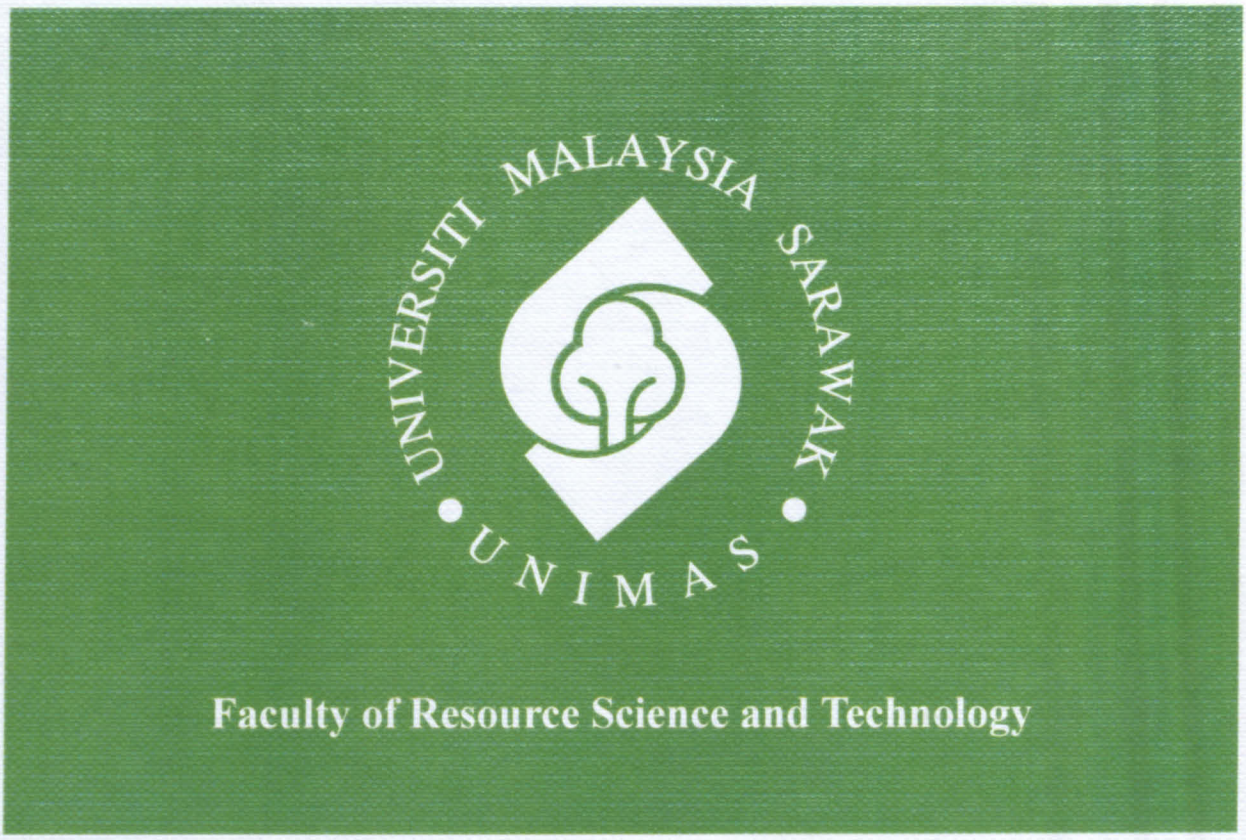

A Study of Selected Fungal Resistance Against Glufosinate

Nor Diana Binti Zulkefli

(50726)

Bachelor of Science with Honours
(Resource Biotechnology)
2018

A Study of Selected Fungal Resistance Against Glufosinate

Nor Diana Binti Zulkefli (50726)

A final project report submitted in partial fulfilment of the
Final Year Project II (STF 3015) Resource Biotechnology

Supervisor: AP Dr Hairul Azman @ Amir Hamzah Roslan

Resource Biotechnology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

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A Study of Selected Fungal Resistance Against Glufosinate

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Abstract

Glufosinate is a contact herbicide which has been widely used in the plantation in order to help prevent plant diseases that caused by pathogenic fungi. However, it becomes a problem when there are some fungi that are naturally resistant towards glufosinate. If those plant pathogenic fungi was not eliminated by usage of herbicide, it may pose threat to worldwide food security. Hence, in this study, fungi that resistant towards glufosinate had been identified. Besides, the maximum level of glufosinate that can be resisted by each type of selected fungi also had been determined in this study. All fungi samples underwent molecular and morphological identification to confirm their identity. Then, the resistance of each fungi was tested by treating the fungi samples with different concentration of glufosinate(0, 4 000, 8 000, 16 000 and 20 000 ppm). Results from this study had shown fungi that were susceptible towards glufosinate were *Aspergillus versicolor, Colletotrichum gloeosporioides, Marasmius cladophyllus, Penicillium pinophilum* and *Neurospora crassa.* The tolerant fungi were *Aspergillus flavus, Cunninghamella bainieri, Penicillium citrinum* and *Aspergillus acuelatus*. Fungi that was resistant towards glufosinate was *Colletotrichum truncantum.*

Keywords: Glufosinate, plant diseases, resistance.

Abstrak

Glufosinate adalah racun rumpai sentuh yang telah digunakan secara meluas dalam ladang untuk membantu mencegah penyakit tumbuhan yang disebabkan oleh kulat patogen. Walau bagaimanapun, ia menjadi masalah apabila terdapat kulat yang mempunyai daya tahan semula jadi terhadap glufosinate. Sekiranya kulat penyebab penyakit tumbuhan itu tidak dihapuskan dengan penggunaan racun rumpai, ia boleh menimbulkan ancaman kepada keselamatan makanan di seluruh dunia. Oleh itu, dalam kajian ini, kulat yang mempunyai daya tahan terhadap glufosinate telah dikenalpasti. Selain itu, tahap maksimum glufosinate yang boleh ditahan oleh setiap jenis sampel kulat terpilih juga telah ditentukan dalam kajian ini. Semua sampel kulat menjalani pemeriksaan molekul dan bentuk struktur untuk mengesahkan identiti mereka. Kemudian, daya tahan setiap kulat diuji dengan merawat sampel kulat dengan kepekatan glufosinate yang berbeza (0, 4 000, 8 000, 16 000 dan 20 000 ppm). Hasil daripada kajian ini menunjukkan kulat yang mudah terkesan dengan glufosinate ialah Aspergillus versicolor, Colletotrichum gloeosporioides, Marasmius cladophyllus, Penicillium pinophilum dan Neurospora Crassa. Kulat yang toleran terhadap glufosinate ialah Aspergillus flavus, Cunninghamella bainieri, Penicillium citrinum dan Aspergillus acuelatus. Kulat yang mempunyai daya tahan terhadap glufosinate ialah Colletotrichum truncantum.

Kata kunci: *Glufosinate, penyakit tumbuhan, daya tahan.*

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mm	Millimetre
w/w	Weight/weight
ppm	Part per million
g	Gram
mg/L	Milligram per litre
kg	Kilogram
g/kg	Gram per kilogram
mg/kg	Milligram per kilogram
PDA	Potato Dextrose Agar
UV light	Ultraviolet light
$M_1V_1 = M_2V_2$	$(Molar_1) \times (Volume_1) = (Molar_2) \times (Volume_2)$
ml	Millilitre
µl	Microlitre
rcf	Relative centrifuge force
Buffer PB	Phosphate buffer
rpm	Rotation per minute
°C	Degree Celcius
DNA	Deoxyribonucleic acid
min	Minute
TAE buffer	Tris base, acetic acid and EDTA buffer
S. D	Standard Deviation
A.G.E	Agarose Gel Electrophoresis

1.0 Introduction

Human food sources are mainly from plants and it is important to secure the survival of healthy plants in order to maintain the continuity of food sources. However, plant pathogenic fungi, will or may cause a nuisance to the crop as it may cause diseases to the crop and this may pose threat to the worldwide food security (Fisher *et al.*, 2012). For instance, the Irish famine in 1845-1852 caused by the *Phytophthora infestans* that infect the potato crops and led to the death of nearly 1 million people (Maranzani, 2013). Glufosinate is a contact herbicide which is effective when it comes to contact with the plant (weeds), as it diffuses into the plant through leaves and stems. Besides eliminate weeds, glufosinate also has antifungal properties.

Hence, to avoid plant diseases, glufosinate can be used to eliminate the plant pathogenic fungi. However, according to Cox (1996), it becomes a problem when in nature, there are some plant pathogenic fungi that are naturally resistant towards herbicide and hence, make it complicated to remove. There were not many studies done on level of resistance of various fungi towards glufosinate as commonly, experiments were only done to compare the herbicidal effect of different types of herbicides. Therefore, in this project, the resistance level of various selected fungi toward glufosinate had been tested. Ten different fungi were used as test subjects. The fungi were *Aspergillus acuelatus, Aspergillus flavus, Aspergillus versicolor, Colletotrichum gloeosporioides, Colletotrichum truncantum, Cunninghamella bainieri, Marasmius cladophyllus, Penicillium citrinum, Penicillium pinophilum* and *Neurospora crassa*. The fungi samples were obtained from Genetic Engineering Laboratory (GEL), UNIMAS.

The fungi were tested with different concentration of glufosinate, which was 0, 4 000, 8 000, 16 000 and 20 000 ppm. There were three replicates of each type of fungi for each different concentration of glufosinate. The growth of fungi on each plate (different concentrations) was observed and evaluated. The growth of fungi was measured until 22 days (reading taken on 2^{nd}, 4^{th}, 7^{th}, 11^{th}, 16^{th}, and 22^{nd} day) using radial growth.

The microscopic and macroscopic views were also recorded. In order to confirm the identity of the fungi samples, confirmation of the fungi identity was carried out, through DNA extraction from each type of fungi samples. After DNA extraction, the DNA was amplified using Polymerase Chain Reaction (PCR) and the PCR products obtained were run on Agarose Gel Electrophoresis (AGE). Then, DNA purification was done to purify the DNA from PCR products before the samples were sent to sequencing.

The objectives of this experiment were to identify the fungal that resistant towards herbicide glufosinate and to determine the maximum concentration of glufosinate that can be resisted by each type of fungi tested. The hypothesis of this experiment was, as the glufosinate concentration increases, the radial growth of fungi decreases. Based on the result of this experiment, the fungi that were susceptible towards glufosinate were *Aspergillus versicolor, Colletotrichum gloeosporioides, Marasmius cladophyllus, Penicillium pinophilum* and *Neurospora crassa*. The tolerant fungi were *Aspergillus flavus, Cunninghamella bainieri, Penicillium citrinum* and *Aspergillus acuelatus*. Then the fungi that can resist towards glufosinate was *Colletotrichum truncantum* only (up to 20 000 ppm of glufosinate).

2.0 Literature Review

2.1 Characteristic of Fungi

Fungus, the singular noun for fungi, is a eukaryotic organism that obtains nutrients by absorbing them directly through its cell walls and digest them externally (Carris *et al.*, 2012). Most fungi have a structure called mycelium, a filamentous thallus, and the mycelium consists of hyphae, microscopic branching tubular cells, that assist in absorbing the nutrients from the foods. The fungal cell wall is made from chitin and glucans, chitosan and other components as well (Kirk *et al.*, 2008). They can be decomposers (brown and white rot), some are beneficial partners in symbiosis (lichen) or they can be parasites or pathogens of other organisms. Fungi are heterotroph as their energy and carbon sources are acquired from another organism (Carris *et al,* 2012). Fungi are an ancient or old group as oldest fossils of fungi are 460 to 455 million years old, from Ordovician (Redecker *et al*, 2000).

Most fungal reproduce by the formation of spores that lack an embryo but they have food reserves needed for germination. Spores are the dispersal or survival unit consist of one or few cells that responsible in germinating and produce new hypha (Carris *et al.*, 2012). The spores may be produced through the asexual or sexual process. According to Griffin (1996), the asexual process involves the process of mitosis (mitospores) only but the sexual process involves a process of mating and meiosis (meiospores) and these processes may need different optimum conditions and environments such as light, moisture, nutrients, and temperature.

2.2 Plant Pathogenic Fungal

Plant pathogens fungi are the parasites but all parasites are not pathogens. Parasites obtain nutrients from the host but do not necessarily cause any symptoms to the host. On the other hand, pathogenic fungi do exhibit symptoms to the host and cause diseases (Carris *et al.*, 2012). Most plant diseases are caused by pathogenic fungal. The plant pathogenic fungi may cause changes to the host plant in terms of the growing style and plant growth regulators level. According to Carris *et al.* (2012), the example of changes caused by the pathogen are cankers, witches broom, galls, stunting and also leaf curl.

The plant pathogenic fungal may pose threat to food security as a major food sources come from plants. Plant pathogenic fungi caused infection in a variety of plants, for instances, *Phytophthora infestans* caused late blight in potatoes, *Magnaporthe oryzae* caused rice blast in rice, *Phakospora pachyrizi* caused rust in soybean, *Puccinia graminis* caused rust in wheat, and *Ustilago maydis* caused smut in maize (Fisher *et al.*, 2012).

2.3 Herbicide

An herbicide is chemical substances used to control the growth of unwanted vegetation by killing the weeds that may disturb and affect the growth of crop plants. The plant pest may affect the growth of crop plants by competing for the water, light, nutrients and also spaces (Schonbeck, 2013). Normally, the herbicide may be applied to the plant before or after the crop has established. There are two types of herbicide which are the selective and non-selective (broad spectrum) as it is only toxic to specific plant species and toxic to any plants that come into contact respectively (Oregon State University, 2017). Example of selective herbicide is the 2,4-Dichlorophenoxyacetic acid and the non-selective herbicide are glyphosate and glufosinate.

2.4 Glufosinate Herbicide

In this experiment, the herbicide used was glufosinate. Several species of the *Streptomycetacea* family do produce glufosinate naturally and this herbicide does show antibacterial and antifungal activity (Tothova et al., 2010). According to Hoerlein (1994), the active ingredient in glufosinate is D,L-Phosphinothricin (D,L-PPT) and its commercial or common name is glufosinate ammonium. It is commercially available under tradenames of Basta®, Buster®, Challenge®, Finale®, Ignite® and Harvest® (Hoerlein, 1994). In this study, glufosinate from Basta® which contained 13.5 % w/w of glufosinate was used. Previously, glufosinate was proved to have a very weak antibiotic action of D,L-PPT but it was proved in another way around by Agrochemical Research Department, even at low concentration, the herbicidal effect of glufosinate still efficient and significant.

According to Hoerlein (1994), glufosinate inhibits the activity of glutamine synthetase, an enzyme which synthesize amino acid glutamine and hence blocks the usual function of the enzyme. According to Krogmann et al., (as cited in Albrecht & Kortekamp, 2009), accumulation of toxic ammonium occurs when glutamine synthetase is inhibited and disrupts electron transport systems and induces production of free radicals. According to Hess's study (as cited in Albrecht & Kortekamp, 2009), free radicals can cause lipid peroxidation and cell death.

2.5 Glufosinate Reduce Fungal Disease

Glufosinate can help in reducing the fungal disease even it is applied before or after the inoculation of fungi. As mentioned by Wang et al. (2003), the effectiveness test of glufosinate was done on two types of the fungal pathogen which were the *Rhizoctonia solani* and *Sclerotinia homoeocarpa* with 560 mgL^{-1} of glufosinate at a rate of 0.56 kg ha^{-1}. The infection on the transgenic grasses, which were the glufosinate-resistant transgenic creeping and velvet bentgrass plants, by *R. solani* and *S. homoeocarpa*, was significantly reduced with the application of glufosinate three hours before or one day after the fungal inoculation (Wang *et al.*, 2003).

Besides, glufosinate also reported to has fungicidal activities to grape pathogens. *Botrytis cinerea*, *Guignardia bidwellii*, *Penicillium expansum*, *Phomopsis viticola* are one of several fungi organisms that putatively pathogenic to the grapevine. These fungi were tested and exposed to glufosinate in an experiment done by Albrecht *et al.* (2009). According to the report, the mycelial growth of fungi was reduced by the herbicidal compound in a concentration-dependent manner. Besides, built-up of secondary inoculum in the same season or primary inoculum for the following year could be reduced by spray applications of glufosinate to the ground which lead to a dispersal of small amounts to non-treated areas (Albrecht & Kortekamp, 2009).

3.0 Material and Method

3.1 Molecular and Morphological Identification of Selected Fungi

3.1.1 Fungi DNA Extraction

Genomic DNA extraction of fungi samples were conducted using GF-1 Plant DNA Extraction Kit (Vivantis). About 10-30 mg of tissue sample was cut into small pieces with a clean scalpel and allowed to freeze in liquid nitrogen. The sample was then grounded into a fine powder with a mortar and pestle. Then, 280 μl of Buffer PL was added to the ground sample for tissue lysis. The tube was mixed by vortexing for 30 sec to obtain a homogeneous solution before adding 20 μl of Proteinase K. According to Epoch Life Science (2009), with some modifications, the solution needed to be incubated in a water bath for 1-2 hours at 65°C. Next, centrifuged the sample at 12,300 rcf for 5 mins to precipitate any soluble/undigested materials. Then, transferred the supernatant containing the DNA into a clean microcentrifuge tube. Next, performed the removal of RNA by adding 40 μl of RNase A (DNase-free, 20mg/ml). Mixed and incubated the sample at 37°C for 5 minutes. Added 2 volumes of Buffer PB into the supernatant to obtain homogeneous solution. Then, allowed incubation for 10 minutes at 65°C. Next, added 200 μl of absolute ethanol and mixed immediately, subsequently followed by loading into a column (max. 900 μl).

Centrifuged the column containing DNA sample at 10,000 rpm for one minute and discarded the flow. Next, performed a column washing with 750 μl Wash Buffer and centrifuged at 10,000 rpm for one minute. The flow was discarded through and again re-centrifuged with the same speed to remove residual ethanol. The last step was DNA elution. The column was transferred into a clean microcentrifuge tube, followed by adding 60 μl of preheated Elution Buffer onto column membrane.

Then, the tube was allowed to stand for 2 minutes before centrifuging at 10,000 rpm for one minute. Stored in the pure DNA obtained at 4°C before proceeding to the next step.

3.1.2 Polymerase Chain Reaction (PCR)

Prior to beginning of the reaction, the PCR mixture was prepared as follows: 2.0 μl of Primer ITS-1, 2.0 μl of Primer ITS-4, 5.5 μl of Nuclease-Free Water, 3.0 μl of DNA template and 12.5 μl of GoTaq® Green Master Mix.

PCR reaction was conducted in a thermocycler where the reaction was carried in a total of 35 cycles.

Table 1: PCR mastermix component and volume.

Reagent	**Volume (μl)**
Primer ITS-1	2.0
Primer ITS-4	2.0
Nuclease-free water	5.5
DNA template	3.0
GoTaq® Green Master Mix	12.5
Total Volume	**25.0**

DNA amplification was run based on the parameter which has been set. According to New England Biolabs (2017), with some modifications, an initial denaturation step at 95°C for 2 minutes will be carried out, followed by 35 cycles of 0.5 minute denaturation at 95°C, 0.5 minute of annealing at 55°C, 1 minute of extension at 72°C, and final extension at 72°C for 10 minutes.

Table 2: Thermal cycling profile for PCR reaction.

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

3.1.3 Agarose Gel Electrophoresis (AGE)

According to Khan Academy (2017), gel electrophoresis was used to separate DNA according to its different fragment size for visualization, purification, and analyzation. The PCR products were separated by AGE on 0.8% agarose gel in 1X TAE buffer. 0.8% agarose gel was prepared by mixing 0.8 g of agarose powder with 80 ml of TAE buffer in Erlenmeyer flask. Then heated the mixture in the microwave for 2 minutes. Poured the heated mixture into agarose gel plate and added 1 µl of ethidium bromide (EtBr).

According to Sigmon and Larcom (1996), EtBr is added to assist in visualizing DNA banding pattern. Then, the comb was put onto the gel and let it solidified. 1 kb ladder was loaded on one side of the gel to determine the DNA pattern and sizes. Electrophoresis was run for 40 minutes at a constant voltage of 80 V. Then, visualized the gel under UV transilluminator.

3.1.4 DNA Recovery

This method was done after getting the clear band of the PCR products, using agarose gel electrophoresis (AGE). This method was used to purify the DNA from the PCR products before sending them for sequencing. This procedure was done using GF-1 DNA Recovery Kit (Vivantis).

Cut the agarose band that contained the desired DNA and placed into a pre-weighted microcentrifuge tube. Determined the net weight of the gel slice and added 1 volume of buffer GB to 1 volume of gel (0.1g = 100 µl). Centrifuged the tube in order to ensure the gel stay at the bottom. Then incubated the tube at 50 °C (until the gel melted completely).

After the gel had melted completely, loaded the gel into the column and centrifuged at 10 000 rpm for 1 minute. The flow was discarded. Then added 750 µl of wash buffer and centrifuged at 10 000 rpm for 1 minute and discarded the flow. After that, the column drying was done by centrifuging the column at 10 000 g for 1 minute. Next, placed the column into a clean microcentrifuge tube and added 50 µl of elution buffer and was let to stand for 2 minutes. After that, centrifuged it at 10 000 g for 1 minute and stored the DNA at -20 °C before sending the samples for sequencing.

3.1.5 Sequencing Analysis

The purified DNA samples were then sent to the 1st BASE Company for sequencing analysis. Sequences were searched against the GenBank database to identify the identical or the closest known deposited sequences in the database using Basic Local Alignment Search Tool (BLAST) analysis.

3.1.6 Morphological Identification

For the morphological identification, macroscopic and microscopic observations were done. Microscopic observations were done by observing the microscope slides of each fungi species used in this experiment, under a microscope. The microscope slides were prepared by dropping a drop of lactophenol onto the slide. The fungi sample was cut and sliced small and put onto the slide. Then, used a coverslip to cover the slide. Covered the slide slowly in order to avoid air bubbles. The fungi samples were viewed at the power of 40 X.

3.2 Test of Glufosinate on Fungi

3.2.1 Fungal Subculture

A total of 10 selected fungal species were obtained from the fungal collection of Genetic Engineering Lab (GEL), UNIMAS. The fungi used were *Aspergillus acuelatus, Aspergillus flavus, Aspergillus versicolor, Colletotrichum gloeosporioides, Colletotrichum truncantum, Cunninghamella bainieri, Marasmius cladophyllus, Penicillium citrinum, Penicillium pinophilum* and *neurospora crassa*. Potato Dextrose Agar (PDA) was used as the growth medium for each fungal colony. A pure fungi culture (5 mm x 5 mm) was sub-cultured inside Petri-dish (90mm), using a sterile blade and forceps inside laminar flow hood. Each Petri-dish was labelled and sealed with parafilm to minimize any contamination. Each fungal species (3 replicates) were incubated at room temperature as a stock culture before subsequently proceed to herbicide treatment.

3.2.2 Glufosinate Treatment on Fungi

In this study, the herbicide used was glufosinate, containing 13.5 % w/w of glufosinate. The fungal resistant was tested using four different concentration of glufosinate, 0 (as control), 4 000, 8 000, 16 000 and 20 000 ppm.

The calculation of the concentrations (ppm) was as follows:

$$\textbf{Ppm} = \frac{\textbf{mass of solute (g)}}{\textbf{Mass of solvent and solute (g)}} \times \frac{\textbf{1 000 000 ppm}}{\textbf{1}}$$

1 mg/L = 1 ppm

1,000,000 ppm = 100%

Conversion from % w/w to ppm

Part per million (ppm) = mg/kg

% (w/w) = weight per 100 g

$$13.5\ \%\ w/w = \frac{13.5\ g}{100\ g} \times \frac{1000 g}{1\ kg}$$

$$= 135\ g/kg$$

$$= 135\ g/kg \times 1000$$

$$= 135\ 000\ mg/kg$$

$$= 135\ 000\ ppm$$

For each type of fungi, the control was PDA without glufosinate, while treatments involved PDA solution mixed with different concentrations of glufosinate as per treatment. Preparation of glufosinate PDA medium was conducted by adding the glufosinate into sterilized PDA medium and mixed thoroughly by continuous gentle swirling of the Schott bottle before pouring the agar into the Petri dishes. Then, the PDA media kept in a sterile laminar flow chamber until the culture medium solidified and dried for about one hour.

The fungal subcultures that had been fully grown, were transferred aseptically using sterile blade and forceps to the center of the glufosinate PDA mediums and control plates. Each fungal type had three replicates for each different concentration (0, 4 000, 8 000, 16 000, 20 000 ppm). The plates were then sealed with parafilm and incubated at room temperature. Radial growth of fungal colony in both control and herbicides PDA plates were measured for 22 consecutive days (2^{nd}, 4^{th}, 7^{th}, 11^{th}, 16^{th} and 22^{nd} day) using centimetre ruler in order to see the effect of glufosinate.