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ISMAIL, B S* and CHONG TET-VUN*

The effects of metsulfuron-methyl on injury and acetolactate synthase (ALS) activity were measured in immature oil palm seedlings under greenhouse conditions. Metsulfuron-methyl sprayed directly onto seedlings caused a whole range of injuries to the 18-month-old oil palm seedlings, but its residue in soil did not affect seedling growth. Chlorosis appeared three weeks after treatment (WAT). The percentage of injury score was found to increase proportionally with herbicide dosage. Metsulfuron-methyl inhibited ALS enzyme in the seedlings. Metsulfuron-methyl at 41 nM could inhibit 50% activity (I_{50}) of the ALS enzyme. Metsulfuron-methyl compound was metabolized to seven metabolites, of which two could not be identified.

Keywords:

metsulfuron-methyl, acetolactate synthase, metabolites

INTRODUCTION

Metsulfuron-methyl is an active ingredient in herbicide Ally®, manufactured by Du Pont. It is widely used in Malaysia for controlling the broad leaf weeds, especially in oil palm and rubber plantations. Like other sulfonylurea herbicides, the mode of action of metsulfuron-methyl is to inhibit the activity of ALS, an enzyme involved in the biosynthesis of branched amino acids, such as valine, leusine and isoleusine (Beyer *et al.*, 1988; LaRossa and Schloss, 1984; Schloss *et al.*, 1988).

It has been reported that sulfonylurea herbicides can be metabolized to other metabolites in plant tissues such as oat, wheat and barley (Anderson *et al.*, 1989; Sweetser *et al.*, 1982; Hutchinson *et al.*, 1984; Beyer *et al.*, 1988). Sulfonylurea tolerance (*e.g.* the tolerance of wheat towards chlorsulfuron) is due to the inactivation and detoxification process within the crop itself. Rapid metabolism in plant tissues is one of the mechanisms which contribute to crop tolerance to sulfonylurea (Beyer *et al.*, 1988; Sweetser *et al.*, 1982).

Metsulfuron-methyl is used in Malaysian plantations to control legume cover crops and broadleaf weeds in palm circles of early immature palms aged between one and 18 months. During weed control, herbicidal damage to oil palm in early stages of growth was observed to be due to the excessive contact or spray drift and this had resulted in phytotoxic symptoms (Wahid and Hassan, 1986). Crop injury symptoms reported in nursery seedlings and immature palms following the use of wrong herbicides, incorrect spraying techniques, and excessive spray drift with high pressure mechanical power spraying, are frond scorching, dieback of spears, bending palms, swelling of frond base and stunted growth (Ismail and Chan, 2000).

Reports on injury levels, particularly as concerns enzyme level, due to the application of metsulfuron-methyl at the immature stages of oil palm growth are very limited. The objective of this study is to investigate the effects of metsulfuron-methyl on the growth of oil palm seedlings, its effect on ALS activity and its metabolites in oil palm seedlings.

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MATERIALS AND METHODS

Materials

The herbicide, (*phenyl*-U-^{14}C) metsulfuron-methyl, specific activity 32.2 μCi mg^{-1}, was supplied by Du Pont, United States. The 17-month-old oil palm seedlings were supplied by the Malaysian Palm Oil Board (MPOB).

Methods

Effects of metsulfuron-methyl on oil palm seedlings. Oil palm seedlings were transferred into larger polybags (50 cm x 60 cm) containing 15 kg loamy clay soil. Seedlings were grown, one each, in 24 bags. The bags were divided into two groups. In one group, the herbicide was sprayed directly onto the fronds of the palms at the dosage required. Seedlings were kept under greenhouse conditions, with light intensity of 800 $\mu Em^{-2}s^{-1}$ and temperature of 27±4°C. One month after transplanting into bigger polybags, the oil palm seedlings were sprayed with either 0 (distilled water as a control), 15 and 30 g ai ha^{-1} of metsulfuron-methyl (Ally®) and 0.25% (v/v) of surfactant (Pulse®) to confirm that the surfactant did not cause injury to the seedlings. As for the second group, similar dosages of the herbicide were sprayed individually by trending onto the soil surface in each bag rather than directly onto the seedlings.

The level of injury of the oil palm was observed with a visual scoring method on 0, 1, 2, 3, 4, 5, 6, 7 and 8 WAT. The level of injury was evaluated in which a score 0 corresponds to the death of the whole palm, score 1 corresponds to death of the frond, score 2 corresponds to collapse of the young frond, score 3 corresponds to appearance of necrosis, score 4 corresponds to appearance of chlorosis, and score 5 corresponds to no observed symptoms of injury. The plants were arranged in a completely random design with three replicates.

Acetolactate synthase extraction and assays. The method used in this enzymatic study represents a modification of the methods suggested by Kwon and Penner (1995), Singh *et al.* (1988), Shaner *et al.* (1984), Chaleff and Mauvais (1984), Westerfield (1945) and Bradford (1976).

The pinnae of the oil palm seedlings (40 g) were ground to a fine powder in liquid nitrogen. Polyvinylpolypyrrolidone (10 g) was added. The powder was added to 10 ml of cold extraction potassium phosphate buffer containing 0.1 M K_2HPO_4, pH 7.5, 1 mM sodium pyruvate, 0.5 μM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 10 μM flavin adenine dinucleotide (FAD) and 10% (v/v) glycerol. The mixture was then stirred and allowed to stand for 30 min at 4°C. The sample was centrifuged at 27 000 g (4°C) for 20 min. The supernatant was brought to 50% concentration with cold saturated $(NH_4)_2SO_4$ and allowed to stand for 1 hr at 4°C. The mixture was then centrifuged at 15 000 g (4°C) for 15 min and the pellet redissolved in cold resuspension buffer (0.1 M K_2HPO_4, pH 7.5, 20 nM sodium pyruvate and 0.5 mM $MgCl_2$) and placed on a Sephadex G-25 PD-10 column to desalt, at 4°C. The desalted enzyme was immediately used for enzyme assays.

The ALS enzyme activity was assayed by mixing 0.1 ml of the desalted enzyme with 1.4 ml of the reaction buffer [25 mM K_2HPO_4, pH 7.0, 25 mM sodium pyruvate, 0.625 μM $MgCl_2$, 0.625 mM thiamine pyrophosphate, 1.25 μM flavin adenine dinucleotide (FAD)] and 5 ml 0.001 ppb metsulfuron-methyl was added. The mixture was incubated for 1 hr at 35°C. The reaction was stopped by the addition of 50 μl 6N H_2SO_4, and the solution was heated at 60°C for 15 min. Then 0.5 ml of 0.5% (w/v) creatine and 0.5 ml of 5% (w/v) α-naphthol freshly prepared in 2.5N NaOH were added to the solution, which was then heated for another 15 min at 60°C. Acetoin content was measured as described by Westerfield (1945), and protein concentration was determined using Bradford's method (Bradford, 1976). The above mentioned step was repeated replacing the concentration of metsulfuron-methyl with 0.01 ppb, 0.1 ppb, 1 ppb and 10 ppb. For control assays, 0 ppb of metsulfuron-methyl was used. The data reported are the averaged treatment means of the five replicates. ALS enzyme activity is presented as a percent of control assays. Data were subjected to an analysis of variance and means were compared by the LSD test at the 5% level of significance.

Identification of the metsulfuron-methyl metabolites in oil palm seedlings. The method used in this study is a modification of the method suggested by Slates (1983), Anderson *et al.* (1989), Harvey *et al.* (1985) and Zhou *et al.* (1994).

Non-labelled and labelled metsulfuron-methyl at 15 g ai ha^{-1} (0.33 μCi / pinnae) was applied to the surface of six pinnae from the third frond of the oil palm seedlings. Other fronds

were treated with 15 g ai ha^{-1} metsulfuron-methyl. The homogeneous pinnae were collected at 0 (*i.e.* immediately after the chemicals dried), 1, 2, 4 and 8 WAT. The pinnae were rinsed with methanol and stored at -20°C until use. The pinnae (15 g) were ground with liquid nitrogen to fine powder. A 100 ml cold (4°C) acetone (80%) and distilled water (20%) were added to the sample, which was then stirred for 30 min. The extraction was filtered by vacuum filtration. The extraction was repeated three times. The extracts were combined and concentrated to near dryness by rotary evaporator at 45°C and then diluted to 100 ml with distilled water. The solution was adjusted to pH 3.0 with 1.0 M phosphoric acid. After adjustment, the extract was sequentially extracted (three times) with 100 ml methylene chloride and 50 ml 1-butanol. The three methylene chloride extracts were combined (methylene chloride fraction), and the three 1-butanol extracts were combined (1-butanol fraction). The aqueous fraction was adjusted to pH 7.0 with 1.0 N NaOH after the final 1-butanol extraction. The three fractions were evaporated to dryness by rotary evaporator at 45°C. The residue of the methylene chloride fraction was redissolved with 2 ml methylene chloride. The residues of the 1-butanol and water fraction were redissolved with 2 ml methanol. All redissolved residues were analysed by HPLC [System: Waters 600, Column: C_{18} Zorbax (25 cm x 3.0 mm, 5 µm)], detector: UV detector at 254 nm, mobile phase: 30% acetonitrile and 70% H_2O (pH 2.2, phosphoric acid), flow rate: 0.5 ml min^{-1}, temperature: 45°C, injected volume: 25 µl, analysis time: 30 min. The effluent was collected every minute and its radioactivity measured with Liquid Scintillation Counter (LSC) and then analysed with GCMS [System Hewlett-Packard 5890 Series II, detector: Hewlett-Packard Mass spectrometer 5971A, column: BPX5 (0.25 µm)], temperature: 70°C for 2 min, and increased to 260°C at 5°C min^{-1}. The percentage of the metabolites was determined based on the total detected radioactivity. The metabolites were identified based on the data reported by Anderson *et al.* (1989) and the molecular weight from the GCMS spectrum.

RESULTS AND DISCUSSION

Effects of Metsulfuron-methyl on Oil Palm Seedlings

Figure 1 shows the injury level of the oil palm seedlings as measured by visual scoring. The metsulfuron-methyl was found to injure oil palm seedlings when in direct contact with the plants. The data showed that injury was positively correlated (r^2 = 0.95) with applied dose. The first symptom was chlorosis, followed by necrosis and finally seedlings death. *Figure 2* shows the injury symptom of oil palm caused by Ally®. Chlorosis appeared at the 3 WAT and fronds died at 7 WAT at metsulfuron dosages of either 15 g ai ha^{-1} or 30 g ai ha^{-1}.

The results showed that the herbicide did not affect the oil palm seedlings when applied on the soil surface. This may be due to the rapid degradation, detoxification or adsorption by the soil organic matter (Beyer *et al.*, 1988; Blair and Martin, 1988; Du Pont, 1997). The compounds were strongly bound by the soil particles and become inactive especially in soil containing high organic matter. The concentration of metsulfuron-methyl residue in soil decreased due to degradation process especially by soil microbes. This may explain why residues of metsulfuron-methyl did not exert any injurious effects on the oil palm seedlings.

Injury symptoms appeared only at 3 WAT. It has already been established that metsulfuron-methyl inhibits amino acid synthesis. Injury occurs within three to eight weeks (Du Pont, 1997). Metsulfuron-methyl does not directly attack plant tissues after the manner of contact herbicides, *e.g.* paraquat (Ashton and Craft, 1973; Summers, 1980). In this study, amino acid synthesis slowly decreased in plants, and injury symptoms appeared at the 3 WAT because no new amino acids were synthesized. The herbicidal action of metsulfuron-methyl is similar to that of other members of the sulfonylurea group. The first symptom is chlorosis at the meristem or the active growing part, followed by necrosis and death.

Effects of Metsulfuron-methyl on ALS of the Oil Palm Seedlings

Figure 3 shows the activity of ALS in the presence of metsulfuron-methyl at different concentrations. The results showed that metsulfuron-methyl inhibited the ALS activity of oil palm seedlings. It has been reported that metsulfuron-methyl inhibits ALS activity in other plants such as pea, wild oat, wild mustard and wheat (Chaleff and Ray, 1984). Metsulfuron-methyl at 0.001 ppb was able to inhibit approximately 18% of ALS activity in the control (102 nM/acetoin/

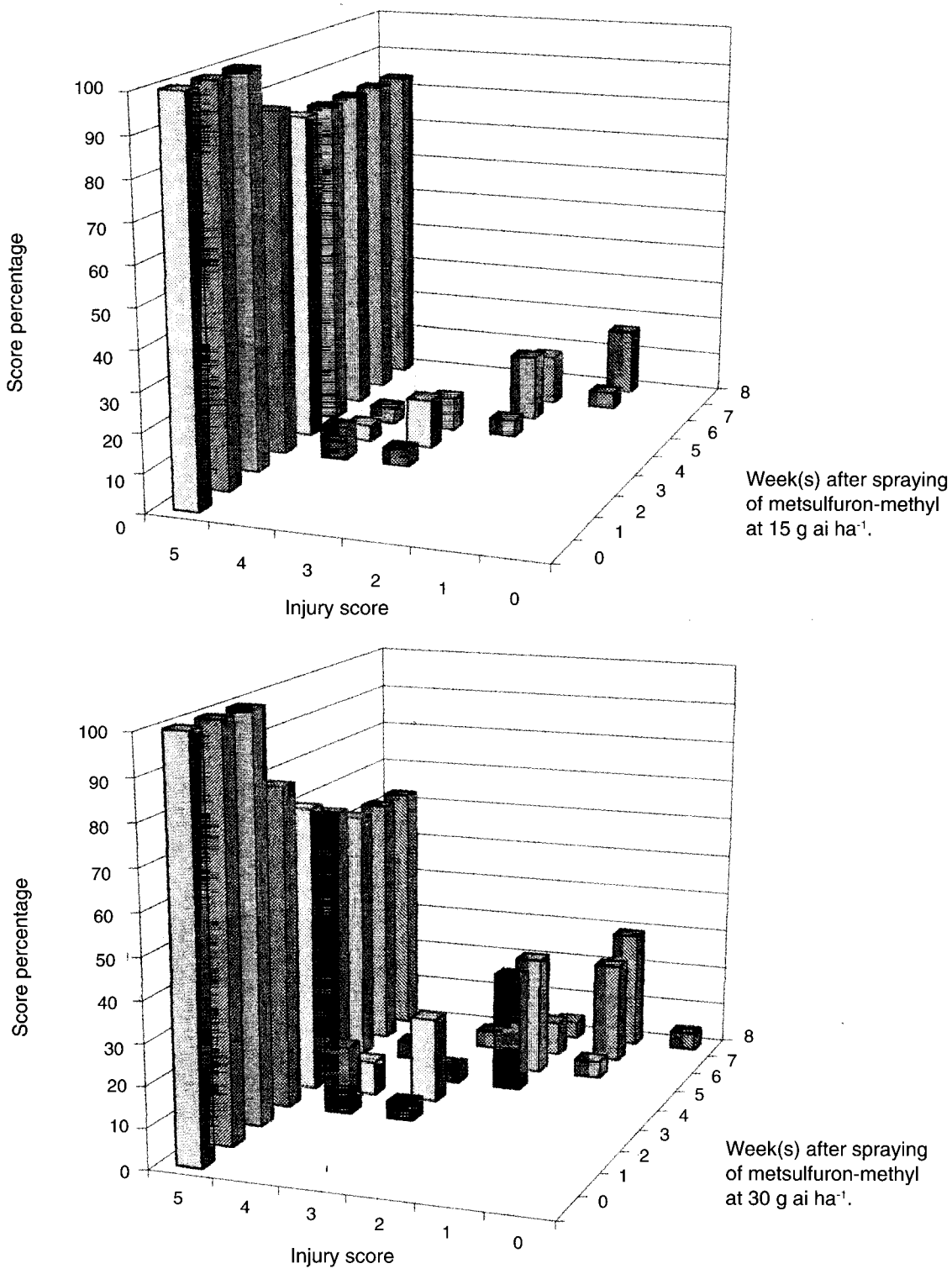


Figure 1. The oil palm seedlings injury level (score 0: whole palm died, score 1: frond died, score 2: young frond fell down, score 3: necrosis appeared, score 4: chlorosis appeared and score 5: no injury symptom appeared).

mg protein). Inhibition of ALS activity approached 99% when the seedlings were treated with 1 ppb metsulfuron-methyl (*Table 1*). The concentration of metsulfuron-methyl required to inhibit 50% (I_{50}) ALS enzyme activity of the control was 41.0 nM (15.64 ppb). The reduction of ALS activity may cause chlorosis due to the inhibition of acid amino synthesis.

The I_{50} obtained for oil palm seedlings was in the range of the I_{50} caused by other member of sulfonylurea group to other plant species. For instance, I_{50} caused by metsulfuron-methyl in wheat was 30 nM and 74 nM caused by chlorsulfuron in *Kochia scoparia* (Kwon and Penner, 1995; Ray, 1984). It has been reported that the I_{50} of oil palm seedlings is much lower

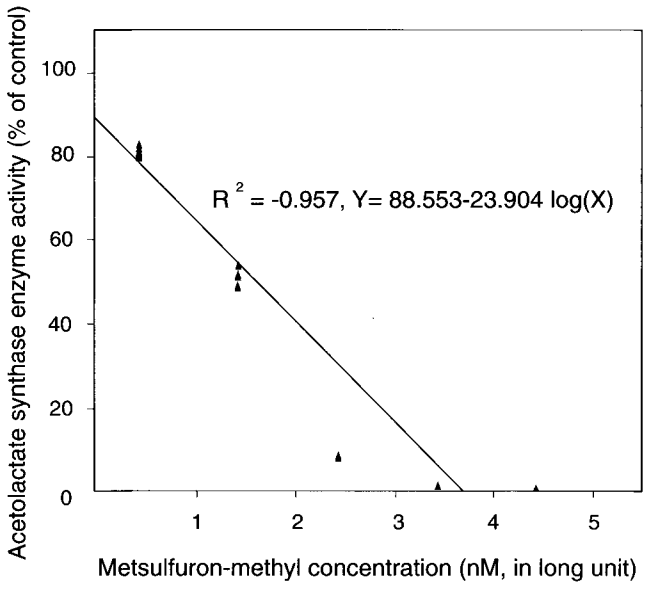


Figure 3. Effect of metsulfuron-methyl on ALS of oil palm seedling.

TABLE I. INHIBITION OF ALS ACTIVITY BY METSULFURON-METHYL

Concentration of metsulfuron-methyl	Activity percentage (% of control ± SD)
Control - 0 nM (0 ppb)	100.00
2.6 nM (0.001 ppb)	81.22 ± 1.36
26.22 nM (0.01 ppb)	51.02 ± 2.00
262.21 nM (0.1 ppb)	8.30 ± 0.35
2622.13 nM (1 ppb)	1.21 ± 0.10
26 221.25 nM (10 ppb)	0.45 ± 0.03

ponents accounting for the highest percentage observed (*Figure 4*). Based on the data reported by Anderson *et al.* (1989), seven metabolites of metsulfuron-methyl in the frond of the oil palm seedlings were identified (*Table 4* and *Figure 5*). However, two other unidentified metabolites detected may be intermediate substances formed in small quantity.

Metsulfuron-methyl was rapidly metabolized to other metabolites in oil palm, as can be seen from the measurements at 0 WAT. The unmetabolized metsulfuron-methyl detected at the 0 WAT was approximately 10% of the total metabolites detected (*Figure 3*). The metabolism rate reduced significantly at 1, 2, 4 and 8 WAT, perhaps indicating oil palm's inability to detoxify the compounds as a result of the inhibition of amino acid synthesis. The rapid metabolism of metsulfuron-methyl also occurred in wheat and

TABLE 2. THE PERCENTAGE OF THE EXTRACTABLE ^{14}C-METSULFURON-METHYL FROM THE PINNAE OF THE OIL PALM SEEDLINGS

WAT	Percentage of the extractable ^{14}C- metsulfuron-methyl (± SD)
0	86.79 ± 6.32
1	81.90 ± 3.37
2	84.04 ± 5.62
4	78.52 ± 0.57
8	81.16 ± 1.58

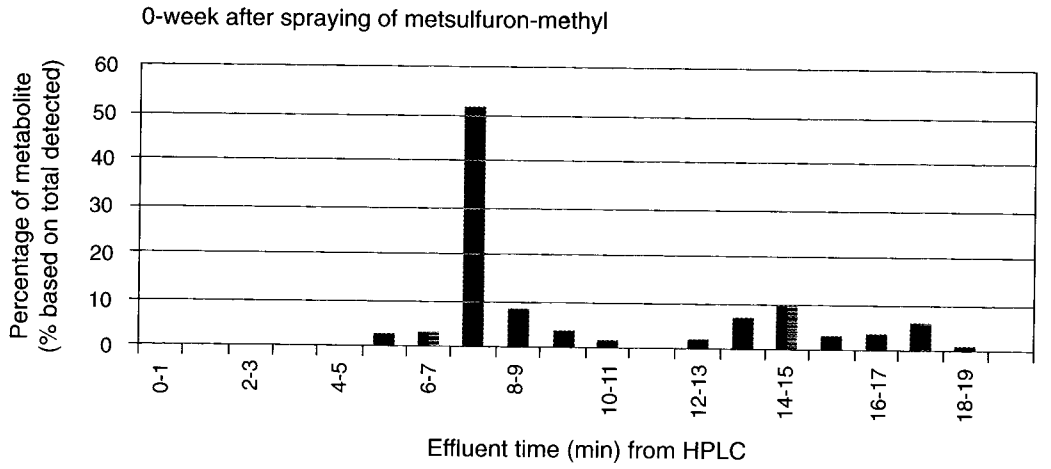
TABLE 3. THE PERCENTAGE OF ^{14}C-METSULFURON-METHYL IN METHYLENE CHLORIDE, 1-BUTANOL AND H_2O FRACTIONS

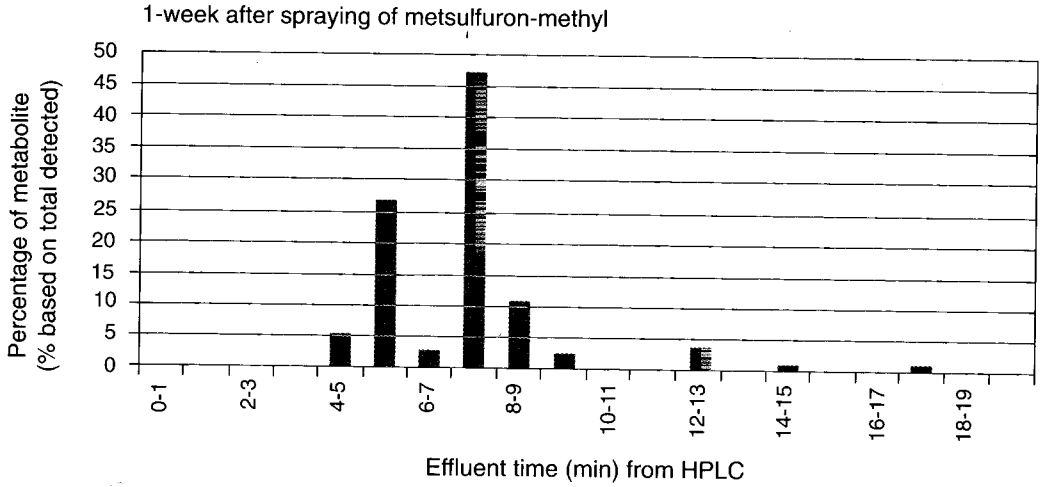
	Percentage of ^{14}C-metsulfuron-methyl (±SD)		
WAT	Methylene chloride	H_2O	1-butanol
0	96.10 ± 0.03	1.04 ± 0.01	2.86 ± 0.04
1	43.82 ± 1.66	44.33 ± 2.58	11.85 ± 0.15
2	72.92 ± 2.80	16.23 ± 1.31	10.85 ± 2.94
4	58.39 ± 0.29	39.79 ± 0.30	1.81 ± 0.02
8	48.71 ± 3.95	49.99 ± 3.85	1.30 ± 0.10

TABLE 4. THE SUGGESTED METABOLITES IN OIL PALM SEEDLINGS

Retention time of HPLC (min)	Molecular weight detected by GCMS	Suggested metabolites (based on Anderson *et al.*, 1989)
4-5	355	Metabolite A1 (401.3)*
5-6	355	Metabolite A1
6-7	355	Metabolite A1
7-8	178	Methyl 2-
8-9		[[[(aminocarbonyl) amino]sulfonyl] benzoate] (258)*
9-10	301	Metabolite B (397)*
10-11	301	Metabolite B
11-12	301	Metabolite B
12-13	549	Metabolite A (558.5)*
13-14	208	Unknown
14-15	169	Methyl (215)* 2- (aminosulfonyl) benzoate
15-16	298	Unknown
16-17	301	Metsulfuron-methyl (381)*
17-18	301	Metsulfuron-methyl
18-19	301	Metsulfuron-methyl
	167	Saccharin (183)*

Note:*molecular weight.





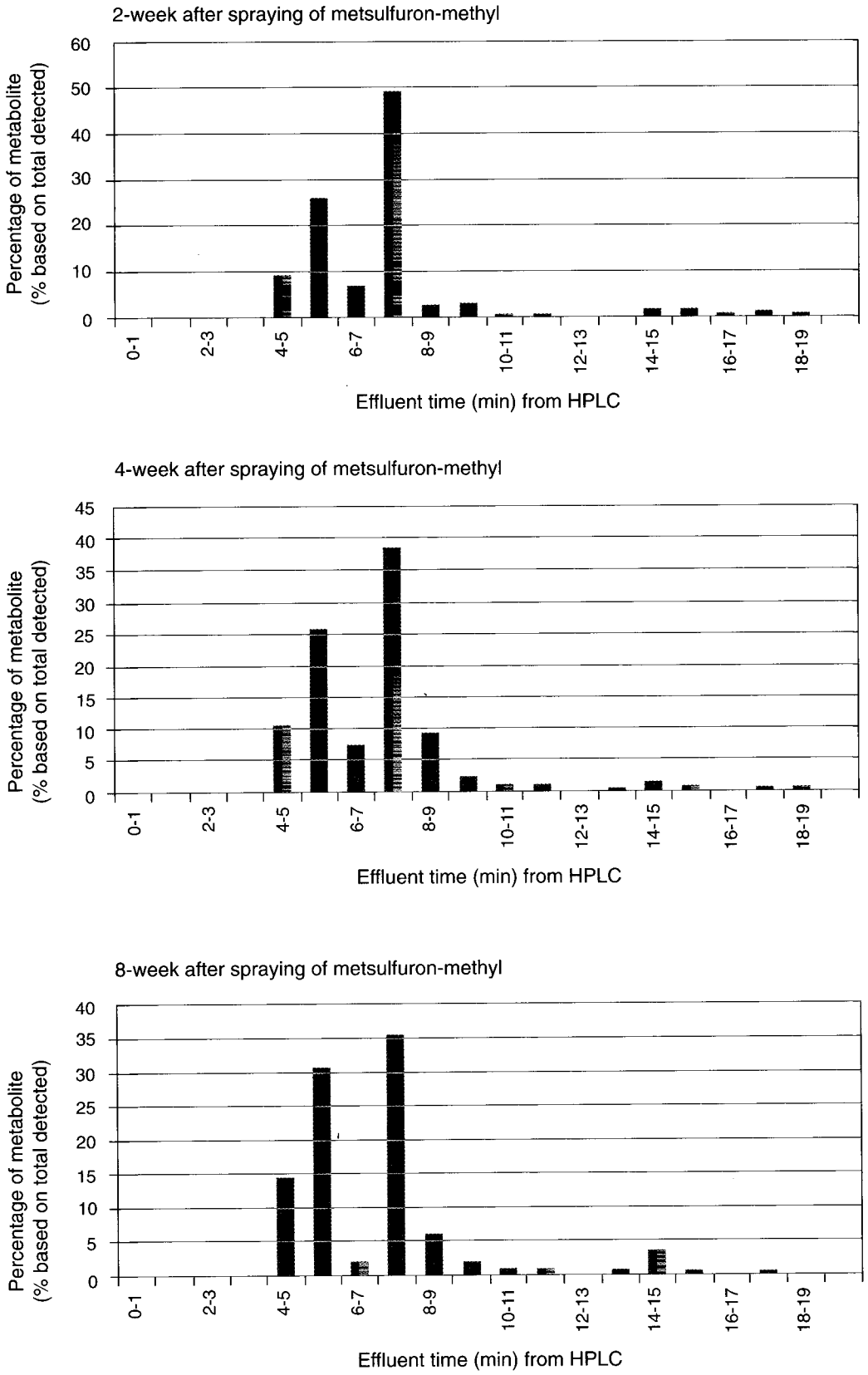
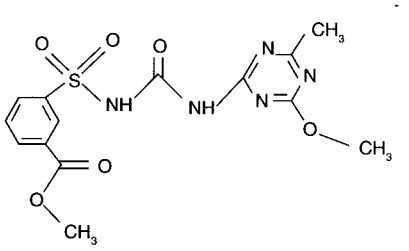
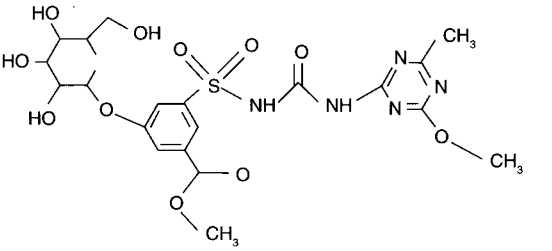


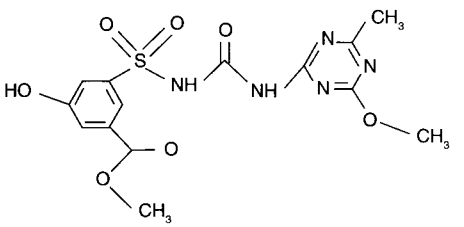
Figure 4. Radioactivity of the metabolites of metsulfuron-methyl in oil palm seedlings detected by HPLC and LSC.



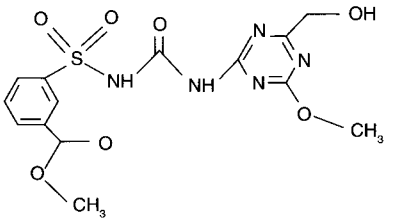
I. Metsulfuron-methyl



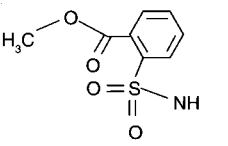
II. Metabolite A



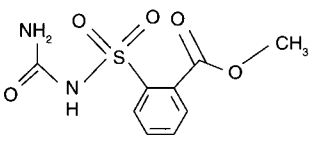
III. Metabolite A1



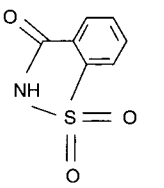
IV. Metabolite B



V. Methyl 2-(aminosulfonyl) benzoate



VI. Methyl 2-[[[(aminocarbonyl) amino]sulfonyl]benzoate]



VII. Saccharin

Source: Anderson *et al.* (1989).

Figure 5. Structures and trivial names for metsulfuron-methyl and suggested metabolites.

barley (Anderson *et al.,* 1989). Most of the previous reports showed that the tolerant plants rapidly metabolize sulfonylurea and *vice versa* (Sweetser *et al.,* 1982). However, the result of this study showed that the high rate of metabolism did not contribute to the tolerance of the oil palm seedlings toward metsulfuron-methyl. Metabolites derived from parent compounds of metsulfuron-methyl may be toxic to the young oil palms. Earlier reports have shown that the metabolites of chlorsulfuron are phytotoxic to susceptible plants such as sugar beet (Beyer *et al.,* 1988; Sweetser *et al.,* 1982).

CONCLUSION

Results of this study clearly showed that metsulfuron-methyl was phytotoxic to young oil palm when it comes in contact with the fronds. In contrast, as soil residue, it did not affect palm growth. Metsulfuron-methyl reduced the ALS activity in immature oil palm, which consequently might inhibit amino acid synthesis leading to plant death. In oil palm seedlings, seven metabolites of metsulfuron-methyl were detected while two others could not be identified. However, the toxicity and the herbicidal effects of these metabolites are not yet known and need further investigation.

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