ISOLATION OF ENDOPHYTIC FUNGI FROM SOME SPECIES OF FABACEAE AND ANTIBACTERIAL ACTIVITY

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Abstract

In the study endophytic fungi, isolated from four species of Fabaceae (Myay-pae, Pe-sin-gon, Pe-Lun and Pe-nauk) collected at Kanpyar Village, Magway Region were undertaken by direct inoculation method. In this investigation, eleven different kinds of fungi TF 1-11 were isolated from collected four species of Fabaceae. TF 1-3 were isolated from Myay-pae, TF 4-6 were isolated from Pe-sin-gon, TF 7-9 were isolated from Pe-Lun, TF 10 and TF-11 were isolated from Pe-nauk. According to the present result indicated that, the surface view and reverse view of endophytic fungi. Five kinds of test organisms (*Escherichia coli, Agrobacterium tumefaciens, Staphylococcus aureus, Pseudomonas fluorescens,* and *Bacillus subtilis*) were used in paper disc diffusion method. In the study of antagonistic property of isolated fungi, it was observed that fungus TF-7 exhibited highest activity on *Agrobacterium tumefaciens*. Therefore, this fungus TF-7 was selected for further investigations. In the fermentation study, it was also found that the optimal ages are 96hrs (24.21 mm) and optimal sizes are 25% (21.91 mm) seed culture at 7 days fermentation. Four fermentation medium (FM 1-4) were used in the fermentation study and it was found that FM-2 showed highest antibacterial activity on the medium. In the preliminary study for the extraction of active compound with four solvents system (Hexane, Chloroform, Dichloromethane and Toluene) were used it was found that chloroform provided highest antibacterial activity on *Agrobacterium tumefaciens*. The selected isolated fungus TF-7 was identified as *Trichoderma hamatum*

Keyword endophytic fungi, microorganism, metabolite.

Introduction

Endophytes are microorganisms that are present in living tissue of various plants (root, fruit, stem, seed, leaf etc) establishing mutual relationship without apparently any symptom of diseases. The endophytic fungi play important physiological and ecological roles in their host life. Endophytic fungi are a good source of antibiotics. Endophytic fungi are also capable to produce antimicrobial metabolites. Endophytic fungi are ubiquitous symbiotic to slightly parasitic microorganisms that live within plant tissues for all or part of their life cycle. *Trichoderma* is a genus of fungi in the family Hypoceraceae that is present in all soils, where they are the most prevalent culturable fungi. Many species in this genus can be characteristized as opportunistic avriulent plant symbionts. The ability of several *Trichoderma* species to form mutualistic endophytic relationships with several plant species. Trichoderma species are economically important for their production of industrial enzymes, antibiotics and their action as biocontrol agents against plant pathogens based onvarious mechanisms such as the production of antifungal metabolites, competition for space and nutrients and mycoparasitism (Howell, 2003).

Materials and Methods

Outstanding Morphological characters of each species was carried by using Dassanayake (1991)

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Table 1 Four species of Fabaceae are collected at Kanpyar Village.

No.	Collected Plants	Scientific Name
1.	Myay pea	*Arachis hypogaea* L.
2.	Pe-sin- gon	*Cajanus cajan* L.
3.	Pe-lun	*Vigna unguiculate* L.,
4.	Pe-nauk	*Vigna radiatus* L

Figure 1 Kanpyar Village Map

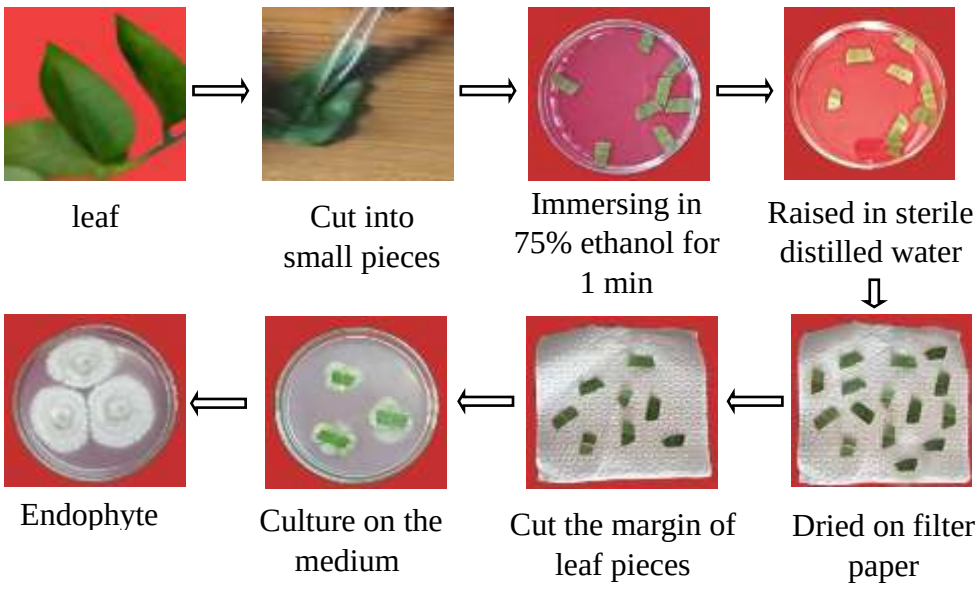


Figure 2 Isolation procedure for endophytic fungi

Medium Used for the isolation of fungi

PGA medium
(Potato Glucose Agar Medium)

Glucose	0.5 g
Yeast	0.1 g
K_2HPO_4	0.001 g
Agar	1.8 g
Poly peptone	0.1g
Potato+Dw	100mL

(after autoclaving chloramphenicol was added to the medium.)

LCA medium
(Low Carbon Agar medium, Ando, 2004)

Glucose	0.2 g
Sucrose	0.2 g
K_2HPO_4	0.1 g
$MgSO_4 7H_2O$	0.05 g
KNO_3	0.1 g
KCL	0.05 g
Agar	1.8 g
DW	100 mL
pH	6.5

(after autoclaving chloramphenicol was added to the medium.)

Study on the Antibacterial Activity of Isolated Fungi

Assay Medium

Glucose	1.0g
Yeast extract	0.2g
Agar	1.8g
DW	100mL
pH	7.0

Seed Medium

Glucose	1.0g
Yeast extract	0.2g
NZ amine type A	0.3g
K_2HPO_4	0.001g
DW	100mL
pH	7.0

Study on the Effect of Age and Size of Inoculum of the Fermentation (Omura, 1985, Crueger and Crueger, 1989)

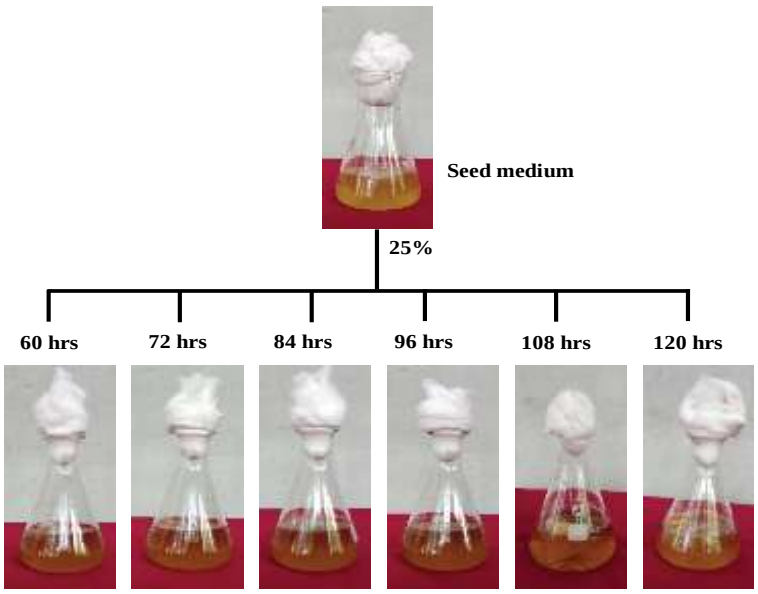


Figure 3 Procedure for the study on the effects of ages of seed culture

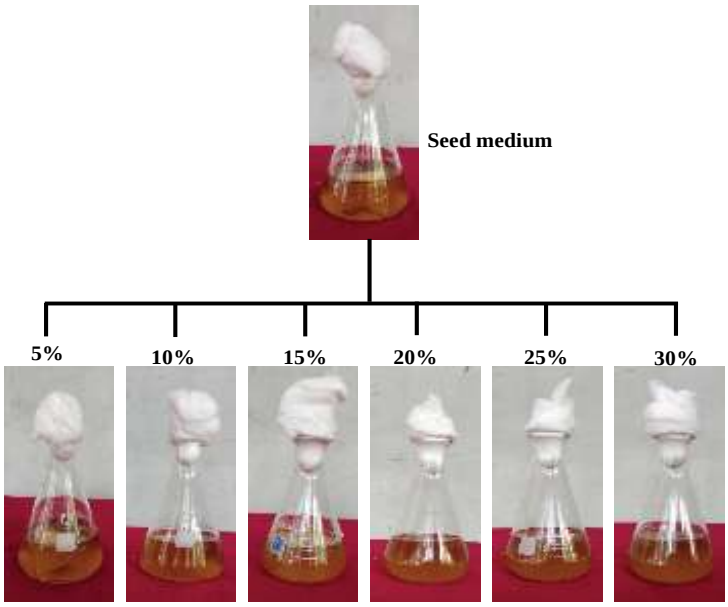


Figure 4 Procedure for the study growth of Fungus TF-07

Preliminary study for the extraction of metabolite (Jain and Pundir, 2011)

In the studies of extraction of metabolite from TF-07, four kinds of solvents (Chloroform, dichloromethane, toluene and hexane). Were utilized and checked with paper disc by assay method.

Identification of Fugus TF-07

The selected fungus TF-07 was cultured on Glucose Yeast Agar (GYA) medium. After 5 days cultured, colony were observed for macroscopic character and microscopic character. The selected fungus was identified followed by Domsch *et al*.,1993.

Results

Isolation of Endophyte Fungi

Scientific Name - *Arachis hypogea* L.
Family - Fabaceae
Myanmar Name - Myay-pe
English Name - Groundnut

Figure 5 Habit of *Arachis hypogaea* L. (Myay-pe)

Annual herb. Stem subteret. Leaves alternate, pinnately compound. Inflorescence axillary, cymose. Sepals (5), pale green, deciduous. Petals 5, papilionaceous, pale yellow,

deciduous. Stamens (10), Monadelphous. Ovary monocarpellary, marginal placentation. Pods oblongoid.

Scientific Name - *Cajanus cajan* L.
Family - Fabaceae
Myanmar Name - Pe-sin-gon
English Name - Red gram

Figure 6 Habit of *Cajanus cajan* L. (Pe-sin-gon)

Annual shrubs. Stem branches. Leaves alternate, trifoliate, pinnately compound. Inflorescence terminal or axillary, cyme. Sepals 5, valvate, green. Petals 5, papilionaceous, white, deciduous. Stamens 10, diadelphous (1+9). Ovary monocapallary, marginal placentation. Pods oblongoid.

Scientific Name - *Vigna unguiculata subsp cylindrica* (L). Walpers *Phaseolus cylindricus* L.,
Family - Fabaceae
Myanmar Name - Pe-lun
English Name - Cow Pea

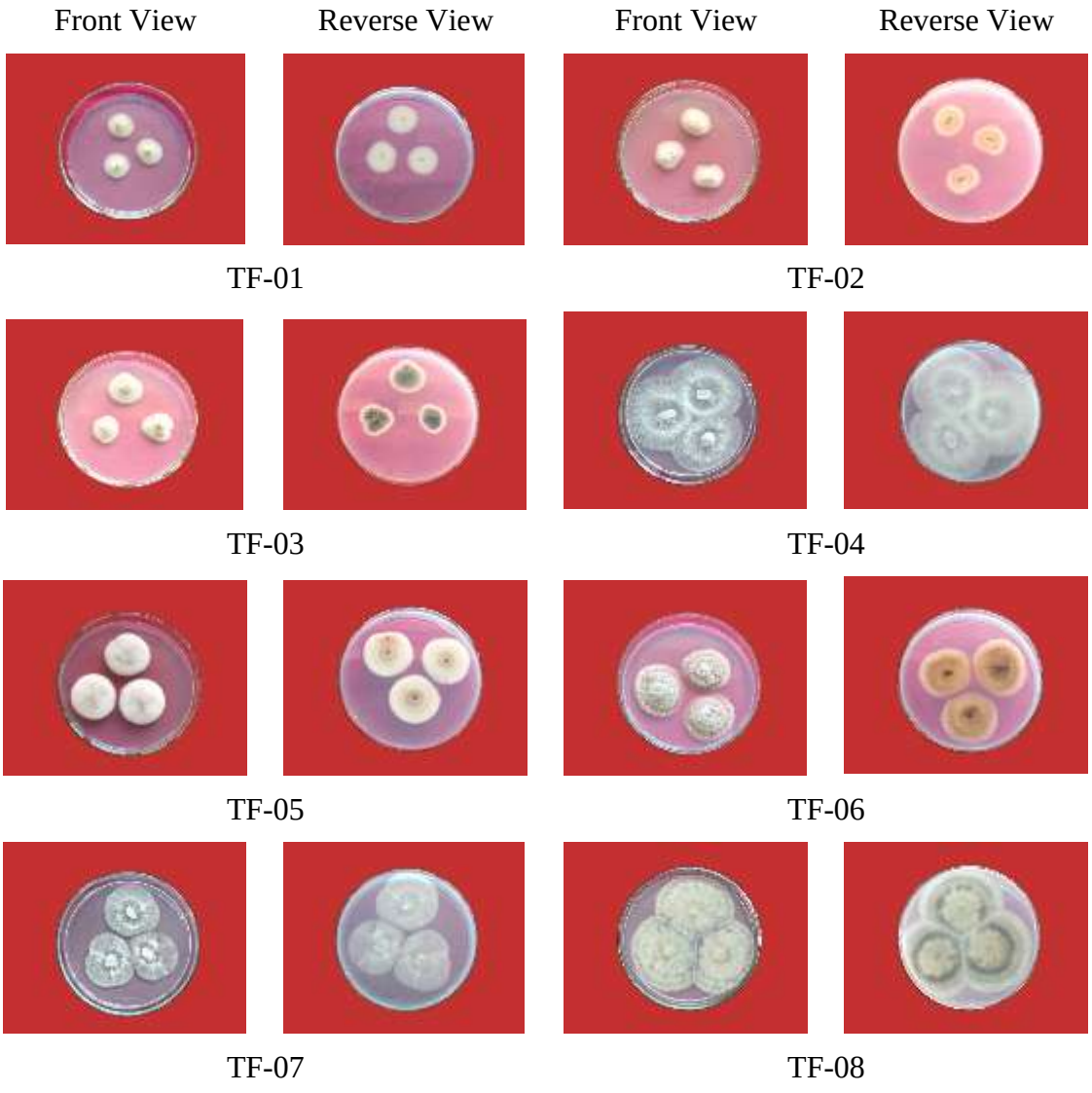
Figure 7 Habit of *Vigna unguiculate* L. (Pe-lun)

Annual herbs, stems subglabrous, stipulate. Leaves trifoliate compound, leaflets ovate-rhomboid. Inflorescences axillary racemes, Flowers blue to purple, papilionaceous; Calyx campanulate; standard suborbicular; wings subdeltoid, blue to purple, keel white stamens 10, diadelphous. Ovary superior, unilocular. Legumes terete. Seeds oblong or reniform.

Scientific Name - *Vigna radiatus* L.
Family - Fabaceae
Myanmar Name - Pe-nauk
English Name - Mung bean

Figure 8 Habit of *Vigna radiatus* L. (Pe-nauk)

Annual erect herb. Stem cylindrical angular. Leaves alternate, trifoliate, pinnately compound. Inflorescence axillary raceme. Sepals 5, valvate, sepaloid, persistent. Petals 5, paoilionaceous, greenish-white. Stamens 10, diadelphous (1+9). Ovary monocapallary, marginal placentation. Pods long and cylindrical.



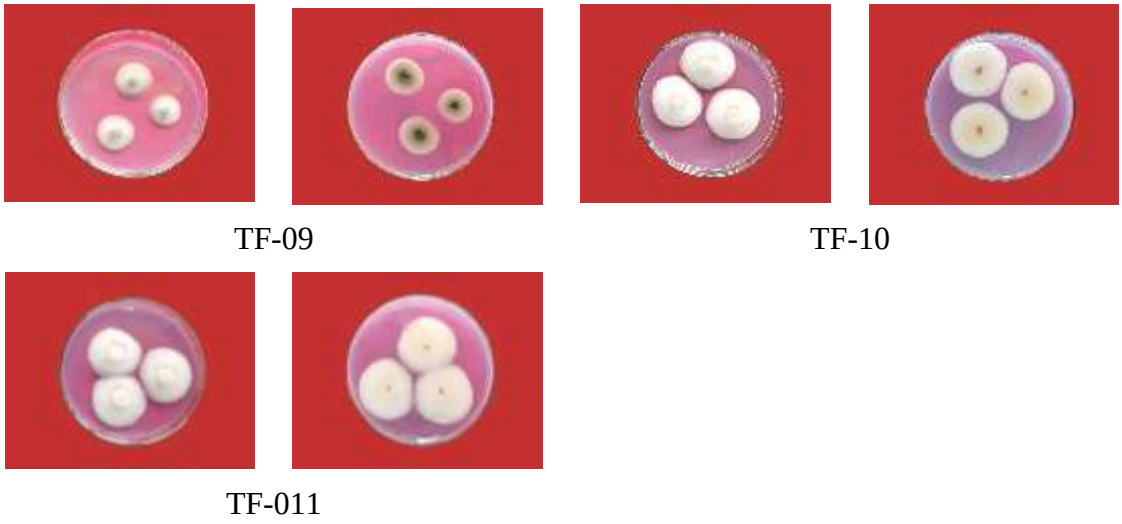


Figure 9 Morphology of fungus TF-01 to TF-11 (7 days old culture on PGA medium)

Table 2 Antibacterial Activities of Isolated Fungi on Test Organisms

Used plants	Isolates	*Escherichia coli*	*Agrobacterium tumefaciens*	*Bacillus subtilis*	*Pseudomonas fluorescens*	*Staphylococcus aureus*
Myay Pea	TF-01	-	18.43	-	-	-
	TF-02	-	16.43	-	-	-
	TF-03	-	12.91	12.96	-	-
Pe-sin-gon	TF-04	-	-	11.00	-	-
	TF-05	-	11.78	-	-	-
	TF-06	-	-	14.98	-	-
Pe-Lun	TF-07		21.39	-	10.89	12.34
	TF-08	-	-	-	-	-
	TF-09	-	12.70	-	-	-
Pe-nauk	TF-10	-	-	-	-	-
	TF-11	-	13.06	-	-	-

Figure 10 Antibacterial activities of isolated fungi TF-07 on *Agrobacterium tumefaciens*

Table 3 The Effect of Ages of Inoculum on Fermentation

Culture Time (hrs)	Activity (Clear zone, mm)
60	18.48mm
72	23.86mm
84	24.06mm
96	**24.21mm**
108	23.49mm
120	20.98mm

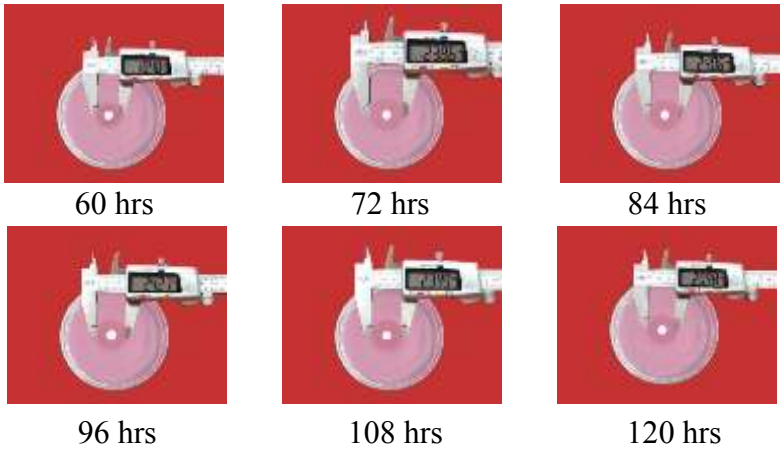


Figure 11 The Effects of Ages of Inoculums on fermentation

Table 4 The Effect of Sizes of Inoculum on Fermentation

Size of inoculum (%)	Antibacterial Activity (Clear zone, mm)
5%	18.02mm
10%	18.78mm
15%	19.70mm
20%	21.69mm
25%	**21.92mm**
30%	18.69mm

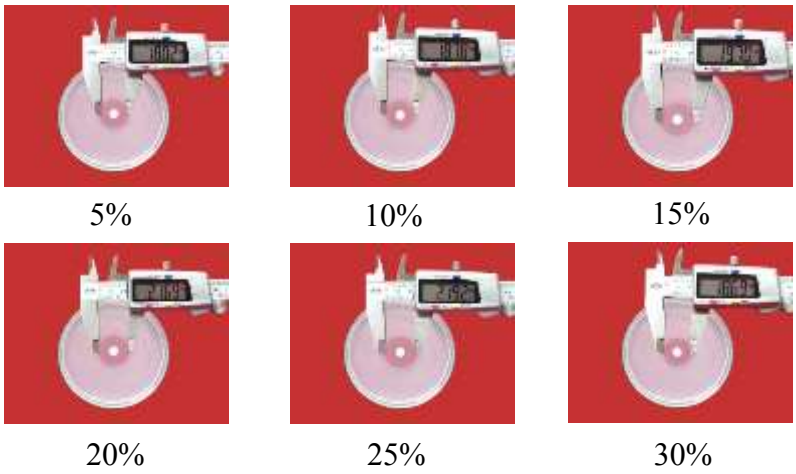


Figure 12 The Effects of Sizes of Inoculums on fermentation

Table 5 Selection of Medium Based on the Results of Antibacterial Activity

Fermentation Medium	Inhibitory Zone (mm)
FM-1	11.56mm
FM-2	20.91mm
FM-3	11.84mm
FM-4	18.89mm
FM-5	12.48mm

According to the results of antibacterial activity, fermentation medium FM-2 was selected for the production of antibacterial metabolite.

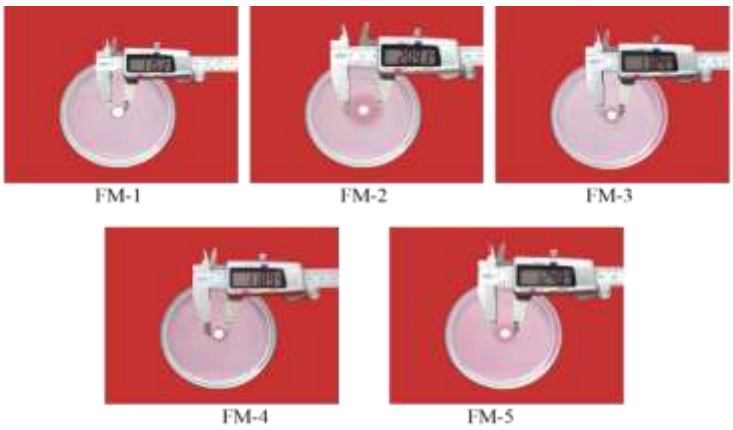


Figure 13 Medium Based on the Results of Antibacterial Activity.

Table 6 Activity of pre-extraction with 4 solvent system

Solvents Used	Activity (mm)	
	Upper Layer	Lower Layer
Hexane	-	18.27
Chloroform	18.42	-
Dichloromethane	18.35	-
Toluene	-	15.39

In this study, it was observed found that Chloroform extract exhibited the highest activity (Table & Figure)

Figure 14 Activity of pre-extraction with 4 solvent system.

Identification of Fungus TF-07

Figure 15 Micrograph of *Trichoderma hamatum* (x 200)

KEY TO GENUS

(Domsch *et al.*, 1993; Klich, 2002; McClenny, 2005; Nyongesa *et al.*, 2015)

KEY TO GROUP

1. Conidium lacking septum…. ……..… Ameroconidium
2. Conidium with 1 septum ….. ……. Didymoconidium
3. Conidium with more than 1 septum and only transverse septa ……..…. Phragmoconidium
4. Conidium body subdivided by intersecting septa in more than one plane .....Dictyoconidium

Ameroconidium

A. Conidiophore not produced
B. Conidiophore not produced or not clear
C. Conidiophores with or without septa develop single, not branched
D. Cpnidiophores with septa develop single and branched

1. Conidia holoblastic
2. Conidia enteroblastic
    i. Phialo-conidium
    ii. Annello-conidium
        - *Paecilomyces*
        - *Penicillium*
        - *Trichoderma* and
        - *Verticillium*
        - According to references key, fungus TF-07 may be the genus of *Paecilomyces, Penicillium, Trichoderma* and *Verticillium.*

Table 7 Comparison between fungus TF-7 and *Paecilomyces, Penicillium, Trichoderma* and *Verticillium*

Genus	Characters
Paecilomyces	Long Phialides
Penicillium	Long Phialides
Trichoderma	Phialides not long and not crowded
Verticillium	Long Phialides
TF-07	Phialides not long and not crowded

According to this comparison (Domsch *et al*, 1993), it was considered that fungus TF-7 may be the group of *Trichoderma*.

Key to the species for Genus *Trichoderma*

(Domsch *et al*, 1993; Klich, 2002; McClenny, 2005; Nyongesa *et al*, 2015)

1. Conidiophores long and thick, side branches mostly short and thick, crowed, short and plump phialide, colonies white ……..…….. 2

Conidiophore and side branches long and slender, phialides not crowed, colony yellowish, bright dull to dark green ……..…..…..... 5

2. Sterile hyphae elongation absent, conidia globose, hyaline…..…….. *T. piluliferum*

   Sterile hyphae elongation present, conidia not globose ……..... 3

3. Conidia green, short ellipsoidal ……. *T. saturnisporum*

   Conidia smooth-walled or finely punctuated……. 4

4. Conidia hyaline, small, 2.4-3.8 x 1,8 x 2.2 mm …… *T. polysporum*

   Conidia white or green, small, 3.8-6.0 x 2.2-2.8 mm…… *T. hamatum*

Based on the references key (Domsch *et al*, 1993; Klich, 2002; Mc Clenny, 2005; Nyongesa *et al*, 2015), and characters, the fungus TF-07 was identified as *Trichoderma hamatum* (Bonord.) Bain. 1906.

Kingdom:	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	Hypocreales
Family	Hypocreaceae
Genus	Trichoderma
Species	*T. hamatum*

Discussion and Conclusion

In the isolation of endophytic fungi, eleven fungus was isolated from the four kinds of Fabaceae plants from Kanpyar Village, Magway Region.

Among them isolated endophytic fungi TF-01, TF-02, TF-03 were isolated from Myay pea, TF-04, TF-05, TF-06 were isolated from Pe-sin-gon, TF-07, TF-08, TF-09 were isolated from Pe-lun and TF-10, TF-11 were isolated from Pe-nauk. Isolated endophytic fungi TF-07 were coinvestigated their antibacterial activities by using paper disc on assay medium for five test organisms.

Isolated fungi, antibacterial activities were excellent growth on *Agrobacterium tumefaciens*. To observe the age of inoculum, six different hours of 60hrs, 72hrs, 84hrs, 96hr, 108hrs, 120hrs and for the size of inoculums 5%, 10%, 15%, 20%, 25% and 30% were used respectively.

In conclusion, the present study described the fungi *Trichoderma hamatum* from some species of Fabaceae collected at Kanypyar Village, Magway Region.

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