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Research Article

Mutation site identification confers glyphosate resistance in goosegrass (*Eleusine indica* L. Gaertn) from Jonggol, Bogor, Indonesia

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ABSTRACT

Glyphosate resistance is a serious problem in weed control, especially in oil palm plantations. However, evaluation of suspected resistant weeds such as Eleusine indica L. Gaertn at the gene level is still scarce in Indonesia. Here, target-site resistance to glyphosate of the EPSPS gene was evaluated. The objective of this study was to identify glyphosate resistance caused by mutation points in goosegrass biotypes from oil palm plantation in Jonggol, West Java, Indonesia. Bio-assay analysis was carried out by planting goosegrass in pots. Glyphosate was applicated using eight-level, namely 0, 0.125x, 0.25x, 0.5x, x, 2x, 4x, and 8x, where x was the recommended dose (972 g ha^{-1}) for three replications. Weed damage was evaluated from plant biomass and then regression analysis was performed to obtain the LD_{50} and resistance index. The E. indica from block V had a reduced sensitivity status with 2.343 value of resistance index, and those from Block II biotype had a sensitive status with 1.588 resistance index. Alignment of the EPSPS gene showed no mutation was observed at two target-points, indicating that increasing resistance of E. indica in the study site could be controlled by other factors. Nevertheless, E. indica of block V biotype had a resistance potential due to the highest LD_{50}. It is necessary to evaluate further the possibility of mutation in other target-points of the EPSPS gene.

Keywords: bio-assay; EPSPS; LD_{50}; resistance index; sequencing; target-site resistance

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INTRODUCTION

Glyphosate is one of the most widely used herbicide active ingredients in weed control. Glyphosate demand increased from 1974 to 2014 by about 71.6% (Benbrook, 2016). Glyphosate is widely used because it is effective to inhibit weed regrowth due to it works systemically and translocates to all plant tissues. Glyphosate is a non-selective herbicide so it is effective in controlling weeds including grasses, sedges, and broadleaves weeds. Glyphosate has a unique mode of action (MOA) because of its ability to inhibit the 5-enolpiruvil-shikimate-3-phosphate synthase (EPSPS) enzyme which acts on the shikimate pathway (Duke, 2017).

Nowadays, the number of weed species that had been reported resistant to glyphosate is 55 species, consisting of 28 species from the monocot and 27 species from the dicot groups (Heap, 2022). In general, repetitive herbicide use of one active ingredient is speculated to cause weed resistance. One of the weeds that had been widely reported as resistant to glyphosate is goosegrass (*Eleusine indica* (L.) Gaertn) (Plaza et al., 2021; C. Zhang et al., 2021). Goosegrass is a monocotyledonous annual weed of the Poaceae. The weed is reported to cause yield losses in cotton up to 50% of the maximum potential yield

(Ma et al., 2015). Glyphosate resistance of goosegrass is also reported in oil palm (Nugraha, 2022; Tampubolon and Purba, 2018) and rubber (Purba et al., 2019) plantations.

Weed resistance was location-specific; so weed biotypes in one location could differ from those in other locations (Kumar et al., 2019). Jonggol Oil Palm Experimental Plantation has reported the incidence of goosegrass resistance to glyphosate recently. The weed resistance occurs in Block II and Block V of the plantation with a resistance index of up to 5 (Nugraha, 2022). In the plantation, glyphosate becomes the main herbicide in the last ten years with herbicide rotations rarely implemented. Based on our data, a continuous application of glyphosate was applied from 2016 to 2022 and in 2021 supplemented with paraquat.

In general, glyphosate resistance occurs through two mechanisms, i.e., target-site resistance (TSR) which is nucleotide changing of herbicide target genes, and non-target-site resistance (NTSR) which is related to secondary metabolic pathways (Domínguez-Valenzuela et al., 2021). The TSR mechanism predominantly occurs in the EPSPS gene through changes in amino acids (non-synonymous mutation), namely: Thr102Ile, Pro106Ser, and Pro381Leu. In addition, nucleotide changes that cause synonymous mutations have also been reported (Franci et al., 2020; Gaines et al., 2019). Mutation at two points of codon no. 102 and 106 (TIPS mutation) was one of the primary causes of resistance to glyphosate because it showed a very high resistance contributing up to 85% against cultivated plants (Han et al., 2017). The existence of TIPS mutation of those codons stimulates the weed to have a resistance index of up to 182, when compared with mutations at other points or single mutations (Yu et al., 2015, p. 201).

In practice, information on resistance mechanisms is important for particular herbicides because it determines the effectiveness of the herbicide application. Moreover, resistance incident implies for the oil palm plantation manager selects other herbicides with different MOA. The objective of this study was to identify the mechanism of target-site resistance associated with the TIPS mutation of *Eleusine indica* to glyphosate herbicide.

MATERIALS AND METHODS

Experiment I: Eleusine indica bio-assay

This research was held from April to September 2022 at Cikabayan Bawah Greenhouse, Department of Agronomy and Horticulture, IPB University, Bogor. The research was conducted using a randomized complete block design (RCBD) with one factor, namely herbicide dose with 8 doses level and 3 replications. Three *E. indica* populations were used (susceptible, Block II and Block V). The suspected resistant *E. indica* seeds were collected from Block II (6°28'25".32 "S, 107°1'42.88 "E) and Block V (6°28'13.68 "S, 107°1'10.04 "E) of the Jonggol Plantation which had been declared as resistant populations in previous research (Nugraha, 2022). Susceptible seeds were collected from the street of IPB Darmaga Campus (6°33'35.7 "S, 106°43'52.9 "E). Therefore, there were 24 experimental units to test weeds from each block of the plantation. Ripe seeds were characterized by brownish-yellow flowers and easily shed seeds from the panicles. Seeds collection was conducted by the plot area from one point with a radius of 100 m and mixed into one sample in a paper bag (Ginting et al., 2015; Stankiewicz-Kosyl et al., 2022).

Seeds were planted in 20 cm diameter pots with a mixture of soil and manure 1:1 (w/w). The selection was carried out by removing non-uniform plants and maintaining the same 15 individual plants. Herbicide application was performed using eight doses namely 0, 0.125x, 0.25x, 0.5x, x, 2x, 4x, and 8x with x being the recommended dose of glyphosate (972 g ha^{-1}). The spray volume used was 400 L ha^{-1}. Glyphosate herbicide was sprayed when the plants reached the stage of four true leaves by arranging the pots in a certain area.

Plant biomasses were harvested 7 days after application (DAA) by cutting all the weed parts at ground level. The green parts of the weed were oven-dried at 80 ℃ for 48 hours (Bilkis et al., 2022). Chlorophyll content was measured using a portable chlorophyll meter (SPAD-502) just before harvesting. The value obtained was then converted to obtain the value of chlorophyll content in the leaves. The formula used was $Y = 0.305\ e^{0.0545X}$, where Y was the total chlorophyll content in mg in each g of fresh leaves and X was the SPAD-502 value (Uddling et al., 2007).

The lethal dose (LD_{50}) value was calculated using regression analysis through the Excel program. The percent damage values obtained were converted into probit values and the dose values were converted into log dose values. The calculation of the LD_{50} value used a linear regression equation of the probit value against the log dose. The linear regression equation used was $Y = aX + b$ where the Y value was the probit value of the percent damage and X was the value of the log dose. The form of the regression equation was then substituted for a value of Y = 5 to determine the log dose value where the weeds died by 50%. The X value was then converted to anti-log to obtain the LD_{50} value. The value of Y = 5 was used because the probit value of 50% is 5 (Guntoro and Fitri, 2013).

The resistance index (RI) was calculated as the LD_{50} value of the resistant population divided by the LD_{50} value of the susceptible population. Weed groups were categorized as sensitive if the RI value was <2; reduced sensitivity (RI = 2.0-2.9); low resistance (RI = 3.0-4.9), moderate resistance (RI = 5.0-9.9); high resistance (RI = 10.0-68.1); and very high resistance (RI > 68.2) (Stankiewicz-Kosyl et al., 2022).

Experiment II: Mutation identification on EPSPS encoding gene

The research was held from September to October 2022 at the Plant Molecular Biology Laboratory 2, Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University. Leaves of 0.1-0.5 g were taken and mashed together with 700 µL of DNA extraction buffer solution [100 mM Tris HCl (pH 8), 1.4 M NaCl, 20 mM EDTA, 2% CTAB, and 1% PVP]. DNA quality was tested using 1% agarose (Turaki et al., 2017). A forward primer with a nucleotide sequence of 5'-GCGGTAGTTGTTGGCTGTGGTG-3' and a reverse primer with a nucleotide sequence of 5'-TCAATCCGACAACCAAGTCGC-3' were used to amplify DNA with a length of 302 bp at the position between 979 base and 1280 base in the gene encoding EPSPS (GenBank accession number QEPD01001275.1).

The PCR amplification used: initial denaturation for 4 minutes at 94 °C, followed by 35 cycles of denaturation temperature at 94 °C for 5 seconds, annealing temperature at 57 °C for 1 minute, and elongation temperature at 72 °C for 1 minute, extension temperature at 72°C for 10 minutes (Ng et al., 2003). The results of the amplification were then electrophoresed using a 1.5% agarose gel and stained using 1 ppm EtBr solution by soaking the gel for 20 seconds and destained with water for 15 minutes. The result was documented using Gel Doc EZTM (Bio-Rad, USA) to see the length of amplified DNA.

Purification of PCR products and nucleotide base sequencing was performed using the Sanger method by the Genetic Science service company. Alignment of nucleotide base sequencing results was performed using the MUSCLE algorithm and visualization of alignment results using the Geneious Prime application.

RESULTS AND DISCUSSION

Experiment I: Eleusine indica bio-assay

Glyphosate application significantly affected chlorophyll contents (Table 1). Chlorophyll concentration in the susceptible population began to show significant differences in 121.5 g ha^{-1} against the 0 g ha^{-1} dose; and decreased further with the addition of treatment doses. Chlorophyll concentration in *E. indica* of Block II and Block V populations began to show significant differences in the treatment of 243 g ha^{-1} and decreased with increasing glyphosate doses. The chlorophyll contents in the Block II and Block V biotypes tended to be higher than those of the susceptible biotype (Table 1). Yellowed leaves occurred due to a decrease in chlorophyll content in the leaves.

Glyphosate could reduce chlorophyll content because this compound inhibits chlorophyll synthesis through a decrease in magnesium content in the leaves (Singh et al., 2020).

Table 1. Chlorophyll contents due to glyphosate treatment.

Glyphosate dose (g ha^{-1})	SPAD-502[a]			Chlorophyll contents (mg g^{-1}) [a]		
	Susceptible	Block II	Block V	Susceptible	Block II	Block V
0.0	26.13a	27.16a	28.77a	1.2668a	1.3405a	1.4633a
121.5	24.79a	27.40a	26.40a	1.1778b	1.3611a	1.2872ab
243.0	16.59b	23.64b	25.07ab	0.7546c	1.1064b	1.2032bc
486.0	14.43c	21.49b	21.61bc	0.6700d	0.9894b	1.0029cd
972.0	7.74d	15.25c	20.61c	0.4653e	0.7007c	0.9495d
1,944.0	6.01e	10.30d	13.48d	0.4235e	0.5412d	0.6392e
3,888.0	3.15f	9.29d	9.58d	0.3627f	0.5076d	0.5166ef
7,776.0	0.44g	1.54e	5.19e	0.3124g	0.3318e	0.4061f

Note: [a] Means within columns followed by different letters are significantly different according to Duncan's multiple range test (DMRT) where P≤0.05. SPAD-502 and chlorophyll contents data of each block were analyzed apart.

The susceptible population of *E. indica* showed the level of damage started to differ significantly at 486 g ha^{-1} glyphosate treatment with 45.28% damage (Table 2). On the other hand, at the same dose, the *E. indica* of Block II population exhibited 19.46% damage and the Block V population showed 25.71% damage. The level of weed damage at the recommended dose of 972 g ha^{-1} showed that the susceptible population was quite influential because it could cause damage of more than 50%, while both Block II and V populations showed potential resistance because their damage was still less than 50%.

Table 2. Percentage of weed damage due to glyphosate treatment.

Glyphosate dose (g ha^{-1})	Percentage of weed damage (%)[a]		
	Susceptible	Block II	Block V
0	1.76e	3.74e	2.88d
121.5	5.86e	8.59e	3.16d
243.0	6.09e	6.91e	11.63d
486.0	45.28d	19.46d	25.71c
972.0	79.43c	37.24c	26.74c
1,944.0	83.80bc	82.03b	53.92b
3,888.0	91.36ab	87.40ab	65.08b
7,776.0	94.54a	92.60a	86.34a

Note: [a] Means within columns followed by different letters are significantly different according to Duncan's multiple range test (DMRT) where P≤0.05. Data of each block were analyzed apart.

The Block V population exhibited the highest LD_{50} value of 1737.24 g ha^{-1} compared to the Block II and susceptible populations (Table 3). The Block II population also showed a higher LD_{50} value compared to the susceptible population. Based on the resistance index scale (Stankiewicz-Kosyl et al., 2022), *E. indica* from Block V was classified as reduced sensitivity status with a resistance index of 2.343 and sensitive status in weeds from Block II with a resistance index of 1.588. The LD_{50} value of weed from the Jonggol Oil Palm Experimental Plantation population was higher than the susceptible population taken from the street of IPB Dramaga. It is probable that selection pressure occurred in Jonggol Oil Palm Experimental Plantation due to the continuous use of glyphosate. Herbicide resistance occurs as the result of the adaptive evolution of weed populations in response to intense selection pressure conducted by herbicide application (Délye et al., 2013; Vila-Aiub, 2019). The susceptible weeds in a population would die when herbicides were being applied, while the resistant weeds in the same population were able to survive and reproduce and therefore the weed population was overwhelmed and enriched by the resistant weeds (Hulme, 2023).

The higher LD_{50} value of *E. indica* from Jonggol Oil Palm Experimental Plantation compared to the susceptible biotype of Darmaga indicates the potential resistance of

goosegrass. The conclusion was evidenced by the acquisition of a resistance index that already showed a reduced sensitivity status of goosegrass in Block V, while goosegrass in Block II still showed a sensitive resistance status. The resistance index obtained might continue to increase when weed control is still carried out using glyphosate without any rotation using different active ingredients (Evans et al., 2016).

Table 3. The lethal dose (LD_{50}) of *Eleusine indica* against glyphosate herbicide and resistance status.

Eleusine indica biotypes	Regression equation[a]	R^2 (%)	LD_{50} (g ha^{-1})[a]	RI	Status[b]
Susceptible	Y = 1.932X - 0.545	86.15	741.58	1.000	Sensitive
Block II	Y = 2.080X - 1.385	95.59	1,177.30	1.588	Sensitive
Block V	Y = 1.451X + 0.300	96.31	1,737.24	2.343	Reduced sensitivity

Note: [a] Abbreviations: Y, Probit value based on the percentage of weed damage; X, Log dose; LD_{50}, effective glyphosate dose needed for 50%, [b] RI-Resistance index classification was based on the scale of (Stankiewicz-Kosyl et al., 2022)

Integrated weed control using various techniques such as mechanical, biological, technical culture, and chemical is another solution to prevent the incidence of resistant weeds. Moreover, the application of chemical control might implement different herbicides with different MOA and types of herbicides both pre- and post-emergence to reduce the incidence of weed resistance. In general crops, the application of crop rotations is effective to reduce selection stress for the evolution of herbicide resistance (Hulme, 2023). However, crop rotation in oil palm plantation is not easy because a life cycle is commonly more than 25 years.

In the present experiment, the bio-assay evaluation confirmed that the *E. indica* biotypes of Block II had not experienced resistance while those in Block V showed the potential for herbicide resistance. Thus, mutation point identification is important because biotypes from Jonggol Oil Palm Experimental Plantation exhibited higher LD_{50} values than the susceptible biotypes.

Experiment II: Mutation identification on EPSPS encoding gene

Amplification of the EPSPS gene produced amplicons around 302 bp in all three *E. indica* biotypes (Figure 1). EPSPS plays an important role in the formation of three essential aromatic amino acids through the cyclic pathway (Dhaniaputri et al., 2022).

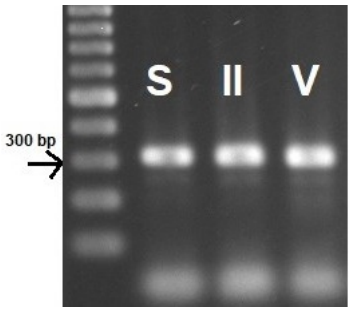

Figure 1. Amplification profile of EPSPS gene at 979-1280 base nucleotide region from *E. indica* biotypes. S-susceptible Darmaga, II and V are Block II and Block V, respectively; Left line-ladder 100 bp.

The results of nucleotide base sequencing showed a base sequence of 229 bp in the *E. indica* biotype from Jonggol Oil Palm Experimental Plantation Block II, and 257 bp in the *E. indica* biotype from Block V. The susceptible *E. indica* biotype shows a readable base length of 302 bp (Figure 2). The length of readable bases in the nucleotide base sequencing process could vary due to the presence of unreadable areas close to the primer binding site or due to differences in DNA quality (Crossley et al., 2020). Low DNA quality during extraction made the number of amplicons low. Increasing the number of amplicons can be done by increasing the number of PCR cycles (Prosser et al., 2016).

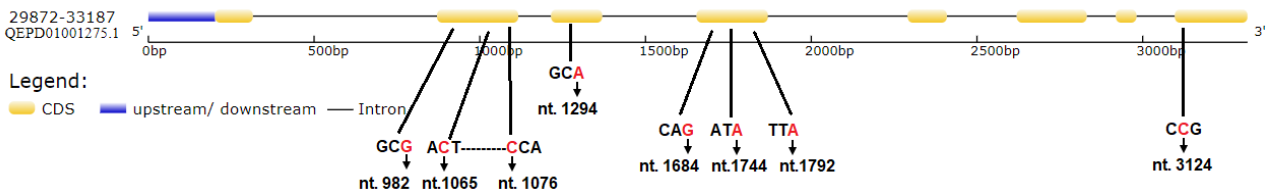

Figure 2. EPSPS gene structure and eight points of most common mutation (Chen et al., 2015; Franci et al., 2020; Li et al., 2022).

The EPSPS gene in *Eleusine indica* species consists of 8 exons and 7 introns with a gene length of 3315 bp starting at 29,872 bp to 33,187 bp of the whole plant draft genome (Zhang et al., 2019). Amplification was performed from base number 979 to base 1280 of the EPSPS gene. The amplicon was located at the second to third exon with a base length of 302 bp. Based on partial sequencing of the EPSPS gene, there was no SNP (Single Nucleotide Polymorphism) found in the amplified area (Figure 3). The SNP positions that have been reported are eight SNPs with five SNPs belonging to the synonymous group and three SNPs belonging to the non-synonymous group. The synonymous group reported amino acid changes at positions Thr102Ile (ACT-ATT), Pro106Ser (CCA-TCA), and Pro381Leu (CCG-CTG). The non-synonymous group occurred at amino acid positions 74, 145, 180, 200, and 216, namely changes in the base sequence GCG-GCA, GCA-GCG, CAG-CAA, ATA-ATC, and TTA-TTG (Figure 2) (Chen et al., 2015; Franci et al., 2020; Li et al., 2022).

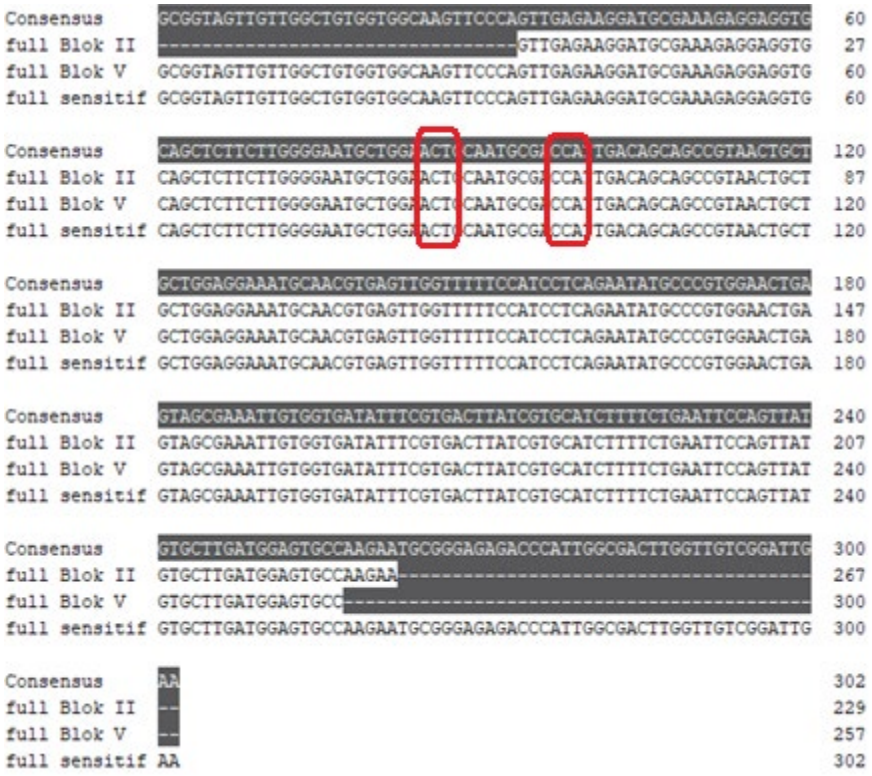

Figure 3. The alignment of EPSPS gene region 979-1280 (302 bp) from three *E. indica* biotypes.

The alignment showed there were no SNPs at two mutation points that have been widely reported (TIPS mutation) as the cause of glyphosate resistance of *E. indica* in both Block II and Block V biotypes, as indicated by red marks (Figure 3). Here, TIPS mutation in the 979-1280 region of the EPSPS gene was absent. The finding is not contrary to the status of *E. indica* of Block V which exhibited 'reduced sensitivity' according to bio-assay

test (Table 3). It is possible that *E. indica* from Block V biotype undergo mutation at other points. Figure 2 shows that in the EPSPS gene, there are eight points that commonly mutate in relation to glyphosate resistance. Therefore, it is interesting to study the presence of potential SNPs at other six points in *E. indica* that were not tested yet in this experiment. It is also possible that *E. indica* of Block V biotype has other resistant mechanisms such as Non-Target-Site resistance that could also cause high LD_{50} values. Nevertheless, the present experiment revealed that the potential resistance of *E. indica* from Block V in Jonggol indicates the need for implementing integrated weed control to reduce the dominance of resistant *E. indica* in the future.

CONCLUSIONS

Expression of *E. indica* Block II and Block V to glyphosate was sensitive and reduced sensitivity based on the resistance index value. The sensitivity of both *E. indica* biotypes from Jonggol Oil Palm Experimental Plantation was not supported by the presence of SNPs at two mutation points (TIPS mutation) in the EPSPS gene. It is interesting to study other mutation points in this gene and other mechanisms of glyphosate resistance in *E. indica*.

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