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Hefa Kemung*, Teatulohi K. Matainaho*, Prem P. Rai^ and Louis R. Barrows*****

*Discipline of Pharmacology, **Discipline of Pharmacy, School of Medicine and Health Sciences, University of Papua New Guinea; ***Department of Pharmacology and Toxicology, Salt Lake City, Utah U.S.A.

^Correspondence author: pprai.prem@gmail.com

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^Correspondence author: pprai.prem@gmail.com

ABSTRACT:

Fungal endophytes are fungi that reside in tissues of healthy living plants offering chemical protection and promoting growth of host plants. The chemistry and biological properties of their secondary metabolites from monoaxenic cultures have been extensively studied, but co-culture techniques using endophytes remain largely unexplored. Co-culture of bacteria with endophytic fungi have shown to be effective, leading to the discovery of novel chemistry while simultaneously addressing the problem of rediscovery of known metabolites from the monoaxenic culture techniques. Forty microliters (40µL) of culture broth consisting of monoaxenic and co-cultures were placed in separate agar wells with 5µg ciprofloxacin as positive control. The co-culture broth from one unidentified endophytic fungus with *B. subtilis* showed a marked zone of inhibition measuring 24.7 ±0.6 mm in comparison to ≥21.0 mm as the sensitivity range for ciprofloxacin against *Escherichia coli* while its monoaxenic culture exhibited no inhibition, but furthermore showed moderate activity (16.7 ± 1.2 mm) against *B. subtilis*. The isolation and chemical characterization of the active component and the mechanism of *B.subtilis* induction is under investigation. It is hoped that this unique Papua New Guinea endophyte will provide broad-spectrum antibiotic to combat the growing global problem of drug resistant infections.

KEYWORDS: Monoaxenic culture, Co-culture, Fungal endophyte, Lianas, Antimicrobial activity

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INTRODUCTION:

Fungal endophytes are fungi that thrive in tissues of virtually all healthy plants for most of their lives. They offer chemically-mediated host protection and resistance to external stress. The chemistry and biological properties of fungal endophytes have been extensively studied in the search for more effective anti-infective agents to combat emerging drug-resistant infectious diseases [1-3].

Co-culture technique is a simple method of culturing two or more microbial strains. This method has shown to effectively enhance chemical yield of present constituents while simultaneously yielding novel defensive chemicals. Molecular studies have demonstrated that genes involved in the biosynthesis of antibiotics by microbes remain silent under classical monoaxenic culture but become activated when whole cell organisms of two or more different strains are cultured together [4-7].

The fungal endophytes in foliar assemblages in Papua New Guinea remain largely understudied and can offer potential lead molecules for drug discovery. The current study was carried out to determine the antibacterial activity of defensive chemicals produced by fungal endophytes isolated from an unknown endemic PNG liana species under monoaxenic and co-culture conditions.

METHODOLOGY:

Sampling

Foliar samples from a mature healthy liana species were randomly collected (diameter, breadth and height (dbh) of ≥10cm at 1.5-2.0m above ground) at Varirata National Park outside of Port Moresby, National Capital District [8-10]. The fresh foliar samples were kept in a cool storage container and transported to the drug discovery laboratory in the School of Medicine and Health Sciences, University of PNG to initiate work on fungal endophytes. Voucher specimens of the host plants were prepared and deposited at the Biological Sciences Herbarium, UPNG.

Surface-sterilization

Surface-sterilization reagents were used to eliminate epiphytes from the foliar samples, and carried out within 48 hours after sampling in field. Methods used were adapted from three different studies [11-13]. Briefly, whole foliar samples were held under running tap water for about 10 minutes to wash out any dirt and debris. Afterwards, a 2mm x 2mm incision were made by sterile surgical blade (size 10) and the cuts dipped sequentially in 70% ethanol (EtOH) for 1 minute, then in 2.6% sodium hypochlorite (NaOCl) for 3minutes and finally twice in sterile double distilled water.

Pure culture

Sterile potato dextrose agar (PDA) supplemented with chloramphenicol (100mg/ 1L of PDA) was used for the isolation and promotion of fungal endophytes while suppressing other forms of endophytes [14-16]. To determine the effectiveness of this surface-sterilization method, aseptically air-dried sterile cuts were printed on control plates that showed absence of growth on control plates [17].

The cuts were left on plates for 3-4 weeks at 25°C, encouraging the outgrowths of fungal endophytes. Contaminations were noticeable as growths protruding from the outer margins of plates and thus discarded. Pure (single) fungal isolates were achieved by continuous sub-culture of growths on freshly made PDA with an incubation period of 3-4 weeks per sub-culture [18]. Active stock cultures were maintained on nutrient agar (NA) and served as the primary source for small-scale fermentation.

Small- scale monoaxenic culture

Agar plugs (5mm x 5mm) were aseptically transferred from the active stock cultures into 100ml of freshly prepared sterile nutrient broth (3.0g beef extract, 5.0g peptone per 1L) in sterile 500ml Erlenmeyer flasks. The fungal isolates were incubated for 3-4 weeks in stationary phase at 25 °C with periodic shaking. Controls of cell-free nutrient broth were used to monitor contamination [2, 19].

Small- scale co-culture with *B. subtilis*

Co-cultures of individual isolates with *B. subtilis* were prepared at the same time as their mono-cultures [20]. Sterile nutrient broth (100ml) was inoculated with single fungal isolates as per the above method. A 24 hour colony of *B. subtilis* grown on Mueller Hinton Agar (MHA) at 37°C was aseptically added into the nutrient broth. The co-culture flasks were left standing at 25 °C for 2 weeks with periodic shaking [2].

Concentration of culture broth

Both culture broths (100ml) were aseptically transferred to new sterile 50ml tubes and centrifuged at 10 000 rpm and 4°C for 10 minutes. The supernatant collected (100ml) were reduced to 3ml through vacuum drying using an Eppendorf concentrator set at 4000rpm and 30°C. Concentrated supernatant were maintained at 4°C for future work [16].

Preliminary Bioassay

Antimicrobial agar-well diffusion assay was carried out in triplicates using supernatants from both the monoaxenic and co-cultures. Test pathogens employed include *Staphyloccocus aureus* ATCC 25923, *B. subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Pseudomonas aerigunosa* ATTC 27853. The standards of Clinical and Laboratory Standards Institute (CLSI) were followed [21]. Briefly, bacterial inoculum matching standard 0.5Mc Farland turbidity was prepared and subsequently swabbed onto

sterile and freshly premade MHA to produce a uniform bacterial lawn. Wells were then aseptically dug using the base of sterile Pasteur pipettes (6mm in diameter) and the supernatant (40µL) added thereafter. Ciprofloxacin which is a broad-spectrum antibacterial agent was employed as positive control. A standard dose of ciprofloxacin (5µg) was obtained through serial dilution in a 96-well plate and transferred onto the pre-dug wells. The plates were placed in incubator at 37.5°C for 24 hours and the zone of inhibition (in mm) measured afterwards.

RESULTS:

The four fungal isolates that were used in this experimental set-up were HK060 3P2GB1A, HK060 2P3C2A, HK060 3P3C1 and HK060 2P3D1A (Figure 1). The antibacterial activity of co-culture 2P3C2A against *E. coli* and *B. subtilis* were significant relative to their monoaxenic culture when grown in nutrient broth showing bacterial sensitivity at 24.7 ± 0.6 mm and 16.7±1.2mm respectively (Tables 1 & 2, and Figure 2). The monoaxenic culture of isolate 3P2GB1A demonstrated a significant zone of inhibition against *E. coli,* measuring 22.0± 1.0 mm comparable to the sensitivity range of ciprofloxacin at ≥ 21.0 mm according to CLSI (Table 1). Antibacterial activity was observed for *E. coli* and *B. subtilis* and hence only the antibacterial results against these 2 bacterial test pathogens were presented in Tables 1 and 2.

Table 1: Antibacterial activity against *E.coli*

		Zone of inhibition (mm)		
	Fungal isolate	Co-culture*	Pure culture¶	Positive control§
1	2P3C2A	24.7±0.6	6.0±0.0#	37.3±0.6
2	3P2GB1A	6.0±0.0	22.0±1.0	34.7±0.6
3	3P3C1	15.0±0.0	6.0±0.0	38.0±0.0
4	2P3D1A	6.0±0.0	6.0±0.0	38.0±0.0

Keys: *Co-culture with *B. subtilis*, § Ciprofloxacin, ¶Pure culture without *B. subtilis*, #6mm represents the diameter of the agar well

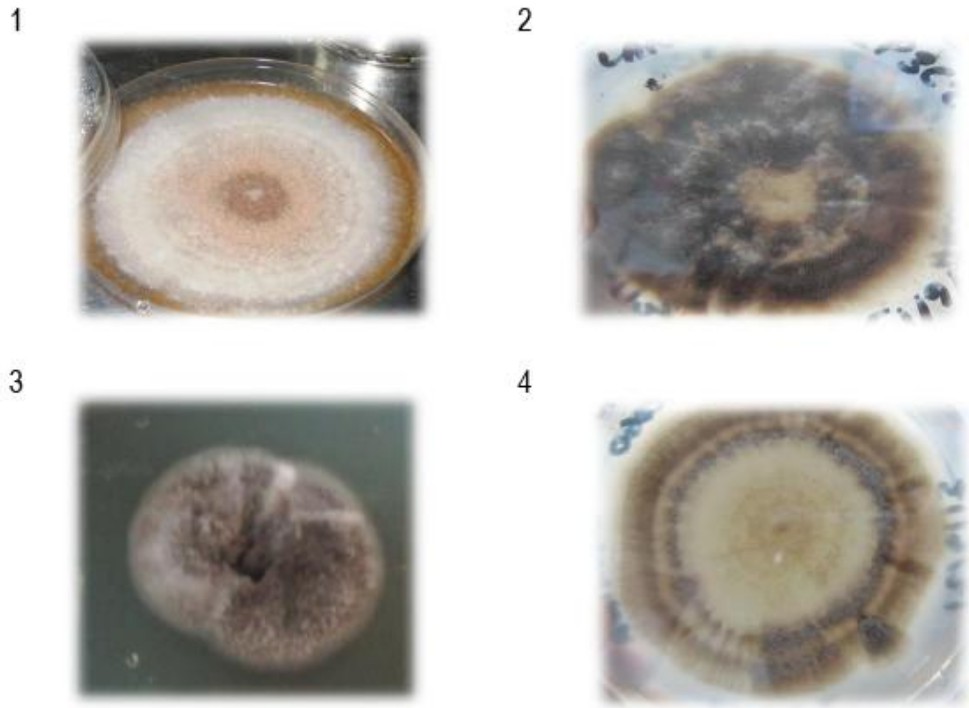

Figure 1: Fungal endophytes: (1- 3P2GB1A 2;(2-2P3C2A);(3- 3P3C1);(4- 2P3D1A)

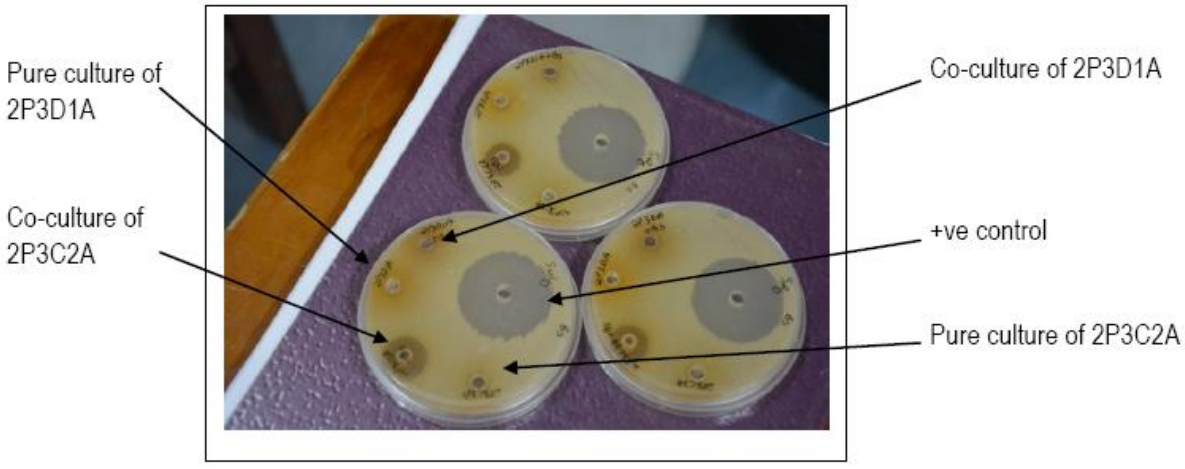

Figure 2: Antibacterial activity of monoaxenic culture and co-culture of 2P3C2A and 2P3D1A against *B. subtilis* shown in triplicates

Table 2: Antibacterial activity against *B.subtilis*

		Zone of Inhibition (mm)		
	Fungal isolate	**Co-culture***	**Pure culture¶**	**Positive control§**
1	2P3C2A	16.7±1.2	6.0±0.0#	36.7±0.0
2	3P2GB1A	6.0±0.0	6.0±1.0	37.3±0.6
3	3P3C1	13.0±0.0	6.0±0.0	38.3±0.6
4	2P3D1A	6.0±0.0	6.0±0.0	38.3±0.3

Keys: *Co-culture with *B. subtilis*, § Ciprofloxacin, ¶Pure culture without *B. subtilis*, #6mm represents the diameter of the agar well

DISCUSSION:

The four fungal endophytes used in this study, have been isolated from the foliar samples of a liana species HK060 (yet to be taxonomically verified). When grown in monoaxenic culture, the fungi 3P2GB1A isolated from midrib segment of HK060, demonstrated significant antibacterial activity against gam-negative *E.coli,* with a zone of inhibition measuring 22.0± 1.0 mm. Extensive reviews have been published describing the diverse chemical and biological properties of fungal endophytes [1,3,12]. Using molecular sequence data of 1403 endophytic strains, Arnold E and Lutzoni F have shown that incidence of foliar endophytes on a latitudinal basis, increases from Canadian arctic to the equatorial zone with less than 1 % to 99% in tissue segments respectively [22]. These findings infer that tropical foliar assemblages are a biodiversity hotspots for rare species of fungal endophytes with unknown host ranges and unrealized biological and chemical potential.

The cell-free supernatant from co-culture 2P3C2A (yet unknown) and bacteria *B. subtilis* exhibited a marked zone of inhibition (24.7 ± -0.6 mm) against *E. coli* when compared to its corresponding monoaxenic culture. When tested against *B. subtilis*, the same co-culture exhibited the greatest zone of inhibition (16.7 ± 1.2 mm). The cell-free supernatant from co-culture 3P3C1 with *B. subtilis* exhibited a zone of inhibition measuring 15.0± 0.0 mm and 13.0mm ±0.0mm against gram-negative bacteria *E.coli* and gram-positive bacteria *B. subtilis* respectively. The marked zone of inhibition demonstrated through co-cultures in this study, may support past studies of bacteria- fungi co-cultures and the discovery of

novel chemical compounds and or accumulation of presently constitutive compounds. *In vitro* culture of endophytic fungi with phytopathogens (bacteria or fungi) has led to the production of previously unsurfaced defensive chemicals. For instance, the co-culture of endophytic fungi *Fusarium tricinctum* with *B. subtilis* strain 168 trpC2 on solid rice medium led to 78-fold increase in the accumulation of constitutively present chemical compounds and spectral analysis identified 3 additional novel compounds namely macrocarpon C, *N*- (carboxymethyl)-anthranilic acid and (-) – citreoisocoumarinol. No such effect was observed with its monoaxenic control [20].

The cell-free supernatant from co-culture of unknown fungi 2P3C2A with *B. subtilis* exhibited significant zone of inhibition compared to its monoaxenic culture. The absence of zone of inhibition observed with the same supernatant against test pathogens *S. aureus* and *P. aerigunosa* may suggest specificity of interaction between the unknown fungi 2P3C2A and *B. subtilis* giving rise to the biosynthesis of defensive chemicals to which only *E. coli* was most sensitive to and to a lesser extent *B. subtilis*. Furthermore, the monoaxenic culture of the unknown endophytic fungi 3P2GB1A exhibited a pronounced zone of inhibition (22.0± 1.0 mm) against *E. coli.* Schroeckh V and colleagues demonstrated that specific intimate physical interaction between whole cell organisms was required to induce the biosynthesis of the dormant archetypal polyketide orsellinic acid (OA) and its derivative lecaronic acid. From 58 streptomycete species, *Streptomycete rapamycinicus* solely triggered the induction of OA in *Aspergillus nidulans* [7].

CONCLUSION:

The results of the study showed that co-culture technique greatly enhanced the antibacterial activity especially of the unknown fungal isolate 2P3C2A when cultured with *B. subtilis*. It also provided insight into antibacterial property of unknown fungal isolate 3P2GB1A. The isolation and chemical characterization of the active component from the co-culture of 2P3C2A with *B. subtilis* and the mechanism of *B. subtilis* induction are currently under investigation. It is hoped that this unique Papua New Guinea endophyte will provide a novel antibiotic to combat the growing global problem of drug resistant infections.

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