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Effect of Selected Herbicides *In Vitro* and in Soil on Growth and Development of Soil Fungi from Oil Palm Plantation

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Abstract

Herbicides are commonly used in integrated weed management programs in oil palm plantation. Their usage not only controls the weed populations but also affects microbial populations especially fungi in soil, and hence modify soil biochemical and biological processes critical for ecosystem functioning. The response of fungal population from oil palm soil exposed to paraquat, glyphosate, glufosinate-ammonium and metsulfuron-methyl at 0.5, 1 and 2 times their recommended field application rates, *in vitro* and in soil environment was assessed in present studies. Herbicides both *in vitro* and in incubated soil caused significant inhibition of fungal growth. Inhibition of fungal growth increased with increased herbicide rates. The degree of growth inhibition by the herbicides tested *in vitro* was in order of paraquat and glufosinate-ammonium > glyphosate > metsulfuron-methyl. Species-specific inhibition and influence of exposure periods were also evaluated and found to vary for fungal species, herbicides and their rates of application. © 2013 Friends Science Publishers

Keywords: Soil fungus; Paraquat; Glyphosate; Glufosinate-ammonium; Metsulfuron-methyl; *In vitro*; Soil microcosm

Introduction

Herbicides are used quite extensively to control weeds in integrated weed management programs in plantation crops in Malaysia. However, when applied to the field, herbicides not only control targeted weeds, but may also have potential residual impact in soil (Zabaloy *et al.*, 2008), and provide considerable exposure of microbes to the herbicides (Pampulha and Oliveira, 2006). Differential toxicity of herbicides in soil may cause changes in microbial community structure and function, and concomitantly influencing soil health and ecosystem processes (Zabaloy *et al*., 2008). Soil fungus is the dominant organism among the soil microbial groups (Chauhan *et al.*, 2006), widely distributed in the upper soil layers (Bridge and Spooner, 2001). Fungi are known to be extremely adaptable in different environments due to their ability to breakdown many complex substrates including herbicides (Das *et al*., 2006).

The toxic effects of herbicides that lead to microbial destruction or shift in the populations would most likely affect agricultural productivity. An ideal herbicide should have the quick ability to be degraded into non-toxic substances that ultimately exert less toxic effects on soil microbes (Araujo *et al*., 2003). The effects of herbicides on soil fungi varied amongst herbicides depending on their application rates (Sebiomo *et al.*, 2011). Some microbial species respond sensitively to a particular herbicide resulting in their reduced number and activity (Hattori, 1973). Depending on the type of herbicide, fungi may be able to degrade herbicides, or are adversely affected (Abdel-Fattah *et al*., 1983; Abdel-Mallek and Moharram, 1986). Herbicides when applied at excessive rates can inhibit or suppress the activities of soil microorganisms (Verma and Mckenzie, 1985). Herbicide effects on fungal growth are specific with respect to herbicide type and dose, microbial species and environmental conditions (Bollen, 1961; Hattori, 1973).

Several herbicides are used in integrated weed management programs in oil palm plantations to control weeds. Among these, paraquat, glyphosate, glufosinte ammonium and metsulfuron-methyl are more common (Chuah *et al.*, 2005; Kuntom *et al*., 2007; Wibawa *et al.*, 2009; Halimah *et al.*, 2010). Determining the impact of herbicides on microbial growth and population is of considerable interest. The assessment of unforeseen consequences on microbial communities especially soil fungi due to use of herbicide is important to provide deeper insight for herbicide risk management in soils from oil palm plantation. The study was undertaken to assess the response of fungal populations exposed to different rates of herbicide application *in vitro* and in soil environment.

Materials and Methods

Herbicide Treatments

Herbicides used in this study were paraquat (Gramaxone®, Syngenta Corporation Sdn. Bhd.), glyphosate (Roundup®,

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Monsanto), glufosinate-ammonium (Basta® 15, Bayer Crop Sciences) and metsulfuron-methyl (Ally® 20 DF, DuPont Agrochemicals). Three rates of 0.44, 0.88 and 1.76 mg a.i. mL^{-1} for each of paraquat and glufosinate-ammonium; 0.88, 1.76 and 3.52 mg a.i. mL^{-1} for glyphosate; 0.015, 0.03 and 0.06 mg a.i. mL^{-1} for metsulfuron-methyl were applied. These application rates represented 0.5, 1 and 2 times (x) their recommended field application rates (paraquat and glufosinate-ammonium, 400 g a.i. ha^{-1}; glyphosate, 800 g a.i. ha^{-1}; metsulfuron-methyl, 15 g a.i. ha^{-1}). The rates were calculated as follows:

$$X \text{ mL } L^{-1} \text{ media} = \frac{\text{Desired field rate (g a.i. ha}^{-1}) \times 1000 \text{ mL}}{\text{Amount of a.i. in formulation (g ai L}^{-1}) \times 450 \text{ L ha}^{-1} \times 1 \text{ L}}$$

Fungal Culture

A total of three fungal species (*Mucor* sp., *Penicillium* sp. and *Aspergillus* sp.) isolated and subsequently identified earlier from the soil of oil palm plantation were studied. Each fungal species was maintained in incubator at 4ºC pure stock culture. The fungal colonies of the pure culture were later sub-cultured on potato dextrose agar (PDA) growth medium in Petri-dish, by transferring a 5 mm diameter using a sterile cork borer. Each Petri-dish was then covered, sealed to avoid contamination and incubated at 25ºC in darkness for 8 days, and then inoculated accordingly to growth medium with herbicide treatments.

In Vitro Study

In vitro study for the effect of herbicides on the fungal colony development was conducted on PDA growth medium in Petri-dish (9 cm diameter). The treatments involved PDA mixed with the herbicides as per treatment, while the control was on PDA without herbicide treatment. The herbicide-PDA medium was prepared by adding the herbicide treatments into sterilized (121ºC, 15 min) PDA medium, and mixed thoroughly on the hotplate and stirrer (Jenway, Bibby Scientific Ltd., UK) before pouring into the Petri-dishes. The Petri-dishes were covered and allowed to dry for 1 h in sterile condition. Fungal subculture of 8 days old was transferred aseptically using sterile inoculation needle to the centre of the herbicide-PDA medium and control plates (marked by perpendicular lines). The plates were then covered and sealed, followed by incubation at 25ºC in darkness.

The effect of herbicides on the fungal species was measured by the radial growth of fungal colony in both control and herbicides-PDA plates for 10 consecutive days using millimeter ruler. The measurements were expressed as inhibition percentage of the colony, calculated using the formula of Pandey *et al.* (1982):

$$\text{Percentage growth inhibition} = \frac{D_C - D_T}{D_C} \times 100$$

Where, D_C is the average diameter of fungal colony in control, and D_T is the average diameter of fungal colony with herbicide treatments.

In Soil Environment Study

Soil was collected from an oil palm plantation at Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia and prepared into soil microcosm (Pampulha and Oliveira, 2006) with minor modifications. Eighty soil cores from the top 0-15 cm layer were collected randomly using auger from underneath the surrounding palms and between the palm rows. The soil cores (totalled approximately 40 kg) were bulked, crumbled and mixed thoroughly to form composite sample and subsequently packed and taken to the laboratory for further processing. The soil was air-dried in laboratory (25ºC; 50% RH) for 24 h before sieving through a 2 mm mesh to remove plant tissue. Characteristics of the soil from oil palm plantation were determined (Table 1). The soil was then analyzed to estimate the moisture content and the moisture holding capacity (MHC). Soils with moisture content of 13% were then mixed together, and 56 ml sterile distilled water was added to attain the moisture level of 18.5%, which was 50% of its maximum MHC. The soil was then placed in sterile glass bottles, each holding 1 kg of soil, with loosely fitting cap to allow gas exchange. The bottles were then incubated in the dark at 25ºC for 10 days to allow time for the adaptation of microorganisms, before adding the herbicide treatments.

The herbicide were applied by spraying 50 mL of respective herbicide solution to the soil in the glass bottles as per treatment by using a hand sprayer, and mixed thoroughly by constant shaking for 5 min. The control bottles were sprayed with the same amount of sterile distilled water. The soil in glass bottles treated with herbicides and control were then transferred into sterile square plastic container (15 cm × 15 cm × 7.4 cm) with lids loosely fitted, forming the soil microcosm. The soil microcosms were then incubated in darkness at 25ºC. Sterile distilled water was added on weekly basis to maintain the constant moisture content in each microcosm.

Soil samples were taken from each microcosm at 2, 4, 6, 10 and 20 DAT (days after treatment) to assess the herbicidal effect on the fungal populations in the soil. Five sub-samples were collected randomly from each microcosm using sterile cork borer (10 mm diameter), and mixed together. Serial dilutions were made from each sample aseptically under laminar flow by suspending 1 g soil in 9 mL of sterile distilled water in a test tube and mixing thoroughly using vortex mixer (Vision Scientific Co. Ltd., Korea) for 30 sec. This process was repeated until the dilutions were made up to 10^{-4} to complete the serial dilutions.

Five drops (10 µL $drop^{-1}$) from each serial dilution of 10^{-2} to 10^{-4} were pipetted out by drop plate method onto PDA growth media, prepared using PDA (Difco, BD, USA) amended with 30 mg L^{-1} streptomycin sulphate (Sigma-

Aldrich, USA) inhibitor added into sterilized (121°C, 15 min) media, and poured into Petri-dish with marker line division at the bottom. These were divided into three sections for enumeration of the fungal populations. The inoculated media plates were covered and allowed to dry. After 1 h the plates were inverted, sealed and incubated in darkness at 25°C. Fungal population in the PDA plates was enumerated by their colony forming unit (CFU). The CFUs were determined after 7 days by counting the visible colonies. The total up of the colonies was used to calculate the CFU g^{-1} dry weight of soil using the formula:

$$\text{CFU/g dry weight of soil} = \frac{\text{Colony-forming unit} \times \text{dilution factor}}{\text{Amount of aliquot} \times \text{dry weight of soil (g)}}$$

Statistical Analysis

The experiments were conducted using complete randomized design (CRD) with five replicates. Data from *in vitro* study were analyzed following 2-way ANOVA between herbicides and each fungal species. Data from soil microcosm were analyzed following 2-way ANOVA between herbicides and each exposure date. Mean separation were done by Duncan's Multiple Range Test (DMRT) using statistical analysis system (SAS Institute Inc., NC, USA). Results were expressed as percentage of fungal growth inhibition with significant difference at $p<0.05$.

Results

Effects of Herbicides *in vitro* on Radial Growth of Fungal Isolates

Treatment of herbicides to growth media *in vitro* significantly restricted the radial growth of the fungal species (*Mucor* sp., *Penicillium* sp. and *Aspergillus* sp.) isolated from the soil of oil palm plantation. Growth inhibition became more severe with increasing concentration of the herbicides. The fungal species grown *in vitro* were most susceptible to paraquat and glufosinate-ammoniun with the highest inhibition between 70% and 100%. Glyphosate was less toxic and inhibited the radial growth of *Penicillium* sp. and *Aspergillus* sp. by18-58%, but *Mucor* sp. was more susceptible with 63-80% inhibition. Metsulfuron-methyl caused minimal inhibition (< 20%) of fungal development (Fig. 1). Among the four herbicides, paraquat and glufosinate-ammoniun were comparable in causing the high inhibition at all concentrations followed by glyphosate; and metsulfuron-methyl was the least inhibitory to the fungal species. At the lowest herbicide treatment of 0.5X recommended field application rate, paraquat and glufosinate-ammoniun caused more than 70% inhibition to the growth and development of fungal species. At this application rate however, glyphosate caused 14 to 60% inhibition and the lowest inhibition to fungal development recorded by metsulfuron-methyl was only 1.8%.

Fungal species showed different degree of sensitivity to different herbicides. Among the three species, the growth of *Mucor* sp. was most affected by all the herbicides. Paraquat and glufosinate-ammoniun caused 80% to 100% inhibition of all fungal species when used at their recommended field application rates. Paraquat when use at either of the three rates caused 100% inhibition to the growth development of *Mucor* sp., whereas glufosinate-ammoniun caused 100% inhibition to *Aspergillus* sp. at 1x and 2x of the recommended field application rates. Glyphosate treatment at 0.5x, 1x, and 2x of the recommended field application rates caused 60% to 80% inhibition to *Mucor* sp., while 20% to 60% inhibition to *Penicillium* sp. and *Aspergillus* sp. In contrast, metsulfuron-methyl caused least inhibition to all the three fungal species that ranged between 0 to 20% at all treatment concentrations. The fungitoxicity of herbicide treatments *in vitro* on the growth and development of fungal species, therefore, ranked in the order of paraquat and glufosinte-ammonium > glyphosate > metsulfuron- methyl.

Effects of Herbicide on Fungal Population in Soil

Fungal colony development in soil was affected significantly by the application of the herbicides. Increased inhibition of the fungal colony development was observed with increased treatment rates of each herbicide (Fig. 2). Inhibition by the herbicide treatments over the exposure periods was highest at 2× recommended field application rate, which ranged from 45 to 80%. The inhibition range at the recommended field application rate (1×) was between 25 and 60%.

Inhibition of fungal colony development among the five exposure periods (2, 4, 6, 10 and 20 DAT) were significantly higher between 2 and 6 DAT, followed by sharp decline onward. Inhibition by paraquat and glyphosate increased with the exposure periods until 6 DAT and declined thereafter. The increased trend of inhibition for glufosinate-ammonium lasted until 4 DAT, whereas metsulfuron-methyl caused gradual decline after the higher initial (at 2 DAT) effect. The highest range of inhibition for paraquat (45%–60%) and glyphosate (50%–75%) were at 6 DAT, whereas for glufosinate-ammonium (45%–80%) at 4 DAT and for metsulfuron-methyl (40%–70%) at earliest sampling date (2 DAT). The inhibition percentage of the fungal colony development, especially at the recommended field application rate (1x), were insignificant among herbicide treatments from 6 DAT onward. At 10 DAT, the inhibition was relatively low at 10% or less for all the treatments. By 20 DAT, no inhibition on the fungal colony by the herbicides was recorded.

Discussion

Herbicide treatments of paraquat, glyphosate, glufosinate-ammonium and metsulfuron-methyl showed significant effects on the fungal growth and development *in vitro* and in soil environment. Significant increase of fungal growth inhibition was observed with increasing herbicide

Table 1: Characteristics of the soil sampled from oil palm plantation, UPM

Soil texture	Soil pH	Soil chemical properties					
		Carbon (%)	Total N (%)	Total P (ppm)	Available K (ppm)	Ca (ppm)	Mg (ppm)
Sandy clay (40% clay, 10% silt, and 50% sand)	4.1 ± 0.01	1.94	0.32	219	104	119	32

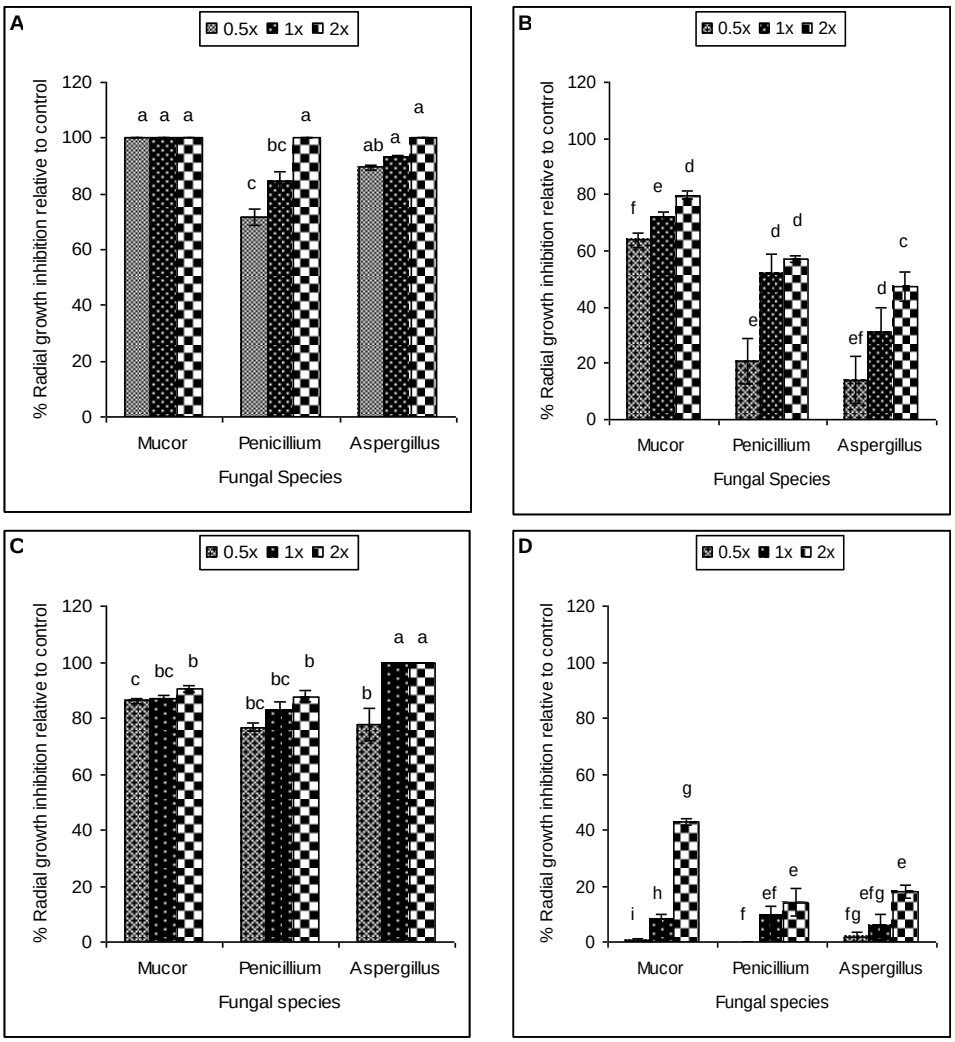

Fig. 1: Effect of herbicide treatments *in vitro* on the radial growth of fungal species at 7 DAT- (A) Paraquat, (B) Glyphosate, (C) Glufosinate-ammonium, and (D) Metsulfuron-methyl

Bars followed by a similar letter in the same fungal species under all four herbicides, are not significantly different by DMRT (P<0.05). Data are presented as mean values (standard error) of five replicates at each fungal species. 0.5×, 1× and 2× represent three herbicide concentrations in terms of the recommended field application rate of each herbicide

concentration from 0.5× to 2× of their field recommended rates, indicating a positive correlation between growth inhibition and treatment rates. Fungal growth inhibition due to the effects of herbicide treatments also varied among fungal species and the types of herbicide. Growth of *Mucor* sp. was inhibited more than that of *Aspergillus* sp. and *Penicillium* sp. Smith and Lyon (1976) explained this was due to the differing abilities of fungal mycelia to absorb herbicides for their utilization. Besides, *Aspergillus* sp. and *Penicillium* sp. were also reported as active degrader of herbicides (Romero *et al.*, 2009).

Paraquat and glufosinate-ammonium were found to be more inhibitory than glyphosate and metsulfuron-methyl, causing 80-100% growth inhibition to the fungal species. In fact, the two herbicides had been reported earlier to be toxic to fungal species. Paraquat was toxic *in vitro* to the radial growth of fungi as *Colletotrichum dermatium*, *Alternaria* sp., *Macrophomina phaseolina* and *Phomopsis* sp. isolated from soybean seeds at a concentration as low as 600 µg a.i. mL^{-1} (Cerkauskas and Sinclair, 1982). Toxicity of glufosinate-ammonium to fungus was reported by Tubajika and Damann (2002),

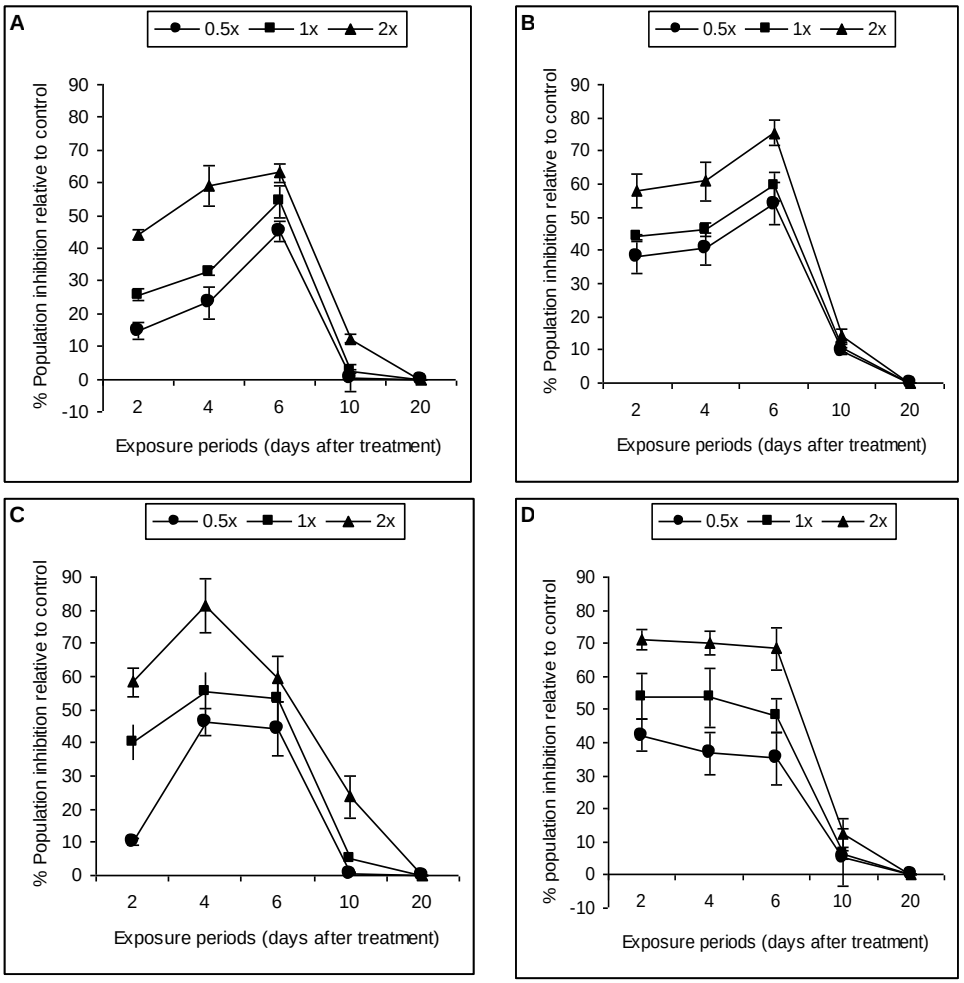

Fig. 2: Effect of herbicide treatments on fungal population in soil - (A) Paraquat, (B) Glyphosate, (C) Glufosinate-ammonium, and (D) Metsulfuron methyl

Bars depict standard error (SE) of mean; Data are presented as mean values (standard error) of five replicates at each exposure period; 0.5x, 1x and 2x represent three herbicide concentrations in terms of the recommended field application rates of each herbicide

where its high concentration (2000 µg mL^{-1}) reduced the growth of *Aspergillus flavus* up to 80%. Strong inhibition of growth in response to glufosinate-ammonium was also observed for *Trichoderma harziamu* and *T. longipilus* (Ahmad and Malloch, 1995) and *Magnaporthe grisea* and *Cochliobolus miyabeanus* (Ahn, 2008). Glyphosate however, showed moderate growth-inhibition effects on fungal species likely to be due to its degradation by microorganisms as source of phosphorus by direct cleavage of the C-P bond producing sarcosine, or *via* intermediate aminomethylphosphonic acid (AMPA) as explained by Malik *et al.* (1989). However, Franz *et al.* (1997) discussed that fungal growth inhibition by glyphosate was due to the blocking of EPSPS enzyme in the shikimic acid pathway that ultimately affected the amino acid synthesis in microorganisms. Other *in vitro* studies conducted on glyphosate reported the growth-inhibitory effects on soil fungi *Fusarium solani*, *Pythium ultimum* and *Trichoderma viridae* at 100 and 140 ppm (Meriles *et al.*, 2006), *Sclerotium rolfsi* at commercial recommended rate of 3.6 g L^{-1} (Westerhuis *et al.*, 2007). On the other hand, metsulfuron-methyl showed the least inhibitory effects to fungal species, which might stem from its low doses and its ability to be degraded by soil fungi (Boschin *et al.*, 2003; Yu *et al.*, 2005; He *et al.*, 2006). Direct exposure of the fungi *in vitro* to the herbicides indicated that paraquat and glufosinate-amonium could be very toxic, whereas glyphosate was moderately toxic, and metsulfuron-methyl had caused the least inhibitory effects to the fungi.

The inhibitory effect of herbicides on growth of the fungus through soil treatment however, was lower than when compared with the direct exposure (*in vitro*). This indicated that herbicides in soil may undergo certain natural processes (biological, chemical and physical) which could reduce its toxicity to the fungal population (Wilkinson and Lucas, 1969). Exposure of soil fungi to herbicide application caused short term growth-inhibitory effects on soil fungal population. The treatment effects on fungal

population growth over the five exposure periods exhibited rapid decreasing trends after 6 DAT so that these reached to zero at 20 DAT. This was expected because the amount of herbicide molecules present in the soil were negligible to have any influence on fungal population that ultimately lead to zero inhibition of fungal growth. The effects of paraquat and glyphosate to growth inhibition of fungi showed increasing trends with passage of exposure time (sampling dates) that reached peak at 6 DAT. This might possibly be attributed to the nature of soil type (sandy clay) of the experimental plot that ultimately reduced the binding of herbicides to soil particles with the prolonged sampling dates (Walker, 1975). Short-term effects on soil microorganisms upon single application of herbicide exposure were reported by Hance (1980) and even when herbicides applied higher than the recommended field rate was found to cause transitory effects on microbial biomass (Das *et al.*, 2006; Weaver *et al.*, 2007). Several studies reported significant inhibition of a great number of cellulytic and pathogenic soil fungi by paraquat (Smith and Mayfield, 1977) and glyposate (Anderson and Kolmer, 2005) in soil. Increased inhibitions of fungal growth by glufosinate-ammonium were observed until 4 DAT with a sharp decline onward. The growth-inhibition by glufosinate-ammonium could be due to negative effects on the dehydrogenase activity of soil fungi as explained by Pampulha *et al.* (2007), and subsequent decline of growth-inhibition likely due to its rapid degradation in soil (Ismail and Ahmed, 1994). However, metsulfuron-methyl effects on fungal growth development were consistent within 6 DAT, with the highest at its earliest exposure period (2 DAT), after which it gradually declined onward. This might be due to the initial bioavailable amount in the soil which gradually underwent hydrolysis and biodegradation, common for metsulfuron-methyl at lower soil pH values (Smith, 1986; El-Ghamry *et al.*, 2000; Zanardini *et al.*, 2002). Degradation rate of sulfonylurea herbicides in soil could also be positively correlated with the size of fungal populations (Voets *et al.*, 1989). Ismail *et al.* (1996) showed that microbial population decreased when the concentrations of metsulfuron-methyl increased during the first 3-9 days after application, depending on soil types.

In conclusion, application of paraquat, glyphosate, glufosinate-ammonium and metsulfuron-methyl at 0.5, 1 and 2 times their recommended field application rates variably inhibited the fungal species, and the extent of inhibition depended on type of herbicide, their rates of application, and period of exposure as well. The study suggested that herbicide applications in oil palm plantations induced transient effects on the growth and development of fungal community in soil.

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