F. fujikuroi*. (M = 100 bp DNA ladder; Lane 1 = P4029π; 2 = P4042π; 3 = A4694π; 4 = A4705π; 5 = B4711π; 6 = B4727π; 7 = N5354π; 8 = N5359π; 9 = M9030π; 10 = M9046π; 11 = J9067π; 12 = J9098π; 13 = Control)

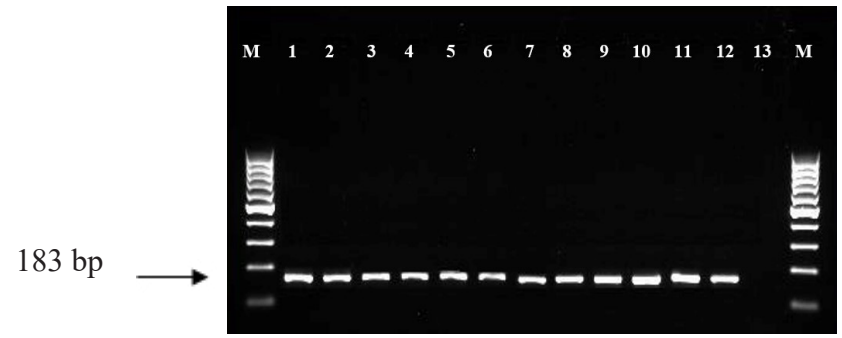

Fig. 2. PCR fragments of *FUM1* gene using FUM1-F/FUM1-R primer of several isolates of *F. proliferatum* and *F. fujikuroi*. (M = 100 bp DNA ladder; Lane 1 = D9110π; 2 = D9111π; 3 = Q9077π; 4 = Q9136π; 5 = S9060π; 6 = S9169π; 7 = K9192π; 8 = K9197π; 9 = P4093π; 10 = P4671π; 11 = A4708π; 12 = A9141π; 13 = Control)

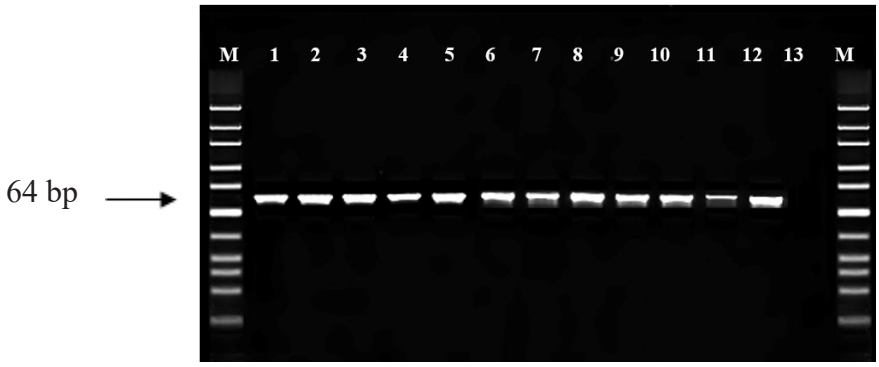

Fig. 3. PCR fragments of *FUM1* gene using FUM1P2-F/FUM1P2-R primer of several isolates of *F. proliferatum* and *F. fujikuroi*. (M = Ultra low range DNA ladder; Lane 1 = P4682π; 2 = A4697π; 3 = B4713π; 4 = N9006π; 5 = N9004π; 6 = N9008π; 7 = M9036π; 8 = J9094π; 9 = J9172π; 10 = D9112π; 11 = D9125π; 12 = 9158π; 13 = Control)

Mycotoxin production by *F. proliferatum* and *F. fujikuroi* isolates from stem rot of *H. polyrhizus* is summarised in Table 2. All tested isolates showed the ability to produce FB_1, MON, and BEA. The production of FB_1, MON, and BEA differed among the isolates, indicating differences in the regulation of mycotoxins and intraspecific variability among the isolates (Gálvez *et al*., 2011; Gálvez *et al*., 2017). According to Rabaaoui *et al*. (2021), *Fusarium* species are characterized by a wide range of interspecific and intraspecific genetic variations, which can also be observed in their mycotoxin profiles.

Table 2. Mycotoxin production by *F. proliferatum* and *F. fujikuroi* isolates recovered from stem rot of *H. polyrhizus*

Isolate number	*Fusarium* species	[a]Mycotoxin concentration (μg/g)		
		BEA	FB_1	MON
P4029π	*F. proliferatum*	37.05 ± 6.16	28.33 ± 8.72	43.72 ± 8.68
P4093π	*F. proliferatum*	2.70 ± 0.13	37.83 ± 6.77	11.92 ± 4.37
A4705π	*F. proliferatum*	3.35 ± 0.42	17.33 ± 8.43	4.05 ± 2.13
A9141π	*F. proliferatum*	2.65 ± 0.55	47.98 ± 8.78	4.06 ± 1.56
B4727π	*F. proliferatum*	2.88 ± 0.56	44.64 ± 7.78	2.52 ± 1.98
N5354π	*F. proliferatum*	0.97 ± 0.04	103.89 ± 9.14	1.68 ± 0.58
N9004π	*F. proliferatum*	1.05 ± 0.07	62.26 ± 7.38	4.16 ± 1.31
N9028π	*F. proliferatum*	0.88 ± 0.11	80.31 ± 8.82	2.37 ± 0.99
M9030π	*F. proliferatum*	24.43 ± 5.13	42.12 ± 5.12	0.48 ± 0.04
M9036π	*F. proliferatum*	1.32 ± 0.42	29.41 ± 4.24	3.74 ± 0.57
J9098π	*F. proliferatum*	1.34 ± 0.42	111.88 ± 8.31	69.55 ± 9.39
J9172π	*F. proliferatum*	1.95 ± 0.17	30.10 ± 2.42	3.27 ± 0.67
D9111π	*F. proliferatum*	3.59 ± 0.11	56.20 ± 8.56	8.95 ± 0.98
D9112π	*F. proliferatum*	0.94 ± 0.09	31.34 ± 4.56	2.88 ± 0.15
Q9020π	*F. proliferatum*	25.62 ± 4.13	76.76 ± 6.55	0.76 ± 0.07
Q9077π	*F. proliferatum*	2.50 ± 1.32	82.52 ± 8.14	35.92 ± 9.18
Q9104π	*F. proliferatum*	4.32 ± 0.67	38.45 ± 3.99	125.35 ± 9.22
S9060π	*F. proliferatum*	4.81 ± 0.13	236.80 ± 8.95	29.69 ± 7.12
S9073π	*F. proliferatum*	1.81 ± 0.84	66.68 ± 5.45	6.07 ± 2.56
K9192π	*F. proliferatum*	2.73 ± 0.43	25.77 ± 5.12	12.07 ± 4.13
K9199π	*F. proliferatum*	1.40 ± 0.06	14.94 ± 2.35	1.95 ± 0.13
K9202π	*F. proliferatum*	2.10 ± 0.21	103.59 ± 9.12	6.62 ± 1.37
P4042π	*F. fujikuroi*	37.05 ± 0.62	28.33 ± 3.64	120.87 ± 7.21
P4671π	*F. fujikuroi*	0.68 ± 1.55	48.50 ± 6.23	13.44 ± 0.67
P4682π	*F. fujikuroi*	7.32 ± 3.24	25.37 ± 4.56	171.56 ± 9.23
A4694π	*F. fujikuroi*	2.01 ± 0.48	23.18 ± 3.21	6.11 ± 1.56
A4697π	*F. fujikuroi*	0.32 ± 0.19	100.55 ± 7.45	5.41 ± 0.48
A4708π	*F. fujikuroi*	3.14 ± 0.13	17.07 ± 3.12	5.56 ± 0.77
B4711π	*F. fujikuroi*	23.45 ± 0.21	58.48 ± 6.98	157.36 ± 9.23
B4713π	*F. fujikuroi*	0.28 ± 0.10	82.65 ± 5.87	9.96 ± 2.14
N5359π	*F. fujikuroi*	2.25 ± 0.32	126.64 ± 8.62	4.81 ± 0.34
N9006π	*F. fujikuroi*	10.48 ± 1.17	24.11 ± 5.44	2.74 ± 0.44
M9046π	*F. fujikuroi*	1.57 ± 0.02	100.80 ± 6.43	58.03 ± 7.24
J9067π	*F. fujikuroi*	8.23 ± 0.91	15.38 ± 2.01	173.47 ± 9.34

Table 2. continued...

J9094π	*F. fujikuroi*	0.66 ± 0.06	35.53 ± 5.23	5.63 ± 1.44
D9110π	*F. fujikuroi*	4.52 ± 0.24	26.90 ± 2.34	128.83 ± 8.76
D9125π	*F. fujikuroi*	70.02 ± 5.30	50.32 ± 5.19	142.33 ± 5.43
Q9136π	*F. fujikuroi*	3.42 ± 0.21	128.63 ± 8.28	163.46 ± 5.64
Q9158π	*F. fujikuroi*	4.57 ± 0.14	29.08 ± 3.12	173.07 ± 5.77
S9164π	*F. fujikuroi*	1.65 ± 0.03	28.25 ± 2.14	63.63 ± 8.11
S9169π	*F. fujikuroi*	21.53 ± 4.56	11.97 ± 1.26	172.22 ± 7.19
S9178π	*F. fujikuroi*	2.83 ± 0.07	60.24 ± 5.76	174.84 ± 6.92
S9181π	*F. fujikuroi*	2.75 ± 0.12	122.04 ± 8.23	6.19 ± 2.01
K9197π	*F. fujikuroi*	0.86 ± 0.02	29.09 ± 3.44	4.70 ± 0.12

[a]Values are presented as mean ± standard deviation.

An association between the presence of the *FUM1* gene and FB_1 production was observed in all isolates tested. The results indicated that all isolates with the *FUM1* gene could produce FB_1, although the concentrations varied. Genes from the *FUM* cluster, especially *FUM1*, are useful markers of fumonisin production ability by *Fusarium* species (Jurado *et al*., 2010; Stepien *et al*., 2011). The detection of marker genes of the fumonisin metabolic pathway is considered a valid approach for detecting toxigenic fungi (Paterson, 2006) and determining their potential for mycotoxin production.

Fusarium proliferatum and *F. fujikuroi* isolates produced FB_1 at various concentrations ranging from 11.97 to 236.80 μg/g (Table 2). FB_1 was not detected in the control corn grit. The isolate of *F. proliferatum* from Sabah (S9060π) produced the highest concentration of FB_1 (236.80 μg/g), whereas the lowest concentration of FB_1 was produced by *F. fujikuroi* (S9169π) also from Sabah (11.97 μg/g).

According to Nelson *et al*. (1991), FB_1 producers can be categorized as low, intermediate, or high producers, based on the FB_1 concentrations. Low, intermediate, and high concentrations are defined as < 50 μg/g, 50–500 μg/g, and > 500 μg/g, respectively. Most isolates of *F. proliferatum* and *F. fujikuroi* were categorized as low or intermediate producers, as 25 isolates (12 isolates of *F. proliferatum* & 13 isolates of *F. fujikuroi*) produced < 50 μg/g FB_1 and 19 isolates (10 isolates of *F. proliferatum* & nine isolates of *F. fujikuroi*) produced 50.32–236.80 μg/g FB_1. None of the tested isolates were high FB_1 producers.

The production of FB_1 by *F. proliferatum* in the present study was generally lower than the level of production previously reported for *F. proliferatum* isolates recovered from other crops, such as asparagus (10–11,499 μg/g; Liu *et al*., 2007), onion (8.61–882.97 μg/g; Dissanayake *et al*., 2009), and maize (248-6,050 μg/g; Reyes-Velazquez *et al*., 2011). This may be because these isolates were recovered from different host plants and different geographical areas.

Fusarium fujikuroi isolates produced 11.97–128.63 μg/g FB_1, which was also lower compared to the amount of FB_1 produced by *F. fujikuroi* isolates from rice (0.386–223.83 μg/g; Cruz *et al*., 2013). Various concentrations of FB_1 produced by *F. fujikuroi* isolates have been reported in other studies, including those of *F. fujikuroi* from rice (Cruz *et al*., 2013).

FB_1 is known to have a phytotoxic effect on plants and can cause physiological damage; growth inhibition; and eventually, plant death (Van Asch *et al*., 1992; Abbas *et al*., 1995; Danielsen *et al*., 1998). Thus, FB_1 produced by *F. proliferatum* and *F. fujikuroi* may play a role in the development of stem rot disease in *H. polyrhizus*. On other crops, such as maize and tomato seedlings, low concentrations of FB_1 may lead to necrosis and other disease symptoms (Lamprecht *et al*., 1994). An association between fumonisin production and high virulence in maize seedlings was reported by Desjardins *et al*. (1995), based on a preliminary genetic study of *G. fujikuroi* mating population A.

Isolates of *F. proliferatum* and *F. fujikuroi* produced MON at concentrations ranging from 0.48 to 174.84 μg/g (Table 2). MON was not detected in the control corn grit. *Fusarium proliferatum* isolate Q9104π produced the highest concentration of MON (125.35 μg/g), whereas the lowest concentration of MON (0.48 μg/g) was produced by *F. proliferatum* isolate M9030π. For *F. fujikuroi*, the highest concentration of MON was produced by isolate S9178π (174.84 μg/g), while the lowest MON concentration was produced by isolate N9006π (2.74 μg/g).

Ten isolates of *F. fujikuroi* produced higher concentrations of MON (> 100 g/g) than the amount produced by *F. proliferatum,* whereas only one isolate produced > 100 g/g of MON. MON is also phytotoxic (Cole *et al*., 1973) but less toxic than FB_1. Similar to FB_1, MON produced by *F. proliferatum* and *F. fujikuroi* may play a role in stem rot development in *H. polyrhizus*. The phytotoxic effects of MON have been reported by Wakulinski (1989). An inhibitory effect of MON has been reported in the leaves and roots of developing wheat seedlings, in which a significant decrease in leaf mass was observed. In duckweed (*Lemna minor*), MON significantly reduces plant growth (Vesonder *et al*., 1992). In subsequent studies, MON was found to play a role in several plant diseases, including the wilt and dieback of ornamental palms (Armengol *et al*., 2005), the wilt of date palms (Abdalla *et al*., 2000), ear rot in maize (Logrieco *et al*., 2002), bulb rot in onion and garlic (Stankovic *et al*., 2007), and head blight

in cereal grains (Bottalico & Perrone, 2002).

BEA production by *F. proliferatum* and *F. fujikuroi* isolates from stem rot of *H. polyrhizus* was relatively low compared to the production of FB_1 and MON, ranging from 0.28 to 70.02 μg/g. BEA was not detected in the control corn grit. The production levels of BEA ranged from 0.88 to 37.05 μg/g for *F. proliferatum* isolates. For *F. fujikuroi* isolates, BEA levels ranged from 0.28 to 70.02 μg/g (Table 2). *Fusarium proliferatum* isolate P4029π and *F. fujikuroi* isolate D9125π showed the highest concentrations of BEA, while *F. proliferatum* isolate N9028π and *F. fujikuroi* isolate B4713π showed the lowest concentrations of BEA. Although produced at low concentrations, this mycotoxin should be regarded as important, as it has been reported to have phytotoxic effects. BEA has been found to affect the viability of tomato protoplasts (Paciolla *et al*., 2004), as well as membrane permeability and ion transport in corn roots (Pavlovkin *et al*., 2006). It has also been associated with diseased onions (Stankovic *et al*., 2007) and date palms (Abdalla *et al*., 2000). Thus, as for FB_1 and MON, BEA may be involved in the development of stem rot disease in *H. polyrhizus.*

CONCLUSION

FUM1 was detected in 44 isolates of *F. proliferatum* and *F. fujikuroi* isolated from stem rot of *H. polyrhizus* in Malaysia, and these isolates were capable of producing FB_1, MON, and BEA at variable concentrations. These mycotoxins may play a role in the development of stem rot disease, as all isolates were pathogenic and caused stem rot in *H. polyrhizus.* Further studies are necessary to determine the association between these mycotoxins and disease development, and particularly to evaluate the synergistic effects of these three mycotoxins on host plants. Further studies are also needed to investigate the occurrence of mycotoxins in fruits, to evaluate the risk of human consumption of affected plants.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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