Fungal Root Endophytes from *Musa* spp. as Biological Control Agents against the Plant Pathogen *Fusarium oxysporum*

Nikki Heherson A. Dagamac, Paolo G. Sogono, Rizza Celina B. Cabalfin, Andre Cedric Y. Adducul and Thomas Edison E. dela Cruz

1 Department of Biological Sciences, College of Science and 2 Research Center for the Natural Sciences University of Santo Tomas España, Manila 1015, Philippines

**Abstract.** Endophytic fungi as biological control agents pose a novel and promising alternative for plant disease management. Thus, our research study aimed to test whether locally isolated banana root endophytes (BRE) can be used as potential biocontrol agents against *Fusarium oxysporum* (Foc). Seventy-five BREs were isolated from roots samples of banana cultivars collected from different sites in Manila and Quezon City. High species richness was observed from the plants collected in Quezon City. Twenty-five selected isolates were screened for their antagonistic interactions against *F. oxysporum* using the dual-culture method, but only three of them inhibited the test fungus either on contact (BRE 18 & BRE 71) or at a distance (BRE 14). Morphocultural characterization identified them as belonging to the genus *Aspergillus*. Further antagonistic testing showed that the three BREs significantly reduced the colony radial growth of *F. oxysporum*, particularly when the BREs were grown 15 days prior to Foc inoculation. Extraction of secondary metabolites was done to test the antifungal properties of the three BREs. The crude culture extracts failed to inhibit the fungus using the paper disk diffusion assay, though they were able to remarkably decrease spore germination. Thus, the isolated banana root endophytes showed potential application for biological control.

**Keywords:** fungal root endophytes, biocontrol agent, antagonistic activities, *Musa* spp., *Fusarium oxysporum*, *Aspergillus* sp.

**INTRODUCTION**

Endophytic fungi occurred in almost all plants and were found in plant parts such as roots, shoots, leaves or stems where they formed internal localized infections [1, 2]. However, many were completely benign and/or may form mutually beneficial relationships with their host plants [3]. In some other cases, their occurrence in plant tissues either elicits no apparent effect in the normal functioning of infected plants, or may confer various benefits to their host, e.g. improved host resistance against herbivores or phytopathogens, increased host plant yield and plant growth, and/or induced plant toler-
ance to stress, heavy metals and drought [4, 5]. Thus, in recent years, endophytic fungal symbionts have been used as novel and promising biological agents for plant protection [6, 7, 8].

Banana is one of the major income-generating commodities in the Philippines [9]. At farm level alone, about 5.9 million Filipinos depend on banana as an important source of income [10]. However, the industry remains plagued by pests and diseases of microbial origin, e.g. the fungus *Fusarium oxysporum*, the causative agent of vascular wilt disease. The fungus infects the banana plant through its roots and further invades and blocks the plant’s water conducting tissues (xylem) resulting to leaf wilting, yellowing and eventually to plant death. Healthy plants can also be infected if grown in contaminated soil [11]. Effective option for managing this disease included the use of agrochemicals. However, excessive use of agrochemicals can lead to health hazards and environmental problems [9, 11, 12]. Other available control measures, e.g. solarization, employment of suppressive soil, use of resistant hybrids, are often costly [13, 14, 15]. Thus, the application of microbial endophytes as biological control agent poses a promising alternative for vascular wilt disease management.

Our research study, then, aimed to test whether locally isolated root endophytes from different banana varieties can be a potential biological agent against the phytopathogen *F. oxysporum*. Specifically, it aimed to isolate, characterize and identify endophytic fungi from the roots of *Musa* spp. and determine their antagonistic and inhibitory activities against *F. oxysporum*. It is hoped that the isolated fungal root endophytes be applied in the future as an alternative method for the management and control of vascular wilt disease.

**EXPERIMENTAL**

**Isolation, characterization and species diversity of banana root endophytes.** Mature asymptomatic roots of different banana varieties, e.g. *Musa acuminata, M. balbisiana* (syn. *M. sapientum*) and *M. textilis*, were collected from the University of Santo Tomas Botanical Garden, Manila and from five sites in Quezon City, namely, Commonwealth Avenue, Lagro, Fairview, University of the Philippines – Diliman, and Manila Water Sewerage System, Diliman. Root samples were stored in a cool box for transport to the laboratory and washed thoroughly with sterile distilled water to remove soil debris prior to surface sterilization.

Initially, surface-sterilization of the banana roots was optimized. Root samples from *M. textilis* collected from UST were immersed in factorial combinations of 1:100 (1%), 1:20 (5%) or 1:10 (10%) commercial bleach solutions (active ingredient = 5.25% NaOCl) for 1, 3 or 5 minutes. After which, roots were immersed in 75% ethanol for 1 minute and were rinsed once for 3 minutes in sterile distilled water. To test the effectivity of the surface-sterilization method, tissue printing was conducted on the surface-sterilized root samples. The root explants were cut into approximately 10 mm length and inoculated at five equidistant points on Potato Dextrose Agar (PDA, High Media) supplemented with 0.2 g L\(^{-1}\) Streptomycin (Sigma) and 0.05 g L\(^{-1}\) Tetracycline (Sigma). The culture plates (30 explants per plant) were incubated at room temperature for 15 days and fungal colonies growing out of the explants were isolated and purified by subsequent subculture. By employing the most effective surface-sterilization method, banana root endophytes (BREs) were isolated from the other banana varieties as previously described and maintained as pure culture.

The endophytic fungi isolates were identified based on their morphocultural characteristics, e.g. colony appearance and spore morphology, by preparing wet mounts and standard Riddell’s slides, i.e. inoculated agar block on glass slide cultured in a moist chamber. Then, the isolated BRE were identified based on literatures and identification keys. The species
Assay for antagonistic activities of isolated BREs against *F. oxysporum*. The test phytopathogen *F. oxysporum*, generously provided by Dr. Teresita Dalisay, Department of Plant Pathology, University of the Philippines – Los Baños, Laguna, and selected banana root endophytes (25 morphospecies, Table 1.0) were initially grown on PDA plates for 7 days. Following incubation, BRE mycelial agar plugs were prepared using a flame-sterilized cork borer (5 mm in diameter) and were placed on one side of the culture medium opposite the plant pathogen. The culture plates in duplicates were incubated at room temperature for 7 days. Antagonistic activities of the fungal isolates were determined based on the type of antagonistic interactions described by Wicklow *et al.* [16]. Fungal root endophytes exhibiting B (mutual inhibition), C (inhibition on contact) or D (inhibition at a distance) type of interactions were further tested for their inhibitory activities against the plant pathogen.

Of the 25 morphospecies tested, three BRE morphospecies were further tested against *F. oxysporum*. Mycelial agar plugs were prepared as previously described and placed at one side of the PDA plates. Opposite the BRE isolates, mycelial agar plugs of *F. oxysporum* were also inoculated at the same time. Another set-up was prepared wherein the BRE isolates were inoculated first on the culture plates and incubated for 15 days prior to the inoculation of the test fungus. As control, unchallenged BRE isolates and *F. oxysporum* were prepared. All culture plates were incubated at room temperature up to 19 days or until contact between the opposing fungi was observed. Starting from day 3, colony radial growth was measured using a Vernier caliper every other day. Percent reduction of colony radial growth was determined using the formula:

\[
\text{percent reduction} = \frac{N_0 - N_1}{N_0} \times 100
\]

where:
- \( N_0 \) = colony radial growth unchallenged *F. oxysporum* (control)
- \( N_1 \) = colony radial growth of *F. oxysporum* with BRE

One-way Analysis of Variance (ANOVA) was done to test for the significant differences between the different fungal strains using SigmaStat (USA).

Production of secondary metabolites by the isolated BREs. The three BRE isolates were initially grown on PDA slants for 7 days. Following incubation, 5 ml sterile distilled water was added to the cultures and the spores and the mycelia were then dislodged with flame-sterilized inoculating loop. The suspensions were poured onto culture bottles pre-filled with 50 ml PDA and incubated for 3 weeks at room temperature. Following incubation, the cultures were frozen for at least 2 hours and were freeze-dried using Heto PowerDry LL 3000 Freeze Dryer (Thermo Electron Corp.). The cultures were macerated manually and soaked overnight with 150 ml buffered ethyl acetate (1000 ml ethyl acetate mixed with 100 ml 1% NaHCO\(_3\)). The filtrates were concentrated in-vacuo (Rotavapor R-200, Büchi). The crude culture extracts were air-dried overnight, weighed, dissolved in 1:1 methanol:acetone and stored inside the refrigerator until needed for the bioassay.

Assay for antifungal activities of the BRE crude culture extracts using spore germination and disk diffusion assays. A spore suspension was initially prepared with 5 ml sterile distilled water from a 9-day old *F. oxysporum* slant culture and was adjusted to a concentration of 5.25 x 10\(^6\) spores/ml using a Neubauer haemocytometer. Then, 50 µl of the prepared spore suspension were pipetted into each well of a 96-well microtiter plates. Concentrations of 15 µl, 30 µl and 50 µl BRE culture crude extracts done in tripl-
cates were added to the spore suspension. The volume in each well was adjusted to a final volume of 200 µl with 0.1% Potato Dextrose Broth (PDB). For the control, wells of the same concentrations of the solvent (1:1 methanol:acetone) and the substrate PDB were prepared. The microtiter wells were incubated at room temperature for 24 hours. After which, the number of germinating and non-germinating spores in each well was counted using a Neubauer haemocytometer under the microscope (HPO). Percent germination was determined using the formula:

\[
\text{percent reduction} = \frac{\text{germinating spores}}{\text{germinating + non germinating spores}} \times 100
\]

*F. oxysporum* spore and mycelial suspensions prepared as previously described were evenly swabbed on PDA plates. To each of the culture plates (in triplicates), 13-mm sterile antibiotic paper discs impregnated with 50 µl BRE crude culture extracts were placed at the center. The culture plates were incubated at room temperature for 5 days. After incubation, the zones of inhibition were measured using a Vernier caliper. Statistical analysis (one-way ANOVA) was done to test for the significant differences between BRE isolates using SigmaStat (USA).

**RESULTS AND DISCUSSION**

Biological control of plant diseases makes use of antagonistic organisms, e.g. endophytic fungi and bacteria. These microorganisms were present naturally inside the host’s tissues, and play a vital role in the plant’s host defense mechanisms. As potential biocontrol agents, endophytes were routinely screened for *in vitro* production of useful compounds or bioactive secondary metabolites [3]. The use of endophytes for biological control is a novel yet effective strategy in controlling plant pests as opposed to the application of chemicals which often results to health hazards and environmental problems [17].

Isolation of root endophytes from three *Musa* cultivars, e.g. *Musa acuminata*, *M. balbisiana* syn. *M. balbisiana* & *M. textilis*, collected in Manila and Quezon City, yielded seventy-five fungal strains (Table 1.0). Surface-sterilization of banana root explants with 1:20 and 1:10 dilution of NaOCl solution for 1, 3 or 5 minutes displayed no bacterial and fungal growth in tissue prints but yielded fungal root endophytes. Thus, the NaOCl concentration (1:20 dilution) and the 1 minute sterilization time was used since it successfully removed root epiphytes and soil borne fungi present on root surfaces, and concurrently minimizing its fumigating effect on the root endophytes. Following isolation and culture, species richness was also computed. Highest species richness (d) was observed from banana plants collected from Lagro (d=8.89) and Commonwealth Ave (d=8.50) in Quezon City. However, the highest number of morphospecies (20) was observed from *M. textilis* collected from UST, Manila (Table 1). Of the 75 fungal isolates, 25 morphospecies were screened for their antagonistic activities using the dual-culture method (Table 2). Known and common soil-borne fungi among the 75 fungal isolates, e.g. *Fusarium* sp., *Trichoderma* sp., *Penicillium* sp., and *Aspergillus niger*, were excluded from the strains tested, though one strain of *Trichoderma* was tested since this genus was shown to be a highly effective biological control agent against various plant diseases [13, 18, 19]. Of the 25 isolates tested, only three exhibited antagonistic activities against *F. oxysporum* based on the types of interaction described by Wicklow *et al.* [16]. BRE 18 and BRE 71 inhibited *F. oxysporum* on contact (Interaction Type C) while BRE 14 inhibited the test pathogen at a distance (Interaction Type D). Most of the BRE isolates displayed mutual intermingling with the plant pathogen (Interaction Type A). Thus, only BRE 14, 18 and 71 were further tested for their antifungal activities. Morphocultural characterization identified these fungal isolates as those belonging to the genus *Aspergillus* (Fig. 1). Fungal colonies formed on PDA were beige to peach to cinnamon with the reverse side yellow. Hyphae were septated and columnar with smooth globose spores (Fig. 1). Several studies have mentioned the presence of antagonistic *Aspergillus* strains as fungal endophytes ob-
tained from rice, mangrove, soybean and corn plants [20, 21, 22, 23].

The 3 BRE isolates inoculated simultaneously with *F. oxysporum* clearly reduced the colony growth of the test fungal pathogen starting from day 11 of incubation (Fig. 2). Measurements taken from day 19 displayed percent colony radial growth reduction equivalent to 32.9% ± 6.9%, 40.2% ± 3.7%, and 31.3% ± 1.4% for BREs 14, 18, and 71, respectively. The pathogen showed stunted, almost

Table 1. Isolated fungal root endophytes from different banana species.

<table>
<thead>
<tr>
<th>Collection Site</th>
<th>Host Plant</th>
<th>Isolated BRE</th>
<th>Species Richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quezon City</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Commonwealth Ave.</td>
<td><em>M. balbisiana</em></td>
<td>1,2,3,4,5</td>
<td>8.50</td>
</tr>
<tr>
<td>2. Lagro</td>
<td><em>M. balbisiana</em></td>
<td>6,7,8,9,10,11,12</td>
<td>8.89</td>
</tr>
<tr>
<td>3. Fairview</td>
<td><em>M. balbisiana</em></td>
<td>13</td>
<td>nd</td>
</tr>
<tr>
<td>4. UP - MWSS</td>
<td><em>M. balbisiana</em></td>
<td>14,15,16,17,18</td>
<td>4.11</td>
</tr>
<tr>
<td>5. UP Diliman</td>
<td><em>M. balbisiana</em></td>
<td>19,20,21,22,23,24,25,26,27</td>
<td>8.06</td>
</tr>
<tr>
<td>Manila City</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. UST</td>
<td><em>M. textilis</em> (plant 1)</td>
<td>35,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54</td>
<td>nd</td>
</tr>
<tr>
<td>2. UST</td>
<td><em>M. textilis</em></td>
<td>65,66,67,68,69,70,71,72,73,74,75</td>
<td>nd</td>
</tr>
</tbody>
</table>

*BRE isolates in bold-type were screened for antagonistic activities.  *nd* = not determined

Table 2. Type of interaction between the BRE isolates and *F. oxysporum*.

<table>
<thead>
<tr>
<th>Type of Interaction Wicklow et al., (1980)</th>
<th>Number of Morphospecies</th>
<th>Isolate Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> mutual intermingling</td>
<td>22</td>
<td>BRE 03,05,07,15,26,37,39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BRE 41,45,47,48,57,58,59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BRE 60,61,62,64,66,69,72,74</td>
</tr>
<tr>
<td><strong>B</strong> mutual inhibition</td>
<td>0</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C</strong> inhibition on contact</td>
<td>2</td>
<td>BRE 18, BRE 71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D</strong> inhibition at a distance</td>
<td>1</td>
<td>BRE 14</td>
</tr>
</tbody>
</table>
Figure 1. Conidiophore and conidial morphology of (a) BRE 14, (b) BRE 18 and (c) BRE 71 (HPO, 40x).

Figure 2. Colony radial growth of *F. oxysporum* grown simultaneously against BRE 14, BRE 18 and BRE 71.

Figure 3. Colony radial growth of *F. oxysporum* grown with BRE 14, BRE 18 and BRE 71. BRE strains were initially cultured for 15 days before inoculation of the test fungus.
no increase in colony radial growth on day 15 when grown against BRE 14 and 18, and on day 17 when grown against BRE 71. The unchallenged \textit{F. oxysporum} (control) exhibited a significantly longer mean colony radial growth than those inoculated with the three BREs. However, no significant differences were observed between the inhibitory activities of the three BREs against \textit{F. oxysporum} (P > 0.05).

Reduced colony radial growth was also observed at a shorter incubation time (5 days) when the BREs were cultured 15 days prior to inoculation of the plant pathogen (Fig. 3). Greater percent reductions in colony radial growth equivalent to 47.5% ± 2.5%, 52.5% ± 0.9% and 42.8% ± 0.5% for BRE 14, 18 and 71, respectively, were observed. The challenged \textit{F. oxysporum} remained viable throughout the incubation period, though growth was observed at a slower rate as compared to unchallenged \textit{F. oxysporum} (data not shown). The fungal root endophytes, BRE 14, BRE 18 and BRE 71, were also able to produce 0.3559 g, 0.2557 g and 0.0266 g of crude culture extract by dry weight, respectively. The crude culture extracts were dissolved with 1:1 MeOH:Acetone in a ratio of 0.05 g dry weight: 0.5 ml solvent which were tested against \textit{F. oxysporum}.

The antagonistic potential of the three BRE crude culture filtrates was further assessed using the paper disk diffusion and the spore germination assay. Results from the paper disk diffusion assay showed the absence of an apparent zone of inhibition against \textit{F. oxysporum} (data not shown). The spore germination assay, on the other hand, showed a remarkable decrease in the germination of spores (Fig. 4). As concentration of the crude culture extracts increased from 7.5% (15 µl) to 25% v/v (50 µl), a corresponding substantial decrease in percent spore germination was observed. Moreover, crude culture extracts of BRE 71 at concentrations of 15% to 25% completely inhibited spore germination (0% germination). These contra-
dicted the results obtained from the paper disk diffusion assay. Perhaps, the bioactive metabolites present in the crude cultured extracts may not be in enough concentration to exhibit clear zones on the test fungus. It is also possible that the three BREs exhibit inhibitory activities only against germinating spores and not on actively growing hyphae or mycelia. Griffin [24, 25] noted that crude extracts may contain inhibitors that suppress spore germination at high conidial densities. No significant differences were also observed between the percent germination of spores treated with PDB and solvent regardless of concentration. However, a significant difference existed between the percent germination of spores treated with PDB and with those treated with BRE crude culture extracts (P > 0.05).

CONCLUSION

In conclusion, a total of 75 banana root endophytes (BRE) were isolated from three local Musa cultivars M. balbisiana, M. acuminata and M. textilis. Only 3 from the 25 isolates tested for antagonistic activities showed inhibition against the test fungus either on contact (Type C = BRE 18 & BRE 71) or at a distance (Type D = BRE 14). Morphocultural characterization identified these isolates as belonging to the genus Aspergillus. Further antagonistic assay showed reduction of colony radial growth of F. oxysporum grown in culture with the three BREs. Percent reduction in colony radial growth was even significantly greater when the BREs were inoculated 15 days prior to the inoculation of the fungal pathogen as compared to simultaneous inoculation of BREs and F. oxysporum. The crude culture extracts failed to inhibit the fungal pathogen using the paper disk diffusion assay, but clearly reduced percent spore germination. Our research study has shown that the three BREs exhibited antagonistic activities against F. oxysporum and may have the potential as biological agents.

REFERENCES

[17] Papar P., T. Dubois, C.S. Gold, E. Adipala, B. Niere and D. Coyne. Inoculation, colonization and


