Effects of glyphosate on the mortality and shikimic acid level in two goosegrass [Eleusine indica (L.) Gaertn.] biotypes

(Kesan glifosat terhadap kematian dan kandungan asid shikimik di dalam dua biotip rumput sambau [Eleusine indica (L.) Gaertn.])

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Key words: Eleusine indica, goosegrass, glyphosate-resistant, shikimic acid

Abstract

The status of glyphosate resistance in Eleusine indica [L.] Gaertn. biotypes collected from two different areas namely, Lenggeng and Bidor as compared to a known susceptible biotype from CCM Bioscience Agrochemicals Research Centre (BRC), Malaysia was determined. Involvement of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the glyphosate-resistant biotype was investigated through the measurement of the shikimic acid content.

Using glyphosate at doses ranging from 0.27–16.00 kg a.i./ha, the Bidor biotype was 7-fold more resistant than the BRC and Lenggeng biotypes. The resistance status of the Bidor biotype was confirmed by a much lower shikimic acid accumulation compared to the BRC and Lenggeng biotypes. There was no significant difference (p >0.05) between shikimic acid accumulation in the leaves and stems at lower doses of glyphosate. At higher doses of glyphosate (1.44–16.00 kg a.i./ha for BRC and Lenggeng biotypes; 7.20–16.00 kg a.i./ha for Bidor biotype), shikimic acid accumulation was detected to be higher in the leaves than in the stems (*p <0.05). While EPSPS in the leaves and stems were both inhibited at lower doses, self-limitation probably impaired glyphosate translocation from the leaves to the stems at higher doses. Therefore, leaves were the likely major inhibition sites at high glyphosate concentrations.

Introduction

Glyphosate (N-[phosphonomethyl]glycine) is a non-selective, broad-spectrum herbicide, used extensively for post-emergence control of annual, biennial and perennial weeds. Glyphosate is absorbed into plants, distributed symplastically and apoplastically from the source organ (leaf) to sink organs (stem, flower, root) (Grossbard and Atkinson 1985). Glyphosate inhibits the sixth enzyme in the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (E.C. 2.5.1.19) preventing the biosynthesis of aromatic amino acids (Duncan et al. 1987; Padgette et al. 1991) which are the precursors of secondary metabolites such as lignin, auxin and tannin (Stafford 1974). It may take 10–20 days after glyphosate treatment for the injury symptoms such as foliar chlorosis and necrosis to be exhibited, before plant death occurs (Ashton and Monaco 1991; Singh and Shaner 1998).
Glyphosate has long been used successfully to control most types of weeds and was considered to be an ideal herbicide until the discovery of a glyphosate resistant weed *Lolium rigidum* Gaudin in 1996 in Australia (Pratley et al. 1999). Then followed reports of resistant *Eleusine indica* in Malaysia (Teng and Teo 1999; Lim and Ngim 2000), *Conyza canadensis* (L.) Cronq. and *Lolium multiflorum* Lam. in Delaware in the United States and in Chile, South America, respectively. Thereafter, two more resistant species were identified in South Africa, namely, *Conyza bonariensis* (L.) Cronq. and *Plantago lanceolata*. Various research studies such as dose response, biochemical, molecular and genetic have been conducted to understand the mode of action of glyphosate and also the mechanism of resistance in these weeds (Heap 2003).

*Eleusine indica*, also known as goosegrass or crow’s foot grass, is one of the most serious weeds found in the tropical and subtropical regions. This weed has caused poor yields in corn, soybean, cotton and vegetable (Gould 1968; Holm et al. 1977). Since the first report of glyphosate-resistant *E. indica* in 1997 at Teluk Intan, Perak (Lim and Ngim 2000), many more locations in Peninsular Malaysia have been reported to be infested by resistant *E. indica* biotypes (Teng and Teo 1999). Higher doses of glyphosate applied repeatedly over a short period are thought to be the main cause for the emergence of the glyphosate-resistant *E. indica* biotypes (Lim and Ngim 2000).

Studies have shown that amplification of the EPSPS gene or its increased expression would cause elevation of the EPSPS enzyme, which can overcome glyphosate inhibition in plants (Holländer-Czytko et al. 1992; Suh et al. 1993) and bacteria (Reinbothe et al. 1993). Substitution of amino acids within the EPSPS substrate binding site has also been shown to alter enzyme affinity for glyphosate, which can cause glyphosate resistance (Padgette et al. 1991). Baerson et al. (2002) and Ng et al. (2003) reported that *E. indica* resistance to glyphosate is due to reduced sensitivity towards glyphosate inhibition that is caused by substitution of the amino acid from proline to serine in the enzyme.

As a result of reduced glyphosate inhibition in the shikimate pathway, accumulation of the pathway intermediate, shikimic acid has been reported to be less in glyphosate-resistant buckwheat (*Fagopyrum esculentum* Moench) and *Galium mollugo* L. (Amrhein et al. 1980), *Glycine max* (L.) Merr. (cv. ‘Williams’) (Singh and Shaner 1998) as well as in *E. indica* (Tran et al. 1999) when compared to glyphosate-susceptible biotypes.

The aim of this study was to explain the response of the different biotypes of *E. indica* after glyphosate treatment, the involvement of EPSPS and the major site of the inhibition by looking at (i) the mortality rate of the biotypes, (ii) the shikimic acid content in both leaves and stems, respectively.

**Materials and methods**

**Seeds**

Seeds of putative susceptible and resistant biotypes of *E. indica* were collected from Lenggeng in Negeri Sembilan and Bidor in Perak, Malaysia, respectively. A known susceptible biotype of *E. indica* from CCM Bioscience Agrochemicals Research Centre (BRC), Melaka, was used as a comparison. All seeds were kept in tight, clean and dry universal bottles and stored at room temperature.

**Chemicals**

Commercially available glyphosate, *Roundup* which consists of 480 g a.i./litre or equivalent to 360 g a.e./litre was obtained from Monsanto (Malaysia) Sdn. Bhd. All other chemicals were obtained from Sigma (USA).

**Planting of Eleusine indica**

Scarified *E. indica* seeds from BRC, Lenggeng, and Bidor were soaked overnight in 0.2% KNO₃ prior to germination to break
seed dormancy and promote germination (Mayer and Poljakoff-Mayber 1982). Seeds were germinated for a week in 9 cm diameter petri dishes overlaid with Whatman No. 1 filter paper, prewetted with 5 mL of distilled water. The seeds were maintained in an incubator at alternate temperatures of 30/20 °C, a 12 h photoperiod and supplemental lighting (50 mE/m²/s). Each seedling was transplanted into a pot (3.9 cm diameter x 5 cm depth) filled with potting soil (Right Grow, Kosas Profil Sdn. Bhd.). Seedlings were watered daily and were grown under greenhouse conditions at a temperature range of 28–35 °C and light intensity of 800 ± 200 mE/m²/s. Glyphosate treatment was carried out when E. indica seedlings reached the five- to seven-leaf stage of development.

**Plant dose-response test**

Glyphosate (Roundup) at doses ranging from 0.27 to 16.00 kg a.i./ha was sprayed at the plants (25 plants per replicate) using a sprayer equipped with a flat-fan nozzle of spraying volume 450 litres/ha by using a moving-nozzle with a flat-fan tip and pressure of 100 kPa. Plant mortality was assessed at 7, 14 and 21 days after treatment (DAT). Control groups of each biotype were not sprayed with glyphosate. There were three replicates for each treatment. The level of resistance for each biotype was determined at the LD₅₀ value obtained using the logistic regression analysis equation (Turner et al. 1992; Pratley et al. 1999):

\[ P = 1/(1 + \exp(-(a + bx))) \]

where \( P \) = mortality proportion, \( x \) = rate of glyphosate, and \( a \) and \( b \) = coefficients for the curve fitted. Analyses were performed by STATISTICA 5.5A (1999).

**Extraction of shikimic acid**

The leaves and shoots (excised above the first leaf pair) from each control and glyphosate treated plants were collected at 2, 7, 14 and 21 DAT. New shoots and tillers, which were generated from the main stem of the surviving resistant biotypes treated with 0.27–2.40 kg a.i./ha of glyphosate were similarly collected at 21 DAT. Shikimic acid extraction was carried out according to Singh and Shaner (1998). Tissue samples of 100–300 mg were frozen in liquid nitrogen and pulverized with a mortar and pestle. The ground material was transferred to a micro centrifuge tube containing 0.25 N HCl (w/v; 1/3) and the mixture was mixed thoroughly. The supernatant was collected for shikimic acid assay after centrifugation at 14 000 rpm for 15 min.

**Determination of shikimic acid**

Shikimic acid content in the plant tissues was determined by the method of Gaitonde and Gordon (1958). Approximately 10–20 mL of the diluted supernatant (usually 10³ dilution) was made up to 3.0 mL with distilled water and then mixed with 0.50 mL of 1 % (w/v) periodic acid to oxidise shikimic acid and allowed to incubate for 3 h at room temperature. Then 0.50 mL of 1 N NaOH was added, mixed and 0.30 mL of 0.1 M glycine was added immediately to stabilise the product formed. The optical density at 380 nm was measured instantly using a spectrophotometer (Model Lambda 12 UV/VIS, Perkin Elmer). The shikimic acid content in the sample was calculated based on a standard shikimic acid curve in the range of 0–14.4 mg. All data were subjected to analysis of variance and the means were compared at \( p = 0.05 \).

**Results and discussion**

**Plant dose-response test**

Glyphosate injuries such as chlorosis and mild necrosis were observed at 7–10 DAT in the susceptible (BRC and Lenggeng) biotypes treated with 0.27–1.44 kg a.i./ha of glyphosate. At these rates, 3–43% of the BRC and Lenggeng biotypes were killed at 21 DAT (Figure 1). At 2.40 and 7.20 kg a.i./ha of glyphosate, both biotypes showed serious necrosis and 92% were killed at 21 DAT. At the highest rate (16.00 kg a.i./ha), 99% mortality of the susceptible biotypes (21 DAT) was observed. For the Bidor
biotype, low doses ranging from 0.27–1.44 kg a.i./ha did not cause any mortality at 21 DAT. Glyphosate at rates of more than 1.44 kg a.i./ha only caused mild chlorosis and slight necrosis on this biotype after 7 DAT. Slight mortality of 29–37% was observed at 2.40 and 7.20 kg a.i./ha and at the highest dose of 16.00 kg a.i./ha, only 74% of this biotype was killed at 21 DAT (Figure 1).

New shoots and short tillers were observed sprouting from the main shoots of the surviving Bidor biotype treated with 0.27–16.00 kg a.i./ha at 21 DAT. Glyphosate is known to stimulate shoot proliferation by reducing auxin production in treated plants (Parker 1976), whereby the auxin is associated with the apical dominance in plants (Clarke 1996). In other words, it is most likely that the effect of the auxin suppressing the growth of the auxiliary buds in *E. indica* was made ineffective by the reduction of the auxin level, which at the sub-lethal doses applied, partially killed the tiller tissues that served as the site for production of the auxin. Teng and Teo (1999) also reported the production by 30 DAT of more new shoots from the resistant *E. indica* after treatment with the sub-lethal dose. Glyphosate treatment was also reported to cause more generated shoots in *Agropyron repens* (L.) Beauv. (Coupland and Caseley 1975).

Logistic regression shows the Bidor biotype to be 7-fold more resistant than the BRC and Lenggeng biotypes. The Lenggeng biotype is however, not significantly different from the BRC biotype (*Table 1*), thus, it is considered a susceptible biotype. Tran et al. (1999) and Lim and Ngim (2000) reported 2–4 fold (early tillering stage) and 8–12 fold (two to three-tiller stage) of resistance in *E. indica*, respectively. From these comparisons, it is noted that a lower resistance level was exhibited in younger plant populations as compared to mature populations.

![Figure 1. *Eleusine indica* mortality due to glyphosate (Roundup) ranging from 0.27 to 16.0 kg a.i./ha at 21 DAT. Curve was fitted into the logistic analysis equation. Open circles (o) each represents mean of three replicates](image)

**Table 1. $L_D_{50}$ values and resistant ratio of BRC, Lenggeng and Bidor biotypes**

<table>
<thead>
<tr>
<th><em>E. indica</em> biotype</th>
<th>$L_D_{50}$ value (kg a.i./ha)</th>
<th>Resistant ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRC</td>
<td>1.1 (1.0–1.2)b</td>
<td>1.0</td>
</tr>
<tr>
<td>Lenggeng</td>
<td>1.2 (1.0–1.4)b</td>
<td>1.1</td>
</tr>
<tr>
<td>Bidor</td>
<td>7.9 (3.8–12.0)a</td>
<td>7.2 6.6</td>
</tr>
</tbody>
</table>

$L_D_{50}$ values (kg a.i./ha) is calculated from the equation: $P = 1/(1 + \exp(-(a + bx)))$

Figures in parentheses are 95% confidence limits $L_D_{50}$ values within the column not followed by the same letter differ significantly as determined by LSD at $p = 0.05$
Shikimic acid level in susceptible and resistant biotypes

There was no significant difference between leaf and stem tissues basal levels of shikimic acid for each BRC and Lenggeng biotypes ($p > 0.05$) (Figures 2 and 3). These levels are also within the range reported in other species such as *Zea mays* L. and *Glycine max* (L.) Merr. (0.07 mg/g fresh weight) (Singh and Shaner 1998), and in *Prunus pensylvanica* and *Populus tremuloides* (0.03–0.10 mg/g dry weight) (Stasiak et al. 1991). This suggested that the shikimic acid basal level is initially low in plants.

The concentration of shikimic acid in the leaves and stems of the control BRC biotype (0.02–0.05 mg/g fresh weight, (Figure 2), was significantly lower than the shikimic acid content of the control in the Lenggeng biotype (0.05–0.08 mg/g fresh weight.) ($p < 0.05$) as shown in Figure 3. Lower shikimic acid accumulation is related to resistance towards glyphosate (Singh and Shaner 1998). Thus, this part of the study proposed that the BRC biotype would be slightly more resistant towards glyphosate as compared to the Lenggeng biotype.

However, the difference between the two
Effects of glyphosate on goosegrass biotypes was not shown in the dose response study (Table 1), which suggested that the shikimic acid assay would be a good method that exhibits differences in quantitative response between biotypes. The dose response study is however, based on qualitative visual evaluation that is very subjective. Glyphosate application history, genetic and geographic variation may have contributed to these differences.

The leaves of the control Bidor biotype however, contained significantly higher basal level of shikimic acid than the two susceptible biotypes, ranging from 0.08 to 0.13 mg/g fresh weight (*p <0.05) (Figure 4). Lower basal activity level of EPSPS in resistant E. indica than in the susceptible biotype (Baerson et al. 2002) may explain the higher basal level of shikimic acid in the resistant biotype, as less shikimic acid could be converted to other.
intermediates by EPSPS. However, Tran et al. (1999) reported no difference in the shikimic acid level before glyphosate treatment between susceptible and resistant *E. indica* biotypes. In another study the control culture cell of glyphosate-resistant *Cichorium intybus* L., was reported to have a higher shikimic acid content of 3.33–7.0 mg/g fresh weight compared to its control susceptible culture (around 0.17 mg/g fresh weight) (Sellin et al. 1992). Having a larger pool of the shikimate pathway intermediates, such as the shikimic acid in the resistant biotype, would be an advantage to overcome glyphosate inhibition (Westwood and Weller 1997).

Generally, increases in shikimic acid concentrations were detected in all biotypes beginning 2 DAT at doses ranging from 0.27–16.0 kg a.i./ha compared to the control. Shikimic acid levels in the leaves and stems of all biotypes changed inconsistently from
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2 DAT to 21 DAT (Figures 2, 3 and 4). Such observations are paralleled with glyphosate treatment that may inhibit the EPSPS, thus blocking the shikimic acid from changing to other metabolites in the shikimate pathway and resulting in the accumulation of shikimic acid in the treated plants.

Reduction of shikimic acid content in the leaves of susceptible biotypes treated with a low dose (0.27 kg a.i./ha) at 21 DAT was consistent with lower injury and mortality rate recorded at 21 DAT. Recovery from glyphosate inhibition has also been reported in plants treated with a low or sub-lethal dose, possibly due to metabolism of shikimic acid (Singh and Shaner 1998), dilution of glyphosate during its translocation from leaves to other sink tissues and new cells, or exudation of glyphosate from the roots (Coupland and Caseley 1979; Rodrigues et al. 1982; Hetherington et al. 1999).

At higher glyphosate doses (0.80–16.00 kg a.i./ha), shikimic acid content in the plants was generally higher at 21 DAT compared to 2 DAT. However, shikimic acid accumulation in tissues treated with lethal doses (7.20 and 16.00 kg a.i./ha) was lower than that at sub-lethal doses (0.80–2.40 kg a.i./ha). At these lethal doses, almost all the plants eventually failed to function, thus contributing to the lower shikimic acid accumulation. The highest level of shikimic acid content in the BRC and Lenggeng biotypes were detected in treated leaves, which were 193 and 158 times higher than that of the control (p <0.05), respectively.

The resistant Bidor biotype treated with 0.27–16.00 kg a.i./ha of glyphosate generally exhibited much lower shikimic acid levels compared to the susceptible biotypes (*p <0.05) (Figure 4). Shikimic acid accumulation was detected in leaves and stems treated with 0.80–16.00 kg a.i./ha beginning 2 DAT compared to the control (*p <0.05). However, less shikimic acid content was observed in these tissues at 21 DAT. These results are expected due to the ability of the Bidor biotype to overcome glyphosate inhibition even at a high dose.

The highest shikimic acid level was detected in leaves from plants treated with 16.00 kg a.i./ha at 14 DAT, this being 111 times higher than that of the control (*p <0.05). The lesser shikimic acid accumulation in the Bidor biotype indicated that its shikimate pathway was partially inhibited by glyphosate as has been reported previously in the Johor E. indica biotype (Tran et al. 1999). This phenomenon may be explained by the reduced sensitivity of EPSPS towards glyphosate that enables shikimic acid in the resistant biotype to succeed the pathway and it is not due to overexpression of the enzyme (Baerson et al. 2002).

Figure 4 shows that there was no accumulation of shikimic acid in new shoots and tillers from the resistant Bidor biotype treated with 0.27–2.40 kg a.i./ha at 21 DAT. This suggests that the shikimate pathway in new shoots and tillers is not inhibited by glyphosate in the resistant biotype and that this may be due to the dilution of the glyphosate in the plants as they grow and via root exudation into the soil (Coupland and Caseley 1979).

The shikimic acid level in the leaves was not significantly different (p >0.05) from the stems of both susceptible biotypes treated with low doses of 0.27 and 0.80 kg a.i./ha at 21 DAT or in the Bidor biotype treated with 0.27–16.00 kg a.i./ha at 21 DAT. Both parts of the plant appear to be equally inhibited by glyphosate at these doses. The shikimic acid level in the leaves was higher than that in the stems (p <0.05) of plants treated with higher doses of 1.44–16.00 kg a.i./ha for the BRC and Lenggeng biotypes at 21 DAT, and 7.20 and 16.00 kg a.i./ha for the Bidor biotype at 14 DAT. This was probably due to the self-limitation mechanism where glyphosate at high doses limits its own mobilisation from leaves to other sink organs. Glyphosate has been shown to eventually accumulate in the leaves, thus making this organ the major
Conclusion
This dose response study has shown that glyphosate is not effective in controlling the Bidor biotype, which was found to be 7-fold more resistant than the BRC and Lenggeng biotypes. Less shikimic acid accumulated in the Bidor biotype compared to the BRC and Lenggeng biotypes and this shows that EPSPS inhibition by glyphosate was evident in the resistant biotype especially when treated with high doses of glyphosate. The major inhibition sites of the glyphosate in the susceptible biotypes are the leaves that also serve as the source organ to carry out photosynthesis to maintain the survival of the plants. The resistant biotype seemed not to be affected and this could be an advantage for recovery from injury by glyphosate.

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References


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Abstrak
Kerintangan dua jenis biotip *Eleusine indica* [L.] Gaertn. terhadap glifosat yang didapati dari dua tempat yang berasingan iaitu, Lenggeng dan Bidor berbanding dengan biotip rentan dari CCM Bioscience Agrochemicals Research Centre (BRC), Malaysia telah dikenal pasti. Pembabitan 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) di dalam biotip rintang terhadap glifosat dikaji melalui pengukuran kandungan asid shikimik.

Dengan menggunakan glifosat pada dos 0.27–16.00 kg a.i./ha, biotip dari Bidor didapati tujuh kali lebih rintang daripada biotip dari BRC dan Lenggeng. Status kerintangan pada biotip dari Bidor telah disahkan dengan penumpukan asid shikimik yang sedikit berbanding dengan biotip dari BRC dan Lenggeng. Pada dos glifosat yang rendah, penumpukan asid shikimik di dalam daun dan batang tidak nyata berbeza (*p > 0.05*). Pada dos glifosat yang tinggi (1.44–16.00 kg a.i./ha untuk biotip BRC dan Lenggeng; 7.20–16.00 kg a.i./ha untuk biotip Bidor), pengumpulan asid shikimik dikeysan lebih tinggi di dalam daun berbanding dengan batang (*p < 0.05*). Manakala EPSPS di dalam daun dan batang direncat pada kepekatan rendah, penghadan-sendiri mungkin telah tidak menyebabkan pengangkutan glifosat dari daun ke batang pada dos yang tinggi. Oleh itu, daun mungkin menjadi tapak perencatan utama pada kepekatan glifosat yang tinggi.