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Identification of Volatile Metabolites from Fungal Endophytes with Biocontrol Potential towards *Fusarium oxysporum* F. sp. *cubense* Race 4

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Abstract: Problem statement: Fungal endophytes are widely studied for their potential as biocontrol agents towards fungal pathogens. *In vitro* assessments usually reveal their antibiosis and mycoparasitism nature, but little is understood regarding their production of volatile metabolites as mechanisms of antagonism. **Approach:** This study explored the potential of fungal endophytes in controlling the pathogen responsible for Fusarium wilt disease. Nine fungal endophytes were tested for their ability to inhibit the growth of the pathogenic *Fusarium oxysporum* F. sp. *cubense* race 4 (FocR4) via production of volatile inhibitory metabolites. The type of volatile metabolites produced were subsequently characterized and identified using the Gas-Chromatography Mass-Spectrophotometry (GCMS). **Results:** Eight of the isolates (BTF05, BTF07, BTF08, BTF15, BTF21, WAA03, WAA02, MIF01) showed positive results with percentages of inhibition varying from 1.43-31.43% while one isolate (ALF01), showed negative result (0% inhibition). Volatile profiles showed that these fungal endophytes produced between 15-47 volatile metabolites per isolate. However, the more volatile metabolites produced by a single endophyte does not indicate better biocontrol potential. Isolate BTF05 produced 47 different volatile metabolites, but has only 8.57% inhibition, compared to isolate BTF21 with 15 metabolites but a percentage of 11.43% inhibition. The potency of the volatile metabolites produced may also influenced the biocontrol potential of the fungal endophytes as some isolates such as BTF08 and MIF01 have only two to three known inhibitory metabolites but have higher PIDG values at 31.43 and 11.43%, respectively. Contrary, isolates WAA02 and WAA03 which has five to six metabolites but PIDG values of less than 3%. **Conclusion:** Fungal endophytes have the ability to produce several types of volatile metabolites to inhibit the growth of FocR4. These volatile inhibitory metabolites can be further extracted and the amount produced ascertained for future manipulation in biological control of FocR4.

Key words: Endophytes, Fusarium wilt, gas-chromatography mass-spectrophotometry, volatile metabolites

INTRODUCTION

Endophytes are most often isolated from symptomless plants of various species (McInroy and Kloepper, 1995). Their association with host plants are known to improve plant growth and vigour (Ting *et al*., 2008), enhance plant nutrient absorption (Chanway, 1996) and to potentially confer disease resistance in plants against pathogen infection (Ting *et al*., 2007). Similar to other soil-borne biocontrol agents, endophytes also produce the following biocontrol activity-production of antimicrobial compounds, competition for colonizing sites and nutrients and stimulation of host defenses; as their mechanisms of inhibition towards various pathogens (Benhamou *et al*., 2000; Larkin *et al*., 1996; Pleban *et al*., 1995).

Of the many mechanisms of pathogen inhibition, production of antimicrobial compounds is the most easily detected and widely studied. Antimicrobial compounds are produced in either volatile or non-volatile forms (Brooks *et al*., 1994; Cazar *et al*., 2005; Chanway, 1996; Chaurasia *et al*., 2005). The non-volatile compounds are often detected using simple plate assay (Chaurasia *et al*., 2005) and the filtrates can be easily extracted, purified and characterized

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(Liu *et al*., 2007). As a result, many non-volatile inhibitory compounds from fungal endophytes have been identified. This includes viridiofungin A secreted by *Trichoderma harzanium* (El-Hasan *et al*., 2009), production of hydrogen peroxide by glucose oxidase enzyme of *Talaromyces flavus* (Naraghi *et al*., 2008) and aflatoxin secreted by *Aspergillus flavus* (Adebanjo and Bankole, 2004).

Contrary, the extraction, characterization and identification of volatile compounds produced by fungal endophytes can only be studied using more sophisticated means, such as with the use of Gas-Chromatography Mass-Spectrophotometry (GC-MS) (Strobel *et al*., 2001). As such, studies on beneficial inhibitory volatile metabolites are relatively new (Chen *et al*., 2008; El-Hasan *et al*., 2009; Fernando *et al*., 2005). Nevertheless, the role of volatile inhibitory compounds in biological control remains to be investigated and may have tremendous potential as the volatile compounds can be entrapped or immobilized in bioformulations and manipulated for applications in the field or for post-harvest disease management (misting, spraying and droplets). Applications using volatile metabolites also exclude the introduction of viable cells, eliminating the unnecessary or accidental introduction of foreign microbes into the environment

In present study, we identified the different types of volatile compounds produced by the fungal endophytes and distinguished between volatiles which may have inhibitory effect towards the fungal pathogen *Fusarium oxysporum* F. sp. *cubense* race 4 (FocR4). Comparisons to literatures also enabled the identification of several compounds, which are established antifungal compounds. The profile of volatile compounds produced by these fungal endophytes can then be used for possible selection and manipulation in biological control implementations in the future.

MATERIALS AND METHODS

Isolate preparation: Nine fungal endophytes (BTF05, BTF07, BTF08, BTF15, BTF21, WAA02, WAA03, MIF01 and ALF01) used in this study were previously isolated from the stem tissues of *Musa* spp. (BTF05, BTF07, BTF08, BTF15, BTF21), grass weeds (WAA02, WAA03), *Mimosa pudica* (MIF01) and *Allamanda* spp. (ALF01). Isolation was performed using methods by (Hallmann *et al*., 1997). The pure cultures obtained were maintained on Potato Dextrose Agar (PDA) (Merck) at room temperature (28±3°C). The fungal endophytes were identified to their genus level by morphological characterization using slide cultures. The fungal pathogen *Fusarium oxysporum* F. sp. *cubense* race 4 (FocR4) from VCG 01213/16 (Bentley *et al*., 1998) was obtained from Prof. Dr. Sariah Meon from University Putra Malaysia as cultures established on PDA.

Detection of inhibitory activity by volatiles produced by fungal endophytes: Double-plate assay was performed to determine the inhibitory potential of fungal endophytes towards FocR4 via production of volatile inhibitory metabolites. The fungal cultures (including FocR4) were first established as pure cultures on PDA plates for 5 days. The PDA plates containing FocR4 were then placed inversely over the plates cultivated with fungal endophytes. The cover lids for the petri dishes were removed and both plates were then sealed together with parafilm. The plates were incubated at room temperature (28±3°C) for 7 days. For control, plates containing fungal endophytes were inoculated instead with plain agar plugs. Growth inhibition of FocR4 by volatiles produced by fungal endophytes was assessed based on the Percentage of Inhibition of Diameter Growth (PIDG). The PIDG values were observed for 7 days and calculated as follows (Trivedi *et al*., 2008):

$$\text{PIDG}(\%) = \frac{\text{D1} - \text{D2}}{\text{D1}} \times 100\%$$

Where:

D1 = Diameter growth of FocR4 exposed to plain agar plug (control)

D2 = Diameter growth of FocR4 exposed to fungal endophytes

The plate assay was conducted in a complete randomized design, with three replicates and repeated once.

Detection of volatile compounds produced: The fungal endophytes were first established as pure cultures on PDA. After 7 days of incubation at room temperature, the volatiles produced by the fungal endophytes were collected using the Solid-Phase Micro-Extraction (SPME) technique (Strobel *et al*., 2001; Ting *et al*., 2007; 2008; Trivedi *et al*., 2008; Wan *et al*., 2008). The SPME technique detects all types of volatile compounds produced at the headspace above the fungal cultures and do not discern between inhibitory and non-inhibitory volatiles. To collect the volatile compounds, a small hole, adequate to accommodate the size of the SPME syringe was first gently forced into the side of the Petri

dish using a sterile needle. The SPME syringe (Supelco), with divinylbenzene/carburen on polydimethylsiloxane fiber material (50/30) (on a 65 µm stable flex fiber), was then inserted through the hole. The syringe was exposed to the volatiles in the headspace for 40 min, allowing volatiles to entrap to the fiber material. The sample in the syringe was then transferred to a GC-MS (Shimadzu GCMS QP2010) for analysis. Volatile samples were analyzed with the following conditions: 40°C for 2 min for initial temperature, gradually increasing to 150 at 20°C min^{-1} and then to 280°C at 100°C min^{-1} and finally programmed at 280°C for 2 min. Helium was used as the carrier gas with a flow velocity of 1 mL min^{-1}. For control, volatile compounds were collected from the headspace of a non-inoculated agar plate. Identification of the volatile compounds was performed by comparing with the National Institute of Standards and Technology (NIST) database on the mass spectrometer (Strobel *et al*., 2001; Ting *et al*., 2007; 2008; Trivedi *et al*., 2008; Wan *et al*., 2008). The GCMS analysis was repeated once.

RESULTS

Inhibition via production of volatile metabolites: Of the nine isolates tested, eight isolates showed inhibitory effect towards FocR4. Five of the eight isolates (BTF05, BTF07, BTF15, WAA02, WAA03) showed weak inhibitory effect towards FocR4 with PIDG values less than 10%, while three isolates (BTF08, BTF21 and MIF01) produced PIDG values more than 10%. The strongest inhibitory effect was produced by fungal endophyte BTF08 with 31.43% (Table 1). In addition, preliminary investigations based on the morphology of the isolates suggested that isolate BTF05 belonged to the class Basidiomycetes, BTF07 is presumptively a *Geotrichum* spp., BTF08 and BTF15 as *Penicillium* spp., BTF21 as a *Botrytis* spp. and MIF01 as a *Cladosporium* spp. The isolates WAA02 and WAA03 were unidentified as these isolates did not produce sporing structures on PDA.

Volatile compounds produced by fungal endophytes: The fungal endophytes produced a variety of volatile metabolites, ranging from 15-47 different volatile metabolites per isolate. To determine potential inhibitory volatile compounds, comparisons were made between the volatile profiles of the fungal endophytes with profiles from control and ALF01 to eliminate non-inhibitory volatiles. An average of six compounds were detected from both profiles of control and fungal endophytes. These compounds were eliminated, as they were considered as compounds non-induced by fungal endophytes. This includes mostly diethyl phthalate, hexane, 2-propanone 1-(1-methylethoxy), pentane 2-methyl, ethyl alcohol and ethyl ether with 29.16, 14.64, 10.45, 5.87, 5.75 and 3.34% peak area, respectively (results not shown). The fungal endophyte ALF01 has 23 compounds, of which two to five of the compounds were also detected in the profiles of other fungal endophytes with varying percentages of peak areas. These include nitrous oxide, 1,3,5-cycloheptatriene, 1,3,5,7-cyclooctratetraene, cyclopropane, 2,4,6-tris (1,1-dimethylethyl) and carbon dioxide (data not shown). These compounds were considered as non-inhibitory as well as their production by ALF01 did not inhibit the growth of FocR4.

As such, only less than half of the volatile metabolites produced by the fungal endophytes have the potential to inhibit FocR4 (Table 1). Results also showed that the more volatiles produced, does not necessary indicate a stronger inhibitory effect. Isolate BTF05 with 29 potential inhibitory volatiles, produced only 8.57% PIDG value. Contrary, isolate BTF08 with only 13 potential volatile inhibitors produced the highest PIDG value of 31.43% (Table 1). This showed that the type of volatiles produced by the isolates may have influenced the inhibitory effect towards FocR4.

Analysis on the volatile profiles showed that several volatile compounds were detected consistently in several isolates with inhibitory effect towards FocR4 (Table 2). The most common volatile compounds were butane 2-methyl, 1-butanol 3-methyl, 1,4-methano-1H-cyclopropa [d] pyridazine, β-butyrolactone and 2-butenedinitrile, found in the most number of fungal endophytes (Table 2). These compounds, except for 1-butanol 3-methyl, were rather potent as with peak areas of less than 10%, they were able to inhibit the growth of FocR4 effectively (up to 31.43% PIDG) (Table 2). Several other compounds known to have antifungal properties were also detected in the profiles of the endophytes. These compounds include glycidol, 2-acetyl-5-methylfuran, acetic acid pentyl ester, 1-propanol 2-methyl, 1-butanol 2-methyl, furan 2-methoxy and α-phellandrene (Table 2). Glycidol and 2-acetyl-5-methylfuran were exclusive to isolate BTF05, while acetic acid pentyl ester and 1-butanol 2-methyl was produced only by isolates BTF07 and WAA03, respectively. The other compounds, 1-propanol 2-meythl, furan 2-methyl and α-phellandrene were detected in more than two isolates.

Analysis based on isolates and the number of compounds produced concur that the more volatiles produced does not necessary lead to better inhibition of FocR4. Isolates BTF08, BTF21 and MIF01 with PIDG

Table 1: Fungal endophytes with their respective PIDG (%) values and number of volatiles compounds detected according to each category

Culture	PIDG (%)	Total volatiles detected	No. of compounds detected in control	No. of compounds detected in ALF01	No. of unidentified compounds	Total potential inhibitory compounds
BTF05	8.57	47	7	4	7	29
BTF07	7.14	35	5	3	9	18
BTF08	31.43	25	5	5	2	13
BTF15	7.14	28	5	4	8	11
BTF21	11.43	15	6	2	0	7
WAA02	2.86	34	9	3	1	21
WAA03	1.43	25	7	2	0	16
MIF01	11.43	31	10	3	5	13
ALF01	0.00	23	6	NA	7	0
Control	NA	23	NA	6	2	0

Note: NA: Not Applicable

Table 2: Volatile compounds detected in the profiles of the respective fungal endophytes and their corresponding peak areas (%)

	Peak areas (%)							
Isolates	BTF05	BTF07	BTF08	BTF15	BTF21	WAA02	WAA03	MIF01
Compounds								
Butane 2-methyl	0.51	x	x	4.56	2.37	x	x	6.45
1-butanol, 3-methyl	9.90	21.24	1.40	x	x	14.47	1.04	x
1,4-methano-1H-cyclopropa[d] pyridazine	0.14	0.31	x	x	x	0.64	0.33	x
β-butyrolactone	x	x	4.77	4.96	3.19	0.54	x	x
2-butenedinitrile	x	x	2.05	1.22	1.88	x	2.19	x
glycidol	0.33	x	x	x	x	x	x	x
2-acetyl-5-methylfuran	1.68	x	x	x	x	x	x	x
acetic acid pentyl ester	x	1.08	x	x	x	x	x	x
1-propanol 2-methyl	2.76	3.76	x	x	x	x	x	3.61
1-butanol 2-methyl	x	x	x	x	x	x	2.95	x
Furan 2-methoxy	x	x	x	x	x	2.55	3.44	x
α-phellandrene	x	x	x	x	x	7.14	3.11	x

Note: x: Not detected

values more than 10%, each produced three, three and two known antifungal compounds, respectively. Contrary, isolates BTF05, WAA03 and WAA02 with six, six and five compounds, respectively, produced less PIDG values (Table 1 and 2).

DISCUSSION

The results from this study showed that fungal endophytes can produce a variety of volatile compounds. However, less than half of these compounds are of importance in biocontrol strategy as most of the volatiles are non-inhibitory in nature. The identification of potential inhibitory compounds for this study is exclusive to FocR4, thus cannot be generalized for the rest of the pathogens. Nevertheless, some of the volatile compounds detected in this study may have a broad-range of antifungal activity as they were reportedly inhibitory towards other pathogens as well. They include 1-butanol 3-methyl and 1-butanol 2-methyl produced by *Gliocladium* spp. which has inhibitory effect towards *Pythium ultimum* and *Verticillum dahliae* (Stinson *et al*., 2003). Butyrolactone was also produced by *Gliocladium roseum* (Strobel *et al*., 2001; 2008) and in *Aspegillus terreus* and has shown inhibitory effect towards the pathogen *Botrytis cinerea* (Cazar *et al*., 2005). Other volatile compounds such as α-phellandrene, acetic acid pentyl ester and 2-acetyl-5-methylfuran, have also been detected in *Penicillium roqueforti* (Jelen, 2002), *Sordoria fimricola* (Wihlborg *et al*., 2008) and *Aspergillus fumigatu* (Frisvad *et al*., 2009), respectively, but their antifungal properties are not well-established.

In addition, since we did not specifically extract and quantify the amount of each of the volatiles produced by the endophytes and their influence on FocR4, we were can only conclude that the presence of these volatiles may have a subsequent impact on the growth of FocR4. Their potency as volatile inhibitors cannot be gauge accurately, although peak area comparisons offered some preliminary understanding. For example, in this study, the volatile 1-butanol 3-methyl had peak areas of 21.24 and 14.47% in isolate BTF07 and WAA02, respectively, but their PIDG values were only 7.14 and 2.86%, respectively. Therefore, 1-butanol 3-methyl is considered as less potent compared to other volatile compounds such as

β-butyrolactone. Nevertheless, potency of volatile compounds is best investigated in a separate study where the individual compounds are elucidated and extracted and the concentration determined for a more accurate analysis.

CONCLUSION

To conclude, our study has shown that fungal endophytes have the ability to produce several types of volatile compounds to inhibit the growth of FocR4. We have also shown that some common antifungal compounds produced by other biocontrol agents are also produced by the fungal endophytes in this study. This suggested that these volatile inhibitory compounds can be exploited for the biological control of FocR4 and may be extended to other various pathogens as well.

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