Isolation of Endophytic Fungi from *Vigna Mungo* (L.) Hepper and Their Antimicrobial Activities

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Abstract

The *Vigna mungo* (L.) Hepper (Mat Pe) plant sample was collected at Thae Phyu Village Hinthada Township, Ayeyawady Region for the isolation of endophytic fungi during June in 2020. The Mat Pe plant was identified by using reference keys (Darwin, R., D. Hooker and D. Jackson.1895). Twelve fungi were isolated from leaf, stem, main root and nodule of Mat Pe plant by using surface sterilization method (NITE, 2004). These fungi were transferred into the Czapek–Doz Agar (CZA) medium and incubated at 27°C for four to seven days. The pure fungi were tested by using seven test organisms for the study of antimicrobial activities. Among them, endophytic fungus TFO-07 showed highest activity against *Agrobacterium tumefaciens* during the confirmatory screening. TFO-07 can kill crown gall disease caused by *Agrobacterium tumefaciens* in plants. Therefore, TFO-07 was selected for the further investigation such as the effect of age and size of inoculum, effect of carbon and nitrogen sources and effect of pH.

Keywords: *Vigna mungo* (L.), endophytic fungi, antimicrobial activities

Introduction

Endophytic microorganisms can be found in virtually every plant on earth. These organisms live in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic. Novel antibiotics, antimycotics, immune suppressants and anticancer compounds are only a few examples of what has been found after the isolation and culturing of individual endophytes followed by purification and characterization of their natural products. The industry and agriculture may also be discovered among the novel products produced by endophytic microbes (Strobel and Daisy, 2004).

Legumes are an important crop, specially with regard to soil fertility, as nodule forming rhizobia will lead to a supply with fixed nitrogen there by enhancing crop productivity also for subsequent cultivation of legumes might also be effectively enriched for other endophytic species which then would impact other crop in rotation (Dudeja *et al.*, 2012). Recently, legumes are becoming interest because they are excellent sources of bioactive compounds, which play a vital role as a nutraceuticals, pharmaceuticals, pesticides and industrial products (Fery, 2002).

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Endophytic microbes from various plants exist in different ecosystems by various investigators reported. Endophytic fungi are the microorganisms that grow inside the plants and are relatively unexplored producers of metabolites useful to pharmaceutical and medicinal industries (Petrini *et al.*, 1992 and Ramasamy *et al.*, 2010). Endophytic fungi are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural and industrial areas (Sowparthani and Kathiravan, 2011).

This study was conducted to investigate some endophytic fungi strains from *Vigna mungo* (L.) Hepper (Mat Pe) plant collected from Thae Phyu Village, Hinthada Township, Ayeyawady Region for the production of antimicrobial secondary metabolites against antimicrobial activities. The aim of the review is to take a comprehensive look at most recent research on antimicrobial activities by isolated endophytic fungi.

Materials and Methods

Collection and Identification of Plant Sample

Vigna mungo (L.) Hepper (Mat Pe) plant was collected from Thae Phyu Village, Hinthada Township, Ayeyawady Region for the isolation of endophytic fungi during June in 2020. Healthy plant sample was collected in plastic bags and pressed, labeled with date and site of collection until isolation procedure was completed. The collected plant sample was recorded by photographs for taxonomic description and identified the specimen according to the available literature such as Darwin, R., D. Hooker and D. Jackson, 1895.

Figure.1 Map of Hinthada Township

(Source: Department of Geography, Pathein University)

Isolation of Endophytic Fungi (NITE, 2004)

The endophytic fungi were isolated from leaf, stem, main root, and nodule of the *Vigna mungo* (L.) Hepper (Mat Pe) plant by using surface sterilization method (NITE, 2004). Plant sample was washed thoroughly in running tap water and air dried before it was processed. The

materials were then surface sterilized by immersing them sequentially in 70% ethanol for 1 minute and then, also immerse 10% sodium hypochloride for 1 minute and rinsed thoroughly with sterile distilled water. Then, with a sterile scalpel, outer tissue was removed and the inner tissues of 0.5 cm size were carefully dissected and placed on sterile tissue paper for dry. The Czapek–Doz Agar (CZA) medium (Sucrose 3.0 g, $NaNo_3$ 0.2 g, K_2HPO_4 0.1 g, $MgSO_47H_2O$ 0.05 g, KCL 0.05 g, $FeSO_47H_2O$ 0.001 g, Agar 1.8 g, Distilled water 100 mL) was used for isolation medium to inoculate the endophytic fungi. After autoclaving, the medium was supplemented with chloramphenicol to suppress bacterial growth. The cut plant parts place on the petri-dish containing isolation medium was incubated at 27°C for three days to seven days until fungal growth appeared.

Antimicrobial Activities of Isolated Fungi (NITE, 2004)

Seven kinds of test organisms such as *Agrobacterium tumefaciens* (NITE-09678), *Bacillus subtilis* (IFO-90571), *Candida albicans* (NITE-09542), *Micrococcus luteus* (NITE-83297), *Salmonella typhi* (AHU-9743), *Escherichia coli* (AHU-5436), *Pseudomonas fluorescens* (IFO-94307) were used in the screening of antimicrobial activities (Table-1).

Antimicrobial activities of isolated pure fungi were investigated by using the agar well diffusion assay method (Collins, 1965). A cut of mycelium from four days old culture of each plate was cultured in a conical flask containing 50 mL of seed medium (Glucose 2.5 g, Yeast Extract 0.8 g, $MgSO_47H_2O$ 0.02 g, K_2HPO_4 0.01 g, Distilled water 100 mL) and incubated at the temperature of 27ºC. After three days, 25 % of seed medium was taken by sterile pipette and poured into another conical flask containing 75 mL of fermentation medium (Glucose 1.5 g, Yeast Extract 0.6 g, Soluble Starch 0.3 g, K_2HPO_4 0.01 g, $MgSO_47H_2O$ 0.02 g, Distilled water 100 mL) and also incubated at the temperature of 27ºC for three to ten days. About 0.2 mL of liquid culture of corresponding test organisms was poured into each corresponding conical flask containing 25 mL of sterilized assay medium (Glucose 0.5 g, KNO_3 0.1 g, Peptone 0.5 g, Agar 1.8 g and Distilled water 100 mL) thoroughly and allowed to solidify. After solidification, a hole with a diameter of 8 mm is punched aseptically with a sterile cork borer or tip. And then, fermented broth was carefully added into well and incubated at room temperature for twenty four hours.

After twenty four hours incubation, the plates were observed for the formation of clear inhibition zone around the agar well. The clear zone was examined by measuring the diameter of the clear zone with the aid of a digital clipper.

Table.1 Test Organisms used in Antimicrobial Activities

No.	Test Organisms	Diseases
1.	*Agrobacterium tumefaciens* (NITE 09678)	Crown gall disease
2.	*Bacillus subtilis* (IFO 90571)	DNA topoisomerase
3.	*Candida albicans* (NITE 09542)	Candidiasis
4.	*Micrococcus luteus* (NITE 83297)	Food spoilage
5.	*Salmonella typhi* (AHU 9743)	Typhoid
6.	*Escherichia coli* (AHU 5436)	Diarrhea
7.	*Pseudomonas fluorescens* (IFO 94307)	Rice pathogen

Results

Collection and Identification of Plant Sample

The plant sample was collected from Thae Phyu Village in Hinthada Township, Ayeyawady Region. It is located at N 17º 40.274´ and E 95º 16.16´. This plant is belonging to the Family Fabaceae.

Scientific Name *Vigna mungo* (L.) Hepper
Synonym *Phaseolus mungo* L.
English Name Black gram, mung bean
Myanmar Name Mat Pe
Family Fabaceae

Outstanding Characters of *Vigna mungo* (L.) Hepper

Annual erect herbs, stems and branches terete, pale green, pubescent. Leaves pinnately trifoliolate compound, stipulate, petiolate, leaflets ovate. Inflorescences axillary racemes. Flower yellow, 0.7 cm to 1.3 cm across at anthesis, papilionaceous, bracteate, pedicellate, bracteolate, bisexual, zygomorphic. Calyx campanulate, tubular with two upper and three lower unequal lobes, persistent. Corolla differentiated into standard, wings and keel. Standard 1.2 cm to 1.6 cm wide; wings about as long as standard; keel spirally coiled with a terminal horn-like appendage. Stamens 10 (9+1), diadelphous, filaments filiform, white; anthers uniform, dithecous, oblongoid, pale yellow, longitudinal dehiscent. Ovary superior, oblongoid, unilocular, marginal placentation; style slender, pale green, hairy; bearded below the oblique stigma. Pods cylindrical, yellowish - brown to black, 4 to 10 seeded. Seeds globoid or cube-like, black, hilum white, non-endospermic.

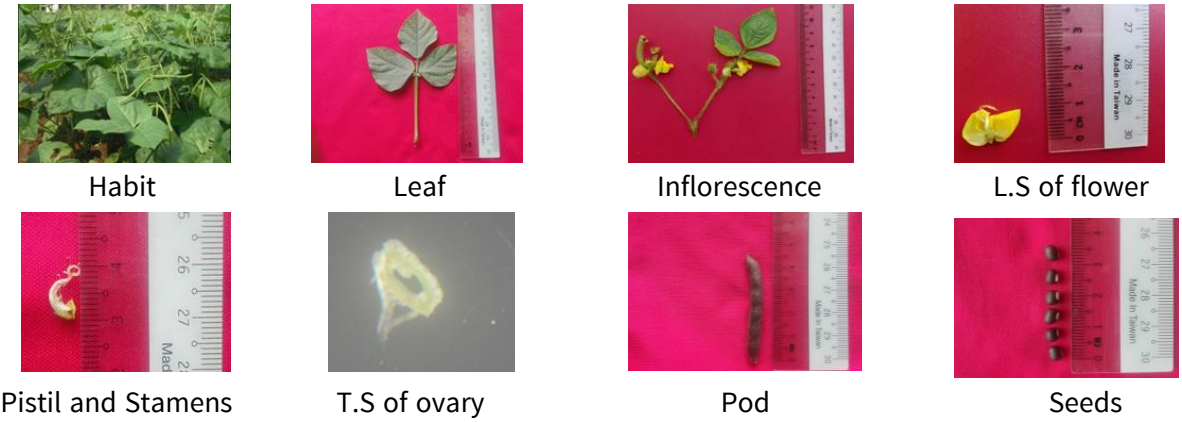

Figure.2 Morphological Characters of *Vigna mungo* (L.) Hepper

Isolation of Endophytic Fungi

Altogether twelve fungi were isolated from leaf, stem, main root and nodule of *Vigna mungo* (L.) Hepper (Mat Pe) plant. These pure culture of isolated fungi were named temporarily as TFO-01 to TFO-12 (Figure-3 to 14).

Figure.3 Colony morphology and photomicrograph of fungus TFO-01

Figure.4 Colony morphology and photomicrograph of fungus TFO-02

Figure.5 Colony morphology and photomicrograph of fungus TFO-03

Figure.6 Colony morphology and photomicrograph of fungus TFO-04

Figure.7 Colony morphology and photomicrograph of fungus TFO-05

Figure.8 Colony morphology and photomicrograph of fungus TFO-06

Figure.9 Colony morphology and photomicrograph of fungus TFO-07

Figure.10 Colony morphology and photomicrograph of fungus TFO-08

Figure.11 Colony morphology and photomicrograph of fungus TFO-09

Figure.12 Colony morphology and photomicrograph of fungus TFO-10

Figure.13 Colony morphology and photomicrograph of fungus TFO-11

Figure.14 Colony morphology and photomicrograph of fungus TFO-12

Table.2 Morphological and Microphical Characters of Isolated Endophytic Fungi

No.	Fungi	Plant parts	Morphological character	Microscopical character
1.	TFO–01	Leaf	Colony gray white colour on both surface and reverse view at 4 days old culture on CZA medium	Aseptate mycelium, conidiosphore long, conidia spherical shape, seven conidia
2.	TFO–02	Leaf	Colony creamy white colour on surface view and white colour on reverse view at 4 days old culture on CZA medium	Aseptate mycelium, conidiosphore long, conidia oblong shape, many conidia
3.	TFO–03	Leaf	Colony white and pale orange colour on surface view and orange colour on reverse view at 4 days old culture on CZA medium	Aseptate mycelium, conidiosphore long, conidia slightly pear shape, many conidia
4.	TFO–04	Leaf	Colony black colour on both surface and reverse view at 4 days old culture on CZA medium	Septate mycelium, conidiosphore long, conidia spherical shape, many conidia
5.	TFO–05	Stem	Colony white colour on both surface and reverse view at 4 days old culture on CZA medium	Aseptate mycelium, conidiosphore long, conidia fusiform to falcate shape, cluster conidia
6.	TFO–06	Stem	Colony cream colour on both surface and reverse view at 4 days old culture on CZA medium	Septate mycelium, conidiosphore long, conidia spherical shape, many conidia
7.	TFO–07	Main root	Colony pale yellow in the center and edge white colour on surface view and pale orange colour on reverse view at 4 days old culture on CZA medium	Septate mycelium, conidiosphore long, conidia elongate shape, cluster conidia
8.	TFO–08	Main root	Colony white colour on both surface and reverse view at 4 days old culture on CZA medium	Septate mycelium, conidiosphore long, conidia spherical shape, many conidia
9.	TFO–09	Main root	Colony white colour on both surface and reverse view at 4 days old culture on CZA medium	Aseptate mycelium, conidiosphore long, conidia produced in coil, elongate shape, many conidia
10.	TFO–10	Nodule	Colony pale brown in the center and edge white colour on surface view and white colour on reverse view at 4 days old culture on CZA medium	Septate mycelium, conidiosphore long, conidia spherical shape, many conidia
11.	TFO–11	Nodule	Colony white colour on surface view and orange colour on reverse view at 4 days old culture on CZA medium	Septate mycelium, conidiosphore long and branch, conidia oblong to cylindrical shape, many conidia
12.	TFO–12	Nodule	Colony pink in the center and edge white colour on surface view and white colour on reverse view at 4 days old culture on CZA medium	Septate mycelium, conidiosphore short, conidia spherical shape, many conidia

Antimicrobial Activities of Isolated Fungi

The antimicrobial activities of twelve fungi were studied by agar well diffusion method. Antimicrobial activities were tested by seven test organisms at six days of fermention period. In the study on antimicrobial activities, all the isolated fungi could not show activities and against seven kinds of test organisms expect TFO-02, TFO-03, TFO-04, TFO-07 and TFO-10 were against *Agrobacterium tumefaciens* (Figure-15 and Table-3). TFO-01, TFO-10 and TFO-12 were against *Bacillus subtilis* (Figure-16 and Table-3). TFO-02, TFO-03, TFO-04, TFO-06, TFO-07 and TFO-10 were against *Micrococcus luteus* (Figure-17 and Table-3).TFO-07 and TFO-10 were against *Escherichia coli* (Figure-18 and Table-3). Among them, antimicrobial activities of isolated fungus TFO-07 showed the maximum inhibitory zone against *Agrobacterium tumefaciens*. Isolated fungus TFO-07 was preserved into slant containing CZA medium for further investigation such as the effect of age and size of inoculum, effect of carbon and nitrogen sources and effect of pH.

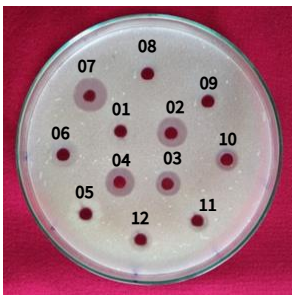

Figure.15 Antimicrobial activities of TFO-02, TFO-03, TFO-04, TFO-07 and TFO-10 against *Agrobacterium tumefaciens*

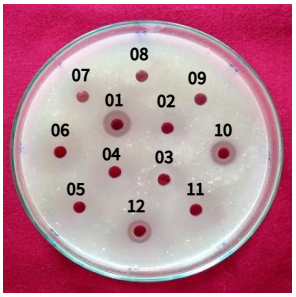

Figure.16 Antimicrobial activities of TFO-01,TFO-10 and TFO-12 against *Bacillus subtilis*

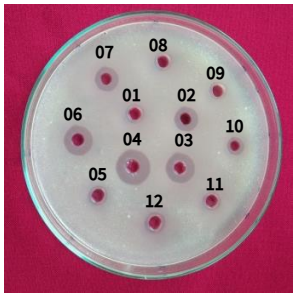

Figure.17 Antimicrobial activities of TFO-02, TFO-03, TFO-04, TFO-06, TFO-07 and TFO-10 against *Micrococcus luteus*

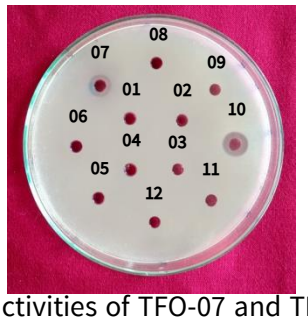

Figure.18 Antimicrobial activities of TFO-07 and TFO-10 against *Escherichia coli*

Table.3 Biological Properties of Isolated Fungi

Isolated fungi	Test Organisms and Antimicrobial Activities Inhibitory Zones, (mm)						
	Agrobacterium tumefaciens	*Bacillus subtilis*	*Candida albicans*	*Micrococcus luteus*	*Salmonella typhi*	*Escherichia coli*	*Pseudomonas fluorescens*
TFO-01	_	17.72	_	_	_	_	_
TFO-02	21.22	_	_	21.93	_	_	_
TFO-03	12.94	_	_	25.74	_	_	_
TFO-04	20.43	_	_	29.60	_	_	_
TFO-05	_	_	_	_	_	_	_
TFO-06	_	_	_	26.23	_	_	_
TFO-07	30.32	_	_	21.93	_	12.10	_
TFO-08	_	_	_	_	_	_	_
TFO-09	_	_	_	_	_	_	_
TFO-10	13.97	14.10	_	12.11	_	15.33	_
TFO-11	_	_	_	_	_	_	_
TFO-12	_	12.84	_	_	_	_	_

6 days fermentation

Discussion and Conclusion

Sharma, (2016) mentioned that endophytes are group of microbes which are related with plants and play indispensable role in defense mechanism and production of secondary metabolites.

In the isolation of fungi, *Vigna mungo* (L.) Hepper (Mat Pe) plant was collected at Thae Phyu Village in Hinthada Township, Ayeyawady Region during June in 2020. Endophytic fungi were isolated by surface sterilization method (NITE, 2004). In this study, twelve fungi were isolated from leaf, stem, main root and nodule of *Vigna mungo* (L.) Hepper (Mat Pe) plant.

Twelve fungi were tested by using seven test organisms (Table-1). In the study of antimicrobial activities, all the isolated microorganisms could not show activities and against seven kinds of test organisms except TFO-02, TFO-03, TFO-04, TFO-07 and TFO-10 were against *Agrobacterium tumefaciens* (Figure-15 and Table–3). TFO-01,TFO-10 and TFO-12 were against *Bacillus subtilis* (Figure-16 and Table-3). TFO-02, TFO-03, TFO-04, TFO-06, TFO-07 and TFO-10

were against *Micrococcus luteus* (Figure-17 and Table-3). TFO-07 and TFO-10 were against *Escherichia coli* (Figure-18 and and Table-3).

The endophytic fungi are important in biotechnology as new pharmaceutical compounds, secondary metabolites agents of biological control and other useful characteristics could be found by further investigation of endophytes (Sowparthani and Kathiravan, 2011).

In the study of antimicrobial activities, endophytic fungus TFO-07 showed highest activity against *Agrobacterium tumefaciens* during the confirmatory screening. TFO-07 can kill crown gall disease caused by *Agrobacterium tumefaciens* in plants. Therefore, TFO-07 was selected for further investigation. Further investigation will focus on the effect of age and size of inoculum, effect of carbon and nitrogen sources and effect of pH.

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References

Collins, C.H. 1965. **Microbiological methods**. Buttrworth & Co., Publisher ltd., London.

Darwin, R., D. Hooker and D.Jackson. 1895. **Index Kewensis Plantarum Phanerogamarum**. Clarendon Press. Oxford.

Dudeja,S.S., R.Giri, R.Saini, P.Suneja-Madan and E. Kothe. 2012. **Interaction of endophytic microbes with legumes**.Journal of Basic Microbiology, 52,248-260.

Fery,F.L. 2002. **New opportunities in *Vigna*. In: J. Janick and A Whipkey, Trends in new crops and new uses**. ASHS Press, Alexandria, VA P 354.

Petrini, O., T. Sieber, L. Toti and O. Viret. 1992. **Ecology metabolite production and substrate utilization in endophytic fungi**.Natural Toxins, 1(3), 185-196.

Ramasamy, K., S.M. Lim, A.B. Bakar, N. Ismail, M.S. Ismail, M.F. Ali, J.F.F. Weber and A.L.J.Cole.2010. **Antimicrobial and cytotoxic activities of Malaysian endophytes**.*Phytothear. Res*, *24(5)*, 640-643.

Sharma,S. 2016. **Endophytic fungi and its role in plant life cyc**le. IJARIIE-ISSN (O), 2 (3): 2395-4396.

Sowparthani,K and G. Kathiravan. 2011. ***In-vitro* antibacterial screening of ethyl acetate extract endophytic fungi isolated from *Phyllanthus amarus* (Schum & Thonn) against pathogenic bacterial strains**. JPBMS, Vol.10, Issue 10.

Strobel,G and B. Daisy. 2004. **Bioprospecting for microbial endophytes and their natural products**. Microbiol Mol Biol Rev.67(4): 491-502.

NITE (National Institute of Technology and Evaluation). 2004. **Surface sterilization and baiting method**.