OPTIMIZATION OF SOME FERMENTATION PARAMETERS, IDENTIFICATION OF THE ENDOPHYTIC FUNGUS MHA-10 AND EXTRACTION OF CRUDE EXTRACT

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Abstract

Endophytic fungi is a rich source of novel organic compounds with interesting biological activities and a high level of structural diversity. In the present study, endophytic fungus MHA-10 was isolated from the leaves of *Dioscorea birmanica* Prain and Burkill. The pure fungal culture was inoculated in fermentation medium with different growth parameters. In the present investigation, the effect of pH was studied by varying pH 3, 4, 5, 6, 7, 8 and 9. The maximum antibacterial activity was recorded at pH 7 (21.86 mm, inhibitory zone) against *Bacillus subtilis*. Antibacterial activity was observed at different temperatures such as 20ºC, 25ºC, 30ºC, 35ºC and 40ºC. Maximum antibacterial activity by fungal isolate MHA-10 was recorded at 30ºC (19.84 mm, inhibitory zone) against *Bacillus subtilis*. In the comparison of static and shaking culture, the antibacterial activity of shaking culture (20.89 mm, inhibitory zone) was more than that of static culture (19.01 mm, inhibitory zone). Maximum antibacterial activity reached at 5 days fermentation period (22.38 mm, inhibitory zone) against *Bacillus subtilis.* In the paper chromatography study, ethyl acetate is suitable for the extraction of crude extract from the fermented broth. Based on the macroscopical and microscopical characters, fungus MHA-10 was identified as *Trichoderma* sp.

Keywords: endophytic fungi, growth parameters, *Trichoderma* sp.

Introduction

Endophytes are microorganisms that are present in living tissues of various plants, establishing mutual relationship without apparently any symptom of diseases (Strobel and Daisy, 2003). It has been known that endophytic fungi are important source of bioactive compounds (Pan *et al.,* 2008).

Several environmental factors, such as temperature, pH and incubation period, play a major role in the production of antimicrobial agents (Lin *et al.,* 2010).

pH is also a very important in parameter because inappropiate pH may change overall physiological and physical environment of microorganisms resulting in decrease in the production of desired product (Verma and Debnath, 2017).

Temperature is known to influence directly the overall growth and development of any organism. It affects the physiology and subsequently the synthesis of various metabolites (Pandey *et al.,* 2005).

Paper chromatography is one of the types of chromatography procedures which run on a piece of specialized paper. It is planar chromatograph system wherein a cellulose filter paper acts as a stationary phase on which the separation of compound occurs. Extraction is a separation process consisting in the separation of a substance from a matrix.

Thin layer chromatography is a method for analyzing mixtures by separating the compounds in the mixture. TLC can be used to help determine the number of components in a mixture, the identify of compound and the purity of a compound (Geiss, 1987).

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Many species in the genus *Trichoderma* can be characterized as opportunistic avirulent plant symbionts (Harman *et al.*, 2004). The fungal *Trichoderma* species inhabiting healthy tissues of host plants as endophytic fungi (Wu *et al.*, 2011).

The aim and objectives of this research were to find out the effect of pH, temperature, shaking and static and fermentation period on the antibacterial activity of fungal isolate MHA-10, to investigate extraction of crude extract from endophytic fungus by using solvent system and to identify the selected fungal isolate MHA-10.

Materials and Methods

Isolation of endophytic fungi

Endophytic fungi MHA-10 was isolated from the leaves of *Dioscorea birmanica* Prain and Burkill, which was collected from Mawlamyine University Campus.

The leaves were thoroughly washed in running tap water. Then the leaf segments were surface sterilized by immersion in 70% ethanol for 1 min and rinsed in sterile distilled water for 1 min. And then the materials were immersed in 70% ethanol for 30 seconds and finally rinsed in sterile distilled water for 1 min and blot-dry.

The surface sterilized leaf segments were evenly spaced in petridishes containing isolation medium (glucose 1.0 g, yeast extract 0.5 g, $MgSO_4$ 0.01 g, K_2HPO_4 0.01 g, agar 1.8 g, distilled water 100 mL) amended with 250 mg/L chloramphenicol. The petridishes were incubated at room temperature and monitored every day for the growth of endophytic fungal colonies from leaf segments. The hyphae, which grew out from leaf segments were isolated and brought into pure culture. The isolated endophytic fungi were identified down to genus level using standard manuals.

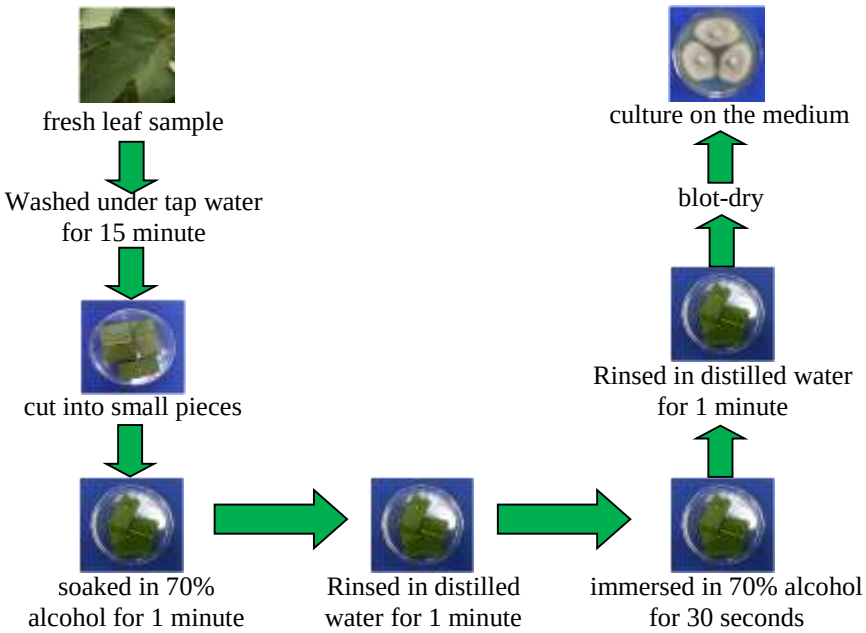


Figure 1 Procedure for isolation of endophytic fungi (BRBDC, 2015)

Fermentation of isolated fungus MHA-10

The isolated fungus MHA-10 was incubated into conical flask containing seed medium (glucose 1.0 g, yeast extract 0.5 g, $MgSO_4$ 0.001 g, KNO_3 0.1 g, distilled water 100 mL) for 3 days. After incubation, the seed culture (30%) was transferred into the conical flask containing

fermentation media (glucose 1.5 g, yeast extract 1.0 g, $MgSO_4$ 0.001 g, KNO_3 0.1 g, distilled water 100 mL). The fermentation period was 3-7 days.

Optimization of some parameters for maximum production of bioactive compounds

Effect of pH on the antibacterial activity of fermentation broth of fungus MHA-10 against *Bacillus subtilis*

The optimization of pH of the fermentation broth for antibacterial activity was done by carrying out the fermentation at seven different pH values viz, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0.

Effect of temperature on the antibacterial activity of fermentation broth of endophytic fungus MHA-10 against *Bacillus subtilis*

The optimization temperature for antibacterial activity was carried out at five different temperatures viz. 20ºC, 25ºC, 30ºC, 35ºC and 40ºC.

Effect of static and shaking condition

250 mL conical flask containing 100 mL of the fermentation medium was incubated on the shaker (150 rpm). Another flask was incubated under static condition without shaking (Figure 2).

Figure 2 Comparison of Static and Shaking culture of fermented broth

Paper chromatography (Tomita, 1988)

Paper chromatography was carried out to extract the crude extract from the fermented broth by the method of Tomita, 1988. The purpose of paper chromatography is to extract the crude extract using suitable solvent systems.

Solvents employed in Paper chromatography

1. 20% NH_4Cl
2. Water saturated n-BuOH
3. n-BuOH-acetic acid-water (3:1:1)
4. Water saturated ethyl acetate

Preparation of PPC

20% ammonium chloride, n-butanol, 3 n-butanol: 1 acetic acid; 1 water and ethyl acetate were used as solvents. The fermented broth samples (100 µL) were applied on the papers and allowed to dry. The paper was immersed in each solvent. Then, bioautography was done to check the antibacterial activity of each. Each paper was placed on assay agar plates. After one hour, they were peel off and kept at over one night. Finally based on R_f value, optimum solvent will be chosen.

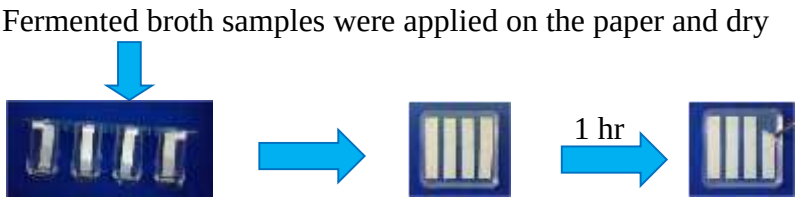


Figure 3 Preparation of paper chromatography

Extraction of the crude extract from fermented broth of the fungus MHA-10

Fermented broth of the fungus MHA-10 was filtered with filter paper to separate the mycelia and the filtrate. To the filtrate equal volume of ethyl acetate was added, shaked well for 30 min and the organic layer was separated and collected (Figure 4).

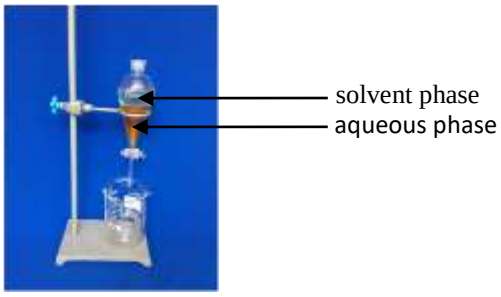


Figure 4 Extraction of the crude extract with ethyl acetate

Thin layer chromatography and Bioautographic Overlay Assay

(Touchstone, 1992 and Aszalos, 1980)

The obtained ethyl acetate extract samples (20 µL) were applied on the TLC plate and allow to dry. The TLC plates were developed in the solvents of chloroform and chloroform-methanol mixture (9:1, 8:2, 7:3) and Hexane and Hexane-ethyl acetate mixture (9:1, 8:2, 7:3). Then, bioautography was done to check the antibacterial activity of each. Each TLC plate was placed on assay agar plates, and then the plates were incubated for 24 hours. In this case, the inhibitory zone was measured yielding an R_f value for the corresponding antibacterial compound. The R_f value can be calculated as

$$R_f \text{ value} = \frac{\text{Distance of compound from origin}}{\text{Distance of Solvent front from origin}}$$

Results

Isolation of Endophytic Fungi

Fungus MHA-10 was isolated from the leaves of *Dioscorea birmanica* Prain and Burkill.

Habit

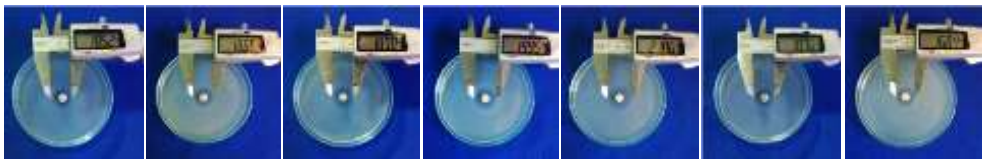
Figure 5 *Dioscorea birmanica* Prain and Burkill

Effect of pH on the antibacterial activity of fermentation broth of fungus MHA-10 against *Bacillus subtilis*

In the present study, pH 7 (21.86 mm) showed the highest antibacterial activity against *Bacillus subtilis* (Table 1, Figure 6).

Table 1 Effects of different pH on the antibacterial activity of selected fungus MHA-10 against *Bacillus subtilis*

pH	inhibitory zone, mm
3	18.52
4	18.66
5	18.98
6	19.95
7	**21.86**
8	18.76
9	16.84



pH-3 pH-4 pH-5 pH-6 pH-7 pH-8 pH-9

Figure 6 Effects of different pH on the antibacterial activity of selected fungus MHA-10 against *Bacillus subtilis*

Effect of temperature on the antibacterial activity of fermentation broth of endophytic fungus MHA-10 against *Bacillus subtilis*

Maximum antibacterial activity was recorded at incubation temperature 30ºC (19.84 mm) and it was followed by 25ºC (17.15 mm) (Table 2, Figure 7).

Table 2 Effect of temperature on the antibacterial activity of fungus MHA-10 against *Bacillus subtilis*

Temperature (ºC)	inhibitory zone, mm
20	14.24
25	17.15
30	**19.84**
35	16.23
40	13.98

20ºC 25ºC 30ºC 35ºC 40ºC

Figure 7 Effect of temperature on the antibacterial activity of selected fungus MHA-10 against *Bacillus subtilis*

Table 3 Differences between Static and Shaking activities of selected fungal isolate MHA-10 against *Bacillus subtilis*

Fermentation condition	Inhibitory zone, mm
Static	19.01
Shaking	**20.89**

The antibacterial activity of shaking culture (20.89 mm) was more than that of static culture (19.01 mm).


Static Culture

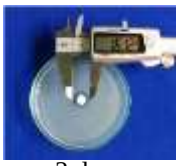

Shaking Culture

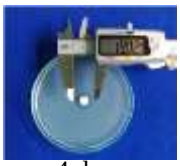
Figure 8 Differences between Static and Shaking activities of MHA-10 against *Bacillus subtilis*

Table 4 Time course of fermentation for the antibacterial activity against *Bacillus subtilis*

Fermentation period (days)	Inhibitory zone, mm
3 day	16.12
4 day	19.02
5 day	**22.38**
6 day	20.30
7 day	19.12
8 day	18.30
9 day	17.63
10 day	16.50

Fermentation was carried out with 84 hrs age and 30% size of inoculum, pH 7.0, 30ºC and FM-1 (glucose 2.0 g, yeast extract 1.0 g, $MgSO_4$ 0.001 g, KNO_3 0.1 g, distilled water 100 mL)


3 days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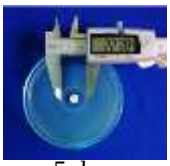
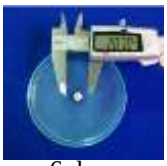
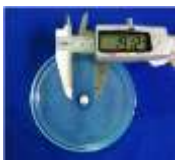
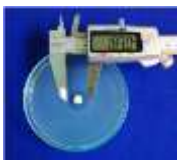

5 days


6 days


7 days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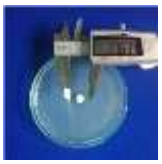

9 days

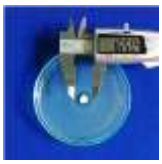
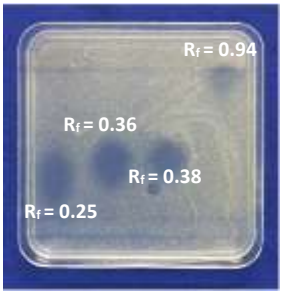

10 days

Figure 9 Antibacterial activity on fermentation period against *Bacillus subtilis*

Paper chromatography to extract crude extract

It was observed that R_f values are 0.25 in 20% NH_4Cl, 0.36 in n-butanol, 0.38 in n-butanol: acetic acid: water (3:1:1) and 0.94 in ethyl acetate. According to the R_f value (Figure 10) it was considered that active compound could be extracted with ethyl acetate.



1. 20% NH_4Cl
2. Water saturated n-BuOH
3. n-BuOH-acetic acid-water (3:1:1)
4. Water saturated ethyl acetate

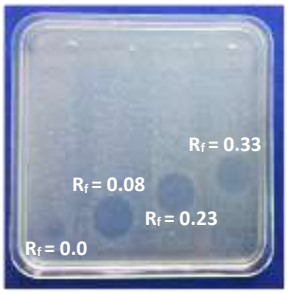
Figure 10 Paper Chromatography Bioautographic Assay

Thin layer chromatography

Thin layer chromatographies were developed by chloroform and chloroform methanol mixture (9:1, 8:2 and 7:3) and hexane and hexane ethyl acetate mixture (9:1, 8:2 and 7:3). The results were shown in Figure 11 and 12.

1. Chloroform only
2. Chloroform-methanol (9:1)
3. Chloroform-methanol (8:2)
4. Chloroform-methanol (7:3)

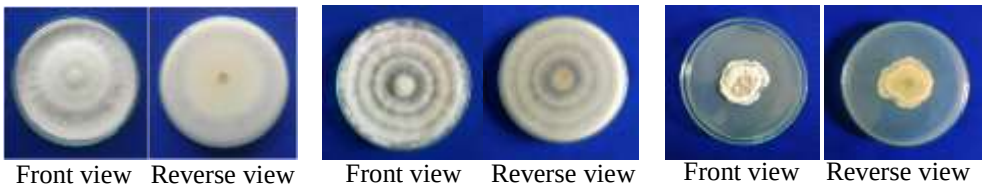
Figure 11 Thin Layer Chromatography with chloroform and chloroform-methanol mixture



1. Hexane only
2. Hexane-ethyl acetate (9:1)
3. Hexane-ethyl acetate (8:2)
4. Hexane-ethyl acetate (7:3)

Figure 12 Thin Layer Chromatography with Hexane and Hexane-ethyl acetate mixture

Identification of the fungus MHA-10



Front view Reverse view on PDA medium

Front view Reverse view on MEA medium

Front view Reverse view on YEA medium

Front view Reverse view on CA medium

Front view Reverse view on OA medium

Figure 13 Macroscopical character of fungus MHA-10

Table 5 Macroscopical characters of MHA-10 on different media

Medium	colour		size
	front	reverse	
MEA	white	pale yellow	9.0 × 9.0 cm
	1-2 concentric ring		
YEA	white	pale yellow	4.1 × 3.8 cm
	with concentric ring		
CA	white	white	9.0 × 9.0 cm
OA	white with dull green patch	white with dull green patch	9.0 × 9.0 cm
PDA	white	pale yellowish	9.0 × 9.0 cm

PDA - Potato Dextrose Agar, MEA - Malt Extract Agar
CA - Corn Agar, OA - Oatmeal Agar,
YEA - Yeast Extract Agar

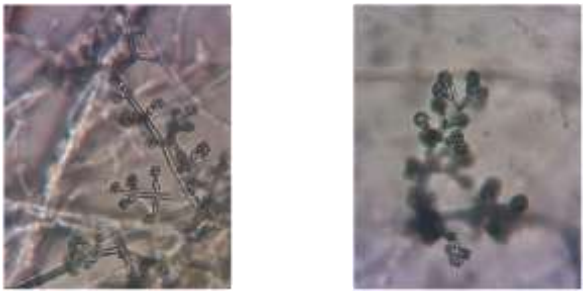


Figure 14 Photomicrograph of MHA-10 (X 400)

Figure 15 Chlamydospore X 400

Macroscopical characters of MHA-10

Colonies grow rapidly and mature in 7 days. On potato dextrose agar, the colonies are wooly. From the front, the color is white. As the conidia are formed, scattered yellow-green patches become visible. These patches may form concentric rings. Reverse is yellowish white.

Microscopical characters of MHA-10

Hyphae are hyaline, septate. Conidiophores are hyaline, branched and display a pyramidal arrangement. Phialides are hyaline, flask-shaped, and inflated at the base. They are attached to the conidiophores at right angles. The phialides are arranged in clusters. Conidia are one-celled and round in shape. They are smooth-walled and grouped in sticky heads at the tips of the phialides. The color of the conidia is green.

These above characters of strain MHA-10 are similar to those of *Trichoderma* (Barnett, 1969).

Key to the genus *Trichoderma* (Barnett, 1969)

A1 Mycelium coenocytic, septa infrequent or absent; conidia present ---------- (conidial PHYCOMYCETES)

* A2 Mycelium not coenocytic, with frequent septa; conidia normally present, except in a few genera ---------- (FUNGI IMPERFECTI) ---------- B1

* B1 Conidia and conidiophores not produced within a pycnidium or acervulus ---------- (MONILIALES) ----------C2

B1 Prasitic on soil-inhabiting rhizopods or nematodes ----------(ZOOPAGALES)

B2 Not parasitic on small, soil-inhabiting animals ----------(MUCORALES)

C1 Conidia more or less coiled or spirally curved, hyaline or dark ----------(parts of Moniliaceae, Dematiaeae and Tuberculariaceae)

* C2 Conidia not coiled ----------D1

* D1 Both conidia and conidiophores (if present) hyaline or brightly colored; conidiophores not united into sporodochia or synnemata (Moniliaceae) ----------E1

D2 Conidiophores forming a sporodochium

* E1 Conidia 1 celled, globose to short cylindrical ----------F2

E2 Conidia more or less globoid, aquatic

F1 Conidiophores absent or reduced to phialides or peg-like sterigmata

* F2 Conidiophores present, although sometimes short ----------G2

G1 Cells of conidiophore not differing greatly from the catenulate conidia

* G2 Conidiophore and its branches distinct from conidia ----------H2

H1 Conidiophores simple or sparingly branched; phialides, if present, not tightly clustered

* H2 Conidiophores mostly branched, sometimes simple, phialides, if present, in groups or clusters ----------I2

I1 Conidia catenulate

* I2 Conidia not catenulate ----------J2

J1 Large, conspicuous, rough-walled chlamydospores present

* J2 Large, rough-walled chlamydospores absent ----------K1

* K1 Conidia produced apically on phialides or branches of conidiophore ---------- L2

K2 Conidia attached both at apex and on sides of conidiophores or its branches

L1 Branches of conidiophore verticillate (at least the larger conidiophores)

* L2 Branches of conidiophore not verticillate, irregular ----------M2

M1 Aquatic on submerged leaves ----------Dimorphospora

* M2 Not aquatic ----------N1

* N1 Conidia held in heads by slime drops ----------O2

N2 Conidia not in slime drops, dry

O1 Conidiophore brush-like, similar to *Penicillium* ----------*Gliocladium*

* O2 Conidiophore branches spreading ----------*Trichoderma*

Classification of endophytic fungus MHA-10

Kingdom	Fungi
Phylum	Ascomycota
Class	Hyphomycetes
Order	Moniliales
Family	Moniliaceae
Genus	*Trichoderma*

Discussion and Conclusion

The antibacterial activity of endophytic fungi MHA-10 was observed on different pH (3, 4, 5, 6, 7, 8 and 9). The result indicated that pH 7.0 was suitable for maximum production of antibacterial activity. Similar reports were given by Verma *et al.,* (2017) and Hassan and Bakhiet (2017). Physical factors such as incubation temperature, can exert different effects on the growth and production phases of secondary metabolism (Rizk *et al.,* 2007). In the present study, maximum antibacterial activity was recorded at 30ºC. In the investigation of the static and shaking condition, shaking condition is the optimum condition for MHA-10 fermentation. Similar observations were made by Hassan and Bakhiet (2017).

Fermentation was undertaken with 84 hrs age and 30% size of inoculum, pH 7.0, temperature 30ºC and FM-1 for 8 days fermentation period. Maximum antibacterial activity was recorded at 5 days fermentation period (22.38 mm, inhibitory zone) against *Bacillus subtilis.*

In conclusion, process parameter like pH 7.0, temperature at 30ºC, 5 days of fermentation period under shaking condition were found to be optimum for maximal production of antibacterial metabolite.

In paper chromatography, four kinds of different solvents were used to observe the optimum extraction ability. According to R_f value, ethyl acetate showed the excellent extraction than other. Therefore, solvent No.4 ethyl acetate is suitable for the extraction of crude extract from the fermented broth.

In thin layer chromatography, chloroform and chloroform-methanol mixture (9:1, 8:2, 7:3) and hexane and hexane-ethyl acetate mixture (9:1, 8:2, 7:3) were used. The isolated MHA-10 was identified by studying the colony morphology on PDA medium and microscopic analysis of reproductive structure. The isolate grew rapidly on PDA medium forming a cottony white colony. The front color is white and the reverse color is pale yellowish. Conidiophores are hyaline and branched, conidia are one cell and globose. Conidia occurred in clusters. These characters were in agreement with those mentioned by Barnett (1969) and Kamala *et al.,* (2015). Based on the above observations, the isolate MHA-10 was identified and assigned to the genus *Trichoderma.*

Acknowledgements

I would like to express my gratitude to Dr Si Si Hla Bu, Rector, Pathein University, for her various guidance, suggestion and permissions to do the research. I am also grateful to thanks Dr Than Tun Pro-rector, Pathein University, for his suggestion and advices. I wish to express most sincere gratitude to Dr Wah Wah Lwin, Professor and Head, Department of Botany, Pathein University, for her guidance, invaluable suggestions and comments offered in writing this research. I also wish to express my thank to Dr Min Min Soe, Professor, Department of Botany, Pathein University, for her encouragement and suggestion for this paper. Many thanks are due to my supervisor, Dr Kyaw Kyaw Lwin, Lecturer, Department of Botany, Pathein University, for his advice, encouragement, understanding and cooperation of this research. My thanks are also extended to all of my friends for their understanding and kind help.

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