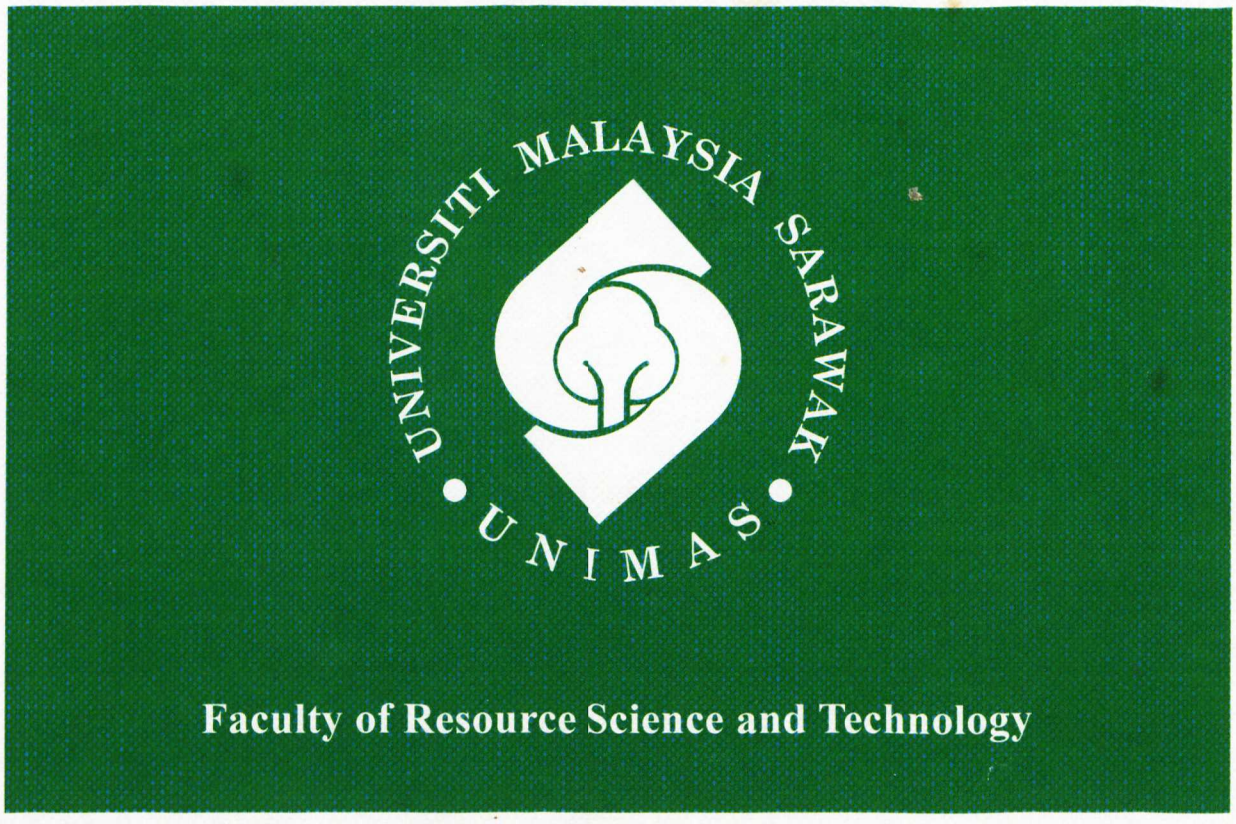

Screening of Selected Fungal Collection for Resistance Against Glyphosate

Fronia Fernanda Dinrark
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Bachelor of Science with Honours
(Resource Biotechnology)
2018

SCREENING OF SELECTED FUNGAL COLLECTION FOR RESISTANCE AGAINST GLYPHOSATE

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51817

A report submitted in partial fulfilment of the Final Year Project 2 (STF3015) Course

Supervisor: AP Dr. Hairul Azman @ Amir Hamzah Roslan

Bachelor of Science with Honours
(Resource Biotechnology)

Faculty Resource Science and Technology
University Malaysia Sarawak
2018

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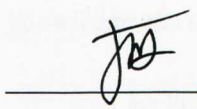

Fronia Fernanda Dinrark

Resource Biotechnology Programme

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

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Screening of Selected Fungal Collection for Resistance Against Glyphosate

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ABSTRACT

Glyphosate is a non-selective broad-spectrum herbicide that commonly been used in the agricultural system to controls the weed populations but also affects microbial populations especially fungi in soil. Plants that were infected by plant pathogenic fungi may affect the crop quality and yield quantity. However, the problem is not all types of fungi are susceptible towards glyphosate because certain types of fungi have different physiological response towards glyphosate. For instance, there are some fungi are resistance towards glyphosate due to the utilization of glyphosate as their food sources and some fungi that are susceptible towards glyphosate due to the interference in fungi shikimate pathway. The significance of this study was to study the physiological response of fungi towards glyphosate. The response of fungi towards glyphosate were monitored by applying a range of glyphosate concentrations (4 000, 8 000, 16 000, and 20 000 ppm) to potato dextrose agar (PDA) medium and following changes in physiological response of ten fungal species for 11 consecutive days in regard to their susceptibility, tolerance and resistance towards glyphosate. The screening of the treatment has been done by *in vitro* and the interaction between glyphosate concentration and the radial growth of ten fungal species. Results showed that susceptible fungi were *Aspergillus aculeatus, Colletotrichum gloeosporioides, Marasmius cladophyllus UMAS MS8,* and *Neurospora crassa*, while the tolerance fungi were *Aspergillus versicolor, Colletotrichum gloeosporioides, Cunninghamella bainieri, Penicillium citrinum,* and *Penicillium pinophilum*, and the only resistance fungi was *Aspergillus flavus.* The fungi also had been identified by morphological and molecular characterization for each of the fungal species.

Keywords: Glyphosate, Susceptible, Tolerance, Resistance, Plant Pathogenic Fungi, Fungal Identification

ABSTRAK

Glifosat adalah racun spektrum luas yang tidak selektif yang lazimnya digunakan dalam sistem pertanian untuk mengawal populasi rumpai dan juga mempengaruhi populasi mikrob terutama kulat di dalam tanah. Tumbuhan yang dijangkiti oleh kulat yang berbahaya boleh menjejaskan kualiti dan kuantiti hasil tanaman. Walau bagaimanapun, tidak semua jenis kulat yang mudah terdedah kepada glifosat kerana kulat yang berlainan jenis mempunyai tindak balas fisiologi yang berbeza terhadap glifosat. Sebagai contoh, ada beberapa kulat yang tidak mudah terdedah terhadap glifosat kerana penggunaan glifosat sebagai sumber makanan mereka dan ada juga beberapa kulat yang mudah terdedah kepada glifosat disebabkan oleh gangguan proses shikimate di dalam metabolisme kulat. Kepentingan kajian ini adalah untuk mengkaji tindak balas fisiologi kulat terhadap glifosat. Tindak balas kulat terhadap glifosat dipantau dengan menggunakan pelbagai kepekatan glifosat (4 000, 8 000, 16 000, dan 20 000 ppm) di dalam media PDA dan pemerhatian terhadap perubahan tindak balas fisiologi oleh semua kesepuluh spesis kulat tersebut dibuat selama 11 hari. Pemerhatian ini dibuat berdasarkan kerentanan, toleransi atau ketahanan kulat tersebut terhadap glifosat. Kajian ini telah dilakukan secara *in vitro* dan interaksi antara kepekatan glifosat dan pertumbuhan jejarian oleh kesemua sepuluh spesis kulat. Hasil kajian menunjukkan bahawa kulat yang mempunyai kerentanan terhadap glifosat adalah *Aspergillus aculeatus, Colletotrichum gloeosporioides, Marasmius cladophyllus UMAS MS8,* dan *Neurospora crassa,* manakala kulat yang toleransi terhadap glifosat adalah *Aspergillus versicolor, Colletotrichum gloeosporioides, Cunninghamella bainieri, Penicillium citrinum,* dan *Penicillium pinophilum,* dan satu-satunya kulat yang mempunyai ketahanan yang tinggi terhadap glifosat adalah *Aspergillus flavus*. Setiap spesis kulat tersebut juga telah dikenal pasti identitinya melalui ciri-ciri morfologi dan molekul.

Kata Kunci: Glifosat, Kerentanan, Toleransi, Ketahanan, Kulat, Pengenalan Kulat

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
Blast	Basic Local Alignment Search Tool
bp	Base pair
DNA	Deoxyribonucleic acid
EPSP	Enolpyruvylshikimate 3-phophate
ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PEP	Phosphoenol pyruvate
ppm	Part per million
S3P	Shikimate-3-phosphate
TAE buffer	Buffer solution containing a mixture of Tris base, acetic acid, and EDTA
UV	Ultraviolet
rpm	Revolutions per minute
EtBr	Ethidium bromide
AMPA	Aminomethyl phosphonic

CHAPTER 1: INTRODUCTION

According to Carris, Little, and Stiles (2012), fungi are an important group of plant pathogens that can cause massive losses in yield and the quality of field crops, fruits, and other edible plant material. This critical issue has affected the food industry where the demand for raw food such as paddy, corn, vegetable and others increase as the human population increase (Yang, Hsiang, Bhadauria, Chen, & Li, 2017). Due to this concern, the usage of herbicides has been used widely in Malaysia especially in oil palm plantation (Zain, Mohamad, Sijam, Morshed, & Awang, 2013a). However, there is a problem where the fungi have varying physiological responses toward the herbicides in term of resistance and susceptibility. Even though a massive amount of herbicide is applied to the plantation it does not kill some of the fungi, but the overuse of herbicide is a threat to the environment and its inhabitants (Smith & Oehme, 1992).

The herbicide that had been used extensively in many countries is glyphosate (N-[phosphomethyl]glycine). Glyphosate is a water-soluble and non-selective broad-spectrum herbicide that can inhibit the growth of fungi as the chemical exerts certain adverse effects to the physiological responses of fungi (Zain *et al.*, 2013a). Glyphosate was developed by Mosanto to help farmers protect their crops from weeds and microbial organisms. This herbicide was applied to the field by randomly which the impact of glyphosate on non-target organisms may be different towards target organisms (Zaller, Heigl, Ruess, & Grabmaier, 2014). In this study, the physiological response of fungi on varying concentration of glyphosate can be determined by the measurement of the radial growth of fungi through *in vitro* approach.

The hypothesis of this study was varying concentration of glyphosate affects the physiological response of the fungi. The first objective of this study was to determine the

resistance of various fungi towards glyphosate. The second objective is to assess the maximum level of fungal response towards varying concentrations of glyphosate.

CHAPTER 2: LITERATURE REVIEW

2.1 Herbicide Resistance

In Malaysia, herbicides have largely replaced mechanical methods to prevent excessive growth of weeds in oil palm plantation (Zain, Mohamad, Sijam, Morshed, & Awang, 2013b). This is because herbicide is more effective and provide economical means of weed control than cultivation, hoeing, and hand pulling (Ware & Whitcare, 2004). Not only that, a study showed that the herbicides also significantly inhibited the development of microbial populations in soil such as bacteria and fungi (Zain *et al.*, 2013b). However, the resistance to herbicide has been growing and is now become the challenges for farmers and implication for the environment (Pannell, Tillie, Rodriguez-Cerezo, Ervin, & Frisvold, 2017).

One of the example that become the challenges for farmers is plant pathogenic fungi that resistance against herbicide. The problem of fungi herbicide resistance is it may inherit the resistance gene to the unwanted plants such as weed and grass. According to Vila-Aiub, Martinez-Ghersa, and Ghersa (2003), fungal symbiotic endophytes, *Neotyphodium spp.* that always infect the cool season grasses has modified the physiology, reproductive biology, and ecology of their hosts. Fungal endophytes are transmitted by vertical and it acts as genetic entities that alter the evolution of herbicide resistance by reducing herbicide effectiveness. Because of this modification, the grasses have a greater resistance towards herbicide and therefore enhanced the susceptibility to plant diseases (Vila-Aiub *et al.*, 2003).

Moreover, plant pathogenic fungi that resistance against herbicide also cause various type of diseases in plants such as rice blast disease by *Magnaporthe oryzae*, grey mould by *Botrytis cinereal*, vascular wilt by *Fusarium oxysporum,* powdery mildews of wheat by *Blumeria graminis*, and anthracnose spots and blights of aerial plant parts by *Colletotrichum*

sp. (Dean *et al.*, 2012). These diseases will affect the production of plants that are very important in the food industry.

2.2 Glyphosate

In 1950, glyphosate was initially discovered by a Swiss chemist, Henri Martin then in 1970, glyphosate was found that it has herbicidal activity by the scientist John E. Franz from Mosanto company which was subsequently patented under the trade name "Roundup". In 1974, Roundup was first commercialized in Malaysia for rubber plantation to control the weed population (Dill *et al.*, 2010).

Glyphosate is a widely used broad-spectrum systematic herbicide and crop desiccant with little harmful to the environment (Tate, Spurlock, & Christian, 1997). The application of glyphosate causes no residual soil activity and does not leach into the non-target areas as it is non-volatile. Glyphosate also is non-toxic to fish, birds, and mammals and showing no bioaccumulation in the food chain as it is biodegraded into natural products. However, only when it used appropriately, glyphosate shows no threat to the environment and its inhabitants (Smith & Oehme, 1992).

2.2.1 Mode of Action

The use of glyphosate in agriculture have modifies the environment which stresses the living microorganisms (Shehata, Schrödl, Aldin, Hafez, & Krüger, 2013). In plants, glyphosate prevents the seed germination by absorbed passively through foliage to areas of meristematic activity which is roots. Moreover, the activity of herbicides also can inhibit the mycelial growth and spore germination of fungi, change the level of phytoalexins, or interfere the physiological processes in plants (Sanyal & Shrestha, 2008)

A studied by Schönbrunn *et al.* (2001) found that the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase is important for the biosynthesis of aromatic amino acids in higher plants, algae, bacterial and fungal. EPSP synthase is a biological target for antimicrobial agents such as glyphosate that effective against fungal and bacterial pathogens. EPSP synthase function is to catalyze the transfer of the enolpyruvyl moiety from phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P) forming the EPSP-S3P complex. Glyphosate is a competitive inhibitor of PEP, that binds more tightly to the EPSP-S3P complex than substrate PEP. Therefore, once glyphosate binds to the EPSPS-S3P complex, the catalysis of the enzyme is inhibited and thus shuts down the shikimate pathway as shown in ***Figure 1*** (Schönbrunn *et al.*, 2001). When the shikimate pathway shut down, the synthesis of aromatic amino acids which are required for the organism to survive also shut down.

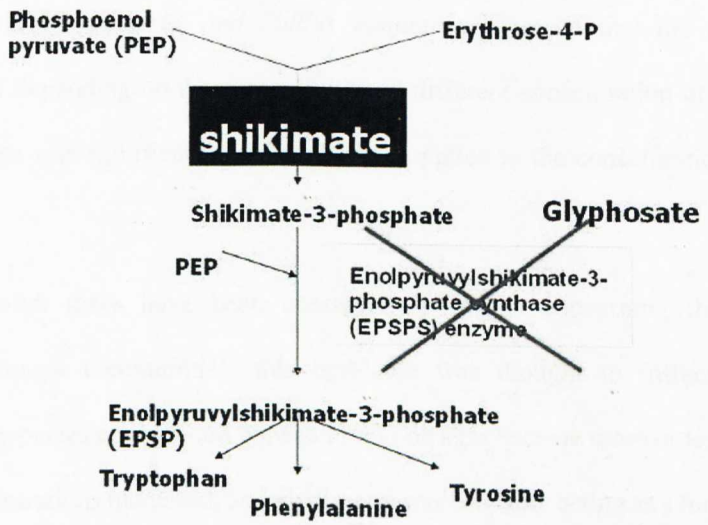

Figure 1. Aromatic amino acid pathway and site of glyphosate inhibition. Reprinted from *Grains Research and Development Corporation*, D. Shaner, 2013, Retrieved from https://grdc.com.au/resources-and-publications/grdc-update-papers/tab-content/grdc-update-papers/2013/02/developing-a-field-assay-kit-to-detect-glyphosate-resistance

2.3 The Response of the Diversity of Fungi Towards Glyphosate

According to Zain *et al.* (2013b), fungi were most affected by glyphosate. However, the impacts of glyphosate on fungi also varied depend on their species, pathogen inoculum, soil properties, the timing of herbicide application, and tillage. For example, *Mycorrhizal* fungi are sensitive to glyphosate, while others such as *Fusarium* fungi are resistance towards glyphosate under certain conditions as glyphosate may serve as nutrient and energy source to the fungi (Schütte *et al.*, 2017).

In the previous study by Lévesque, Rahe, & Eaves (1993), sub-lethal doses of glyphosate had induced susceptibility to *Fusarium oxysporum* in two resistant tomato cultivars and increase the resistant bean to *Colletotrichum lindemuthianum.* However, according to Chakravarty and Sidhu (1987), glyphosate that was tested on five species of ectomycorrhizal fungi which are *Hebcloma crustuliniforme*, *Laccaria laccata*, *Thelophora amcrieana*, *Thielavia terrestris*, *and Suillus tomentosus* showed that the response of individual fungal depending on the susceptibility to different concentration of glyphosate. The fungal growth was significantly reduced when applied to the concentration above 10 ppm.

Even though there have been contradictory results concerning the effect of glyphosate on fungal communities, this herbicide was thought to influence species interactions. Interspecies competition between fungi on agar became more or less intense as glyphosate concentrations increased, and glyphosate was probably acting as a fungal nutrient (Tsui, Hyde, & Hodgkiss, 2001). This is because different fungal species may exhibit different physiological responses towards glyphosate. As for example, Tsui *et al.* (2001) stated that some fungal strains have been shown to utilize glyphosate as a source of phosphorus or carbon.

CHAPTER 3: MATERIALS AND METHODS

3.1 Maintenance of Fungal Cultures

3.1.1 Preparation of Potato Dextrose Agar (PDA) Medium

3.1.1a Preparation of 30 mg/ml Antibiotic Chloramphenicol Stock Solution

To prepare 10 ml of stock solution of antibiotic chloramphenicol, 0.3 g of chloramphenicol powder (DUCHEFA, Netherlands) were weighed and diluted with 10 ml of absolute ethanol that was prepared inside a 15 ml falcon centrifuge tube. Then, the tube was vortexed until the chloramphenicol powder completely diluted. After that, 1 ml of 10 ml of solution was transferred into each 1.5 ml centrifuge tubes and stored at -20 °C for further use.

3.1.1b Potato Dextrose Agar (PDA) medium

Preparation of Potato Dextrose Agar (PDA) medium was conducted by weighing 19.5 g of PDA powder (Merck, Germany). Then, the PDA powder was suspended in 500 ml of distilled water in Schott bottle. The medium was then heated to dissolve completely using hot plate stirrer. After completely dissolved, the medium was sterilized by autoclaving at 121 °C for 15 min.

Next step was conducted inside the sterile laminar flow hood and in sterile condition to avoid any contamination. After the PDA medium was autoclaved, the medium was cooled to handling temperature. Before pouring the medium into the Petri-dishes, 500 µl of the medium was discarded and 500 µl of antibiotic chloramphenicol was added into the medium (for 500 ml of PDA). Then, the mixtures were mixed thoroughly. After thorough mixing, about 10 ml of sterilized PDA medium was poured into the Petri-dishes. These Petri-dishes were kept in a sterile laminar flow hood until the culture medium solidified and dried for

about 30 min. Then, the plates were stored in a refrigerator or it can be used immediately for subculture.

3.1.2 Fungal Subculture

The experiment was carried out with ten selected fungal species (*Aspergillus aculeatus, Aspergillus flavus, Aspergillus versicolor, Colletotrichum gloeosporioides, Colletotrichum truncatum, Cunninghamella bainieri, Marasmius cladophyllus UMAS MS8, Penicillium citrinum, Penicillium pinophilum, and Neurospora Crassa* that were obtained from the fungal collection of Molecular Genetic Laboratory (MGL), UNIMAS.

The fungal colonies of the pure fungi culture were sub-cultured on Potato Dextrose Agar (PDA) growth medium in Petri-dish (90 mm). The sub-culture was performed inside the laminar flow hood to avoid any contamination. 5 mm diameter of fungi plug was aseptically cut and transferred using sterile blade. For each fungal species three replicates were prepared. Then, each Petri-dish was covered and sealed with parafilm to avoid contamination and labelled with the species name and date (the day of sub-cultured). The fungal isolated was then incubated at room temperature in darkness until it was fully grow, and then maintained in refrigerator at 4 °C as a pure stock culture.

3.2 Morphological Identification of Fungal Species

The identification of fungal species was conducted via two methods, macroscopic and microscopic identification. Macroscopic identification was done by examined the colony surface and colony reverse of the fungal cultures. Surface and reverse colony observations were done with regards to the colony appearances, mycelial textures, and pigmentation.

For microscopic identification of the isolated fungi, it was performed via lactophenol blue slide mount. First, lactophenol blue solution (Merck KgaA, Germany) was dropped onto the clean glass slide. A tiny piece of the colony was then transferred onto the lactophenol droplet on the clean slide and the fungi colonies were then tease into very tiny pieces by using sterile iron needle. Then, the preparation was covered with a cover slip. Finally, the wet preparation was examined under microscope with different magnification.

3.3 Molecular Identification of Fungal Species

3.3.1 Fungi DNA Extraction

3.3.1a Preparation of Proteinase K Stock Solution

The stock solution of 20 mg/ml Proteinase K was prepared by weighing 20 mg of Proteinase K (Merck KgaA, Germany) and it was then diluted with 500 µl of TE buffer and 500 µl of 100% glycerol in a clean 1.5 ml microcentrifuge tube. Then, Proteinase K was stored at -20 °C for further use.

3.3.1b DNA Extraction

The genomic DNA was extracted by using GF-1 Plant DNA Extraction Kit (Vivantis). About 10 – 30 mg of tissues sample was cut into small pieces with a sterile scalpel and then the fungi sample was homogenized by allowing to freeze in liquid nitrogen. Then, the fungi sample was grinded into fine powder with a mortar and pestle. After the sample became fine powdery form, 280 µl of Buffer PL was added to the ground sample for tissue lysis. Next, the sample was transferred into a clean 1.5 ml microcentrifuge tube and mixed thoroughly by vortexing the tube for 30 secs to obtain a homogeneous. After mixing, 20 µl of 20 mg/ml Proteinase K was added and the tube was inverted to mix thoroughly. The

sample was then incubated in a water bath at 65 °C for 1 to 2 hrs and mix several times during incubation until the tissue mixture appeared clear.

Next, the sample was centrifuged at 12, 300 rpm for 5 min by using GYROZEN micro centrifuge machine to precipitate any soluble/undigested materials. The supernatant containing the DNA (max. 200 µl) was transferred into a clean 1.5 ml microcentrifuge tube. After that, 2 volumes of Buffer PB was added and mixed thoroughly by inverting the tube several times until a homogeneous solution was obtained and incubated at 65 °C for 10 min. After incubated, 200 µl of absolute ethanol was added and mixed immediately to prevent uneven precipitation of nucleic acid, subsequently followed by transferring the sample into a column (max. 900 µl). The column containing DNA sample was then centrifuged at 10, 000 rpm for 1 min and the flow was discarded. Next, a column washing was performed with 750 µl of Wash Buffer and then it was centrifuged at 10, 000 rpm for 1 min. This column washing was repeated for the second time to remove residual ethanol.

After that, the flow was discarded and centrifuged again with the same speed to remove residual ethanol. The last step was DNA elution. The column was transferred into a clean 1.5 ml microcentrifuge tube, followed by adding 60 µl preheated Elution Buffer onto column membrane. Then, the tube was allowed to stand for 2 min before centrifuged at 10, 000 rpm for 1 min. Finally, the pure DNA obtained was stored at -20 °C for further analysis.

3.3.2 Polymerase Chain Reaction (PCR) Amplification

The pure DNA from the extraction was use as DNA template in the PCR. The PCR mixtures were prepared as in ***Table 1*** and the PCR reactions were carried out in a thermocycler (Major Cycler by MS Major Science, California) with the total of 35 cycles. In this study, primer pairs ITS1 and ITS4 were used to amplify ribosomal internal transcribed spacer (ITS) regions.

Table 1. PCR Master Mix component and volume.

Reagent	Volume (µl)
Primer ITS-1	1.0
Primer ITS-4	1.0
Nuclease-Free Water (Promega by Madison, USA)	5.5
DNA template	1.0
GoTaq® Green Master Mix (Promega by Madison (USA)	7.5
Total volume	**16.0**

The amplification of DNA with the primer pairs ITS1 and ITS4 was performed based on the parameter (***Table 2)*** which has been set where the denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. A final extension step at 72 °C for 10 min was then employed.

Table 2. Thermal cycling profile for PCR reaction.

Parameter	Temperature (°C)	Time (min)	No. of cycles
Initial denaturation	95	2.0	
Denaturation	95	0.5	35
Annealing	55	0.5	
Extension	72	1.0	
Final extension	72	10.0	

3.3.3 Gel Electrophoresis

3.3.3a Agarose Gel

0.8% of Agarose gel was prepared by measuring 0.8 g of Agarose (1st BASE, Singapore) and the agarose powder was mixed with 80 ml of 1X TAE Buffer (Vivantis) in a microwave flask. Then, the mixtures were heated in the microwave for 2 min until the agarose was completely dissolved. Next, the agarose solution was poured slowly into a casting tray and 1 µl of Ethidium bromide (EtBr) was added to the agarose solution. Then,