STUDY ON ANTIFUNGAL ACTIVITY OF ENDOPHYTIC FUNGI ON PATHOGENIC FUNGI FROM SUNFLOWER SEEDS AND IDENTIFICATION OF SELECTED ENDOPHYTIC AND PATHOGENIC FUNGI

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Abstract

In the isolation of endophytic fungi, 5 different plant sources were collected in Pathein Area and10 kinds of endophytic fungi (TP-01,02,03,04,05,06,07,08,09,10) were isolated. For the antifungal activities, the pathogenic fungi were isolated from the seeds of sunflower grown in Hinthada Area. Three kinds of pathogenic fungi (PF-01,PF-02,PF-03) were isolated by serial dilution methods. Ten kinds of isolated endophytic fungi were tested for antifungal activities. Among them, 4 kinds of endophytic fungi (TP-01,08,09,10) showed the antifungal activities (15 mm, 14 mm, 17 mm and 21 mm of inhibitory zones) on pathogenic fungus (PF-03) of sunflower seeds. According to these inhibitory zones, the biggest zone (21 mm) of endophytic fungus from *Polygonum barbatum* L. (suzat-pan) belonging to the family of Polygonaceae was selected for further investigation. Selected endophytic fungus (TP-10) and pathogenic fungus (PF-03) were identified by using reference keys. These selected fungus were important for all farmers because the seed-borne fungi were deterioration in sunflower oil and lost yield.

.**Key words**: isolation of fungi, antifungal activities, identification of fungi

Introduction

Soil, plant parts (living and fallen leaves, leaf litters), dung, insect, fresh water and marine water are the typical materials for microbial sources to isolate the microorganisms (Harayama and Isono, 2002). Plants can be considered as a new isolation source of microorganisms. This means that there is much possibility of findings new microorganisms (Scott and Lori, 1996). The medicinal plants in Myanmar are useful for therapeutic and microorganisms which living inside these healthy plant parts are also useful to produce the metabolites (Saisamon and Nipawam, 2007).

The deterioration in sunflower oil due to seed-borne fungi is of a great importance. Sunflower (*Helianthus annuus* L.), considered as commercial oil crop all over the world, the crop is widely cultivated in Egypt and in many countries all over the world. Sunflower is particularly used for production of

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edible oils as well as for seed consumption. The crop is attacked by numerous seed mycoflora and these pathogens may affect the crop resulting in a reduction of the seed quantity and quality. The direct impact of storage fungi on the economical part of the plant (seed) need further studies to find out the different effects of storage fungi on sunflower oil in order to increase oil yield and crop quality for human consumption and food industries (El-Wakil, 2014).

In continuation of screening programme for biological activities of the higher plants, studied *Polygonum barbatum* L. *Polygonum barbatum* L. is called joint weed, smartweed and knotgrass in Philippine. Its young leaves and shoots are cooked as vegetables. In medicinal properties, the sap of pounded leaves applied to wounds is considered an effective cicatrizant. Seeds are used to relieve colic pains. Roots are used as astringent. In China, leaves and stems are used to wash wounds and ulcers. The sap is applied to wounds as antiseptic. The paste of roots is used for treatment of scabies (Mao, 2012). The leaves of *Polygonum barbatum* L. were used for isolation of endophytic fungi for the antifungal activity.

Aims and objectives of present work are (i) to isolate the endophytic fungi from different plant sources;(ii) to study the isolation of pathogenic fungi from the infected seeds that cause the diseases;(iii) to investigate the antifungal activities of endophytic fungi on pathogenic fungi and (iv) to identify selected endophytic and pathogenic fungi.

Materials And Methods

Isolation of endophytic fungi

Five different plants were utilized in the isolation and they were collected from Pathein Area (Table-1). In the isolation procedure of endophytic fungi (Figure-1), the leaves were washed in running tap water for 15 minutes and sterilized by soaking in 95% alcohol for 15 seconds. Then, the leaves were cut into small pieces and dried on the sterilized tissue paper. After that, cut pieces were incubated on nutrient agar plate (LCA medium) for 3 days to 1 week at room temperature.

Table-1 Plant samples used for screening of endophytic fung

No.	Scientific Name	Myanmar name	Family
1	*Premna corymbosa* Rottle & Willd	Pyae sone	Lamiaceae
2	*Tadehagi triquetrum* (L) H. Ohashi	Lauk thay	Fabaceae
3	*Cissus discolor* Blumi, Bijd.	Tabin-taing- mya-nan	Vitaceae
4	*Gynura procumbens* (Lour.) Merr.	Pya-me-swae	Asteraceae
5	*Polygonum barbatum* L.	Suzat pan	Polygonaceae

The procedure of isolation method for endophytes (Tomita, 1998)

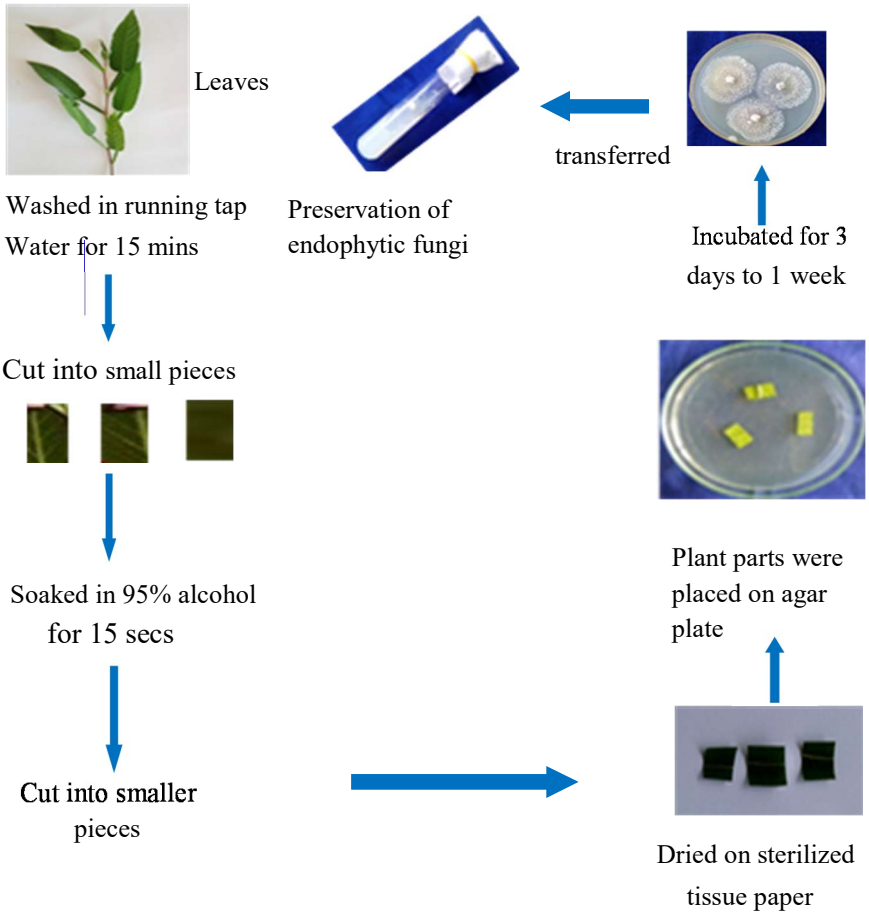


Figure 1. Isolation procedure of endophytes from plant parts

Isolation of pathogenic fungi from dried seeds of *Helianthus annuus* L

Seeds may carry spores of some pathogenic fungi. In such cases, a sedimentation or seed-washing test is useful for detecting spores. Seeds with spores samples were placed in test tube containing 10 ml of 0.85% saline. The aqueous suspension (0.5 ml) was transferred into other test tube containing 4.5 ml of distilled water and then 0.5 ml suspension into 4.5 ml distilled water tube after that 1 ml suspension into 4 ml distilled water. After serial dilution for spores suspension, 1 ml suspension was inoculated onto the nutrient agar plates (glucose 1%, peptone 0.3%, agar1.8%) incubated for 3-7 days at room temperature (Omura, 1985).

Serial dilution method, in biotechnology, Japan (Omura, 1985)

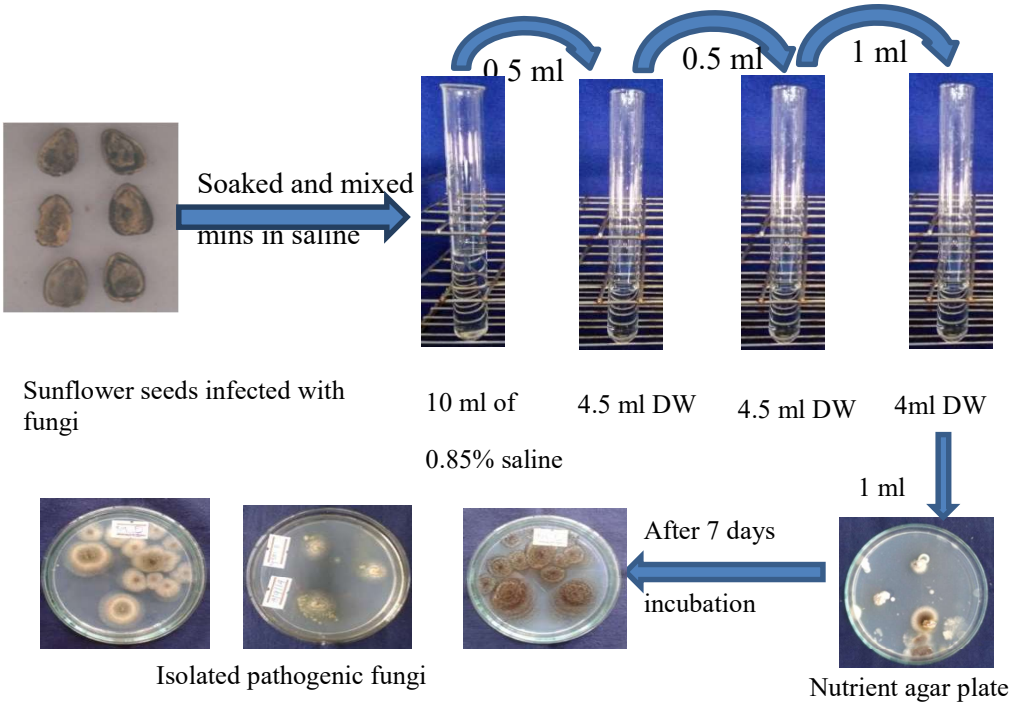


Figure 2. Isolation procedure of pathogenic fungi

Medium used of the isolation of fungi

LCA medium (Ando, 2004)

Glucose	0.1 g
K_2HPO_4	0.1 g
$MgSO_4.7H_2O$	0.02 g
$NaNO_3$	0.2 g
KCl	0.02 g
Yeast Extract	0.02 g
Agar	1.8 g
DW	100 mL

PGA medium (Ando, 2004)

Potato	20.0 g
Glucose	2.0 g
Agar	1.8 g
DW	100 mL

(After autoclaving chloramphenicol were added to the medium)

Preliminary study of the activities of isolated fungi

The isolated fungi were inoculated into the preculture medium (glucose 1%, potato dextrose broth 10%, at pH 7.0) for 3 days at room temperature. After three days, the preculture (1%) was transferred into the fermentation medium (glucose 1%, yeast extract 0.3%, peptone 0.3%, at pH 7.0) and carried out for 10 days by static culture. Then, the fermented broth was used to check the antifungal activity by paper disc diffusion assay method (Suto, 1999). Paper disc having 8 mm in diameter (Advance, Tokyo Roshi Kaisha Co., Ltd., Japan) were utilized for antifungal activity. The paper discs were soaked in fermented broth each and allowed to dry.

Paper disc diffusion assay method (Suto, 1999)

This method was used for the antifungal activity by the pathogenic fungi. The assay medium (glucose 1%, peptone 0.3%, agar 1.8% at pH 7.0) was utilized for these fungi. The pathogenic fungi were inoculated in assay broth for 3 days at room temperature. One percent of pathogenic fungi was added to assay medium and then poured into petridishes. After solidification, the paper discs impregnated with fermented broth samples were applied on the

agar plates and the plates were incubated for 24-36 hrs. Clear zones (inhibitory zones) surrounding the paper discs indicate the presence of bioactive compounds which inhibit the growth of the pathogenic fungi.

Preliminary identification of selected endophytic fungus and pathogenic fungus

The morphological and microscopical characters were observed by the methods of Ando and Inaba (2004) and Barnett (1956): Fungi Imperfecti.

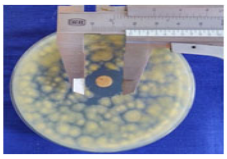
RESULTS

Table -2. Isolated endophytic fungi from plant sources

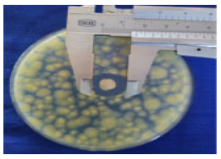
No.	Scientific Name	Myanmar name	No. of isolates	Isolated endophytic fungi
1	*Premna corymbosa* Rottle &Willd	Pyait sone	1	TP-01
2	*Tadehagi triquetrum* (L.) H. Ohashi	Lauk thay	2	TP-02, 03
3	*Cissus discolor* Blumi, Bijd.	Tabin-taing-mya-nan	3	TP-04,05,06
4	*Gynura procumbens* (Lour.) Merr.	Pya-me-swae	1	TP-07
5	*Polygonum barbatum* L.	Suzat pan	3	TP-08,09,10
	Total isolates		10	

Table-3. Antifungal Activities of Isolated Endophytic Fungi

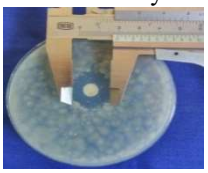
Isolated endophytic fungi	Activities on three pathogenic fungi		
	PF-01	PF-02	PF-03
TP-01	-	-	+(15 mm clear zone)
TP-02	-	-	-
TP-03	-	-	-
TP-04	-	-	-
TP-05	-	-	-
TP-06	-	-	-
TP-07	-	-	-
TP-08	-	-	+(14 mm clear zone)
TP-09	-	-	+(17 mm clear zone)
TP-10	-	-	+(21 mm clear zone)



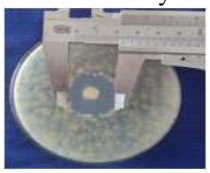
TP-01
(15 mm inhibitory zone)



TP-08
(14 mm inhibitory zone)



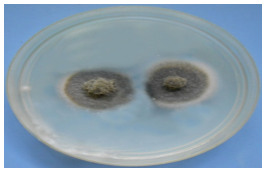
TP-09
(17 mm inhibitory zone)



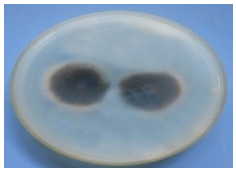
TP-10
(21mm inhibitory zone)

Figure 3. Antifungal activities of endophytic fungi on pathogenic fungi (PF-03)

Morphological and microscopical characters of selected endophytic fungus (TP-10)



Front colour



Back colour

Figure-3 Morphological Character of Selected Endophytic Fungus (TP-10)

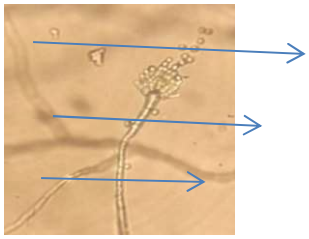
After 3 days of cultivation, it was observed that dark brown colonies were reached 2.5cm diameter at room temperature on PGA medium.

Distinctive character of selected endophytic fungus (TP-10)

conidia

conidiophore

hyphae



Photomicrograph X 100

Distinctive character of selected endophytic fungus (TP-10) was found that hyphae are hyaline, septate and branched; conidiophore brown, unbranched, hyaline at the base, bearing clusters of multi-phialides; conidia hyaline, lacking septum, one-celled, ameroconidium, globose, conidial chain long with many conidia(6-10), borned at the apex of the short phailide.

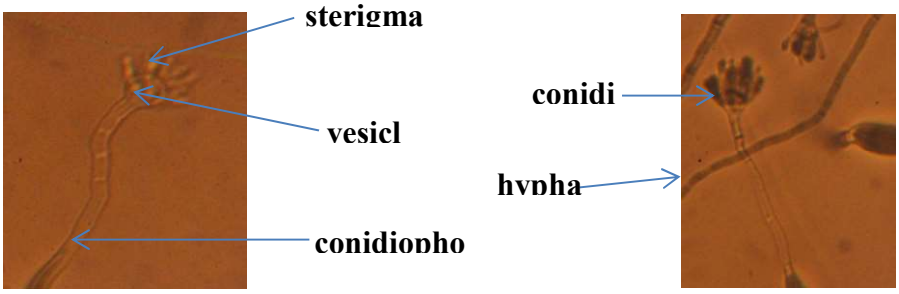
According to the morphological and microscopical characteristics features and based on the references keys of Ando and Inaba (2004), Barnett,(1956),this endophytic fungus was grouped as the fungi imperfecti may be *Aspergillus* sp.

Morphological and microscopical characters of pathogenic fungus (PF-03)

Front colour Back colour

Figure-4 Morphological Character of Pathogenic Fungus (PF-03)

Distinctive character of selected pathogenic fungus (PF-03)



Distinctive character of pathogenic fungus (PF-03) was found that hyphae are septate and hyaline;conidiophore with septum and hyaline, simple, long and ends in a dome–shaped multinucleate head called the vesicle, subglobose, club-shaped; sterigmata are tubular outgrowths from the vesicle, bearing cluster of 4-6 phialides; conidia hyaline, one celled, amerospore, globose.

According to the morphological and microscopical characteristics features and based on the references keys of Ando and Inaba (2004), Barnett, H.L., (1956), this pathogenic fungus was grouped as the fungi imperfecti may be *Aspergillus* sp.

Disscussion and Conclusion

In the course of the isolation for antifungal metabolites producing microorganisms, ten fungi were isolated from five different kinds of plant leaves collected at Pathein Township, Ayeyarwady Region. One fungi (TP-01) was isolated from the leaf of *Premna corymbosa* Rottle & Willd.; two

fungi (TP-02, TP-03) were isolated from the leaf of *Tadehagi triquetrum* (L.) H. Ohashi.; three fungi (TP-04, TP-05, TP-06) were isolated from the leaf of *Cissus discolor* Blumi, Bijid..; one fungi (TP-07) was isolated from the leaf of *Gynura procumbens* (Lour.)Merr. and three fungi (TP-08, TP-09, TP-10) were isolated from the leaf of *Polygonum barbatum* L. by surface sterilization method. For the antifungal activities, the pathogenic fungi were isolated from the seeds of sunflower (*Helianthus annuus* L. belonging to Asteraceae) grown in Hinthada Area. Three kinds of pathogenic fungi (PF-01, PF-02 and PF-03)were isolated by serial dilution method.

In order to find new antimicrobial metabolites, it is very important to set up an effective screening which has a unique target and deals with unique microorganism (Phay, 1997). During the study of antifungal activities, isolated endophytic fungi, TP-01 showed the antifungal activities (15 mm of inhibitory zone) on pathogenic fungus (PF-03) of sunflower seeds; TP-08 against 14 mm, TP-09 showed 17 mm and TP-10 showed 21 mm clear zones of antifungal activities on pathogenic fungus (PF-03). Among them, endophytic fungus TP-10 showed more highly antifungal activities (21 mm clear zone) against pathogenic fungus (PF-03) than other isolated fungi, these endophytic fungus (TP-10) from *Polygonum barbatum* L. (suzat-pan) belonging to the family of Polygonaceae. Therefore this strain TP-10 was selected for further investigation of identification.

In the identification of selected endophytic and pathogenic fungi, antifungal metabolite producing selected endophytic fungus TP-10 was grouped and keyed out fungi imperfecti. Based on the macroscopical - microscopical characters and the reference keys, this fungus TP-10 was grouped as the fungi imperfecti may be *Aspergillus* sp. However, identification of pathogenic fungus PF-03, according to the morphology and microscopical charasteristics features and based on the references keys, this pathogenic fungus (PF-03) was grouped as the fungi imperfecti may be *Aspergillus* sp. These selected fungi benefit for the against of infected seed-borne fungi. These investigations clearly indicate that the selected endophytic fungus, the genus *Aspergillus* sp. is useful for the production of antifungal substances. The type of antifungal agents produced by these research will be investigated as well.

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