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Research Article

Characterization of *EiEPSPS* Genes from *Eleusine indica* (L.) Gaertn. Ecotypes Differing in Glyphosate Resistance

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ABSTRACT

Goosegrass (*Eleusine indica* (L.) Gaertn.) is among the most noxious weeds, particularly during the early growth stages of oil palm. Glyphosate is the most widely used herbicide globally for controlling *E. indica* by targeting the EPSPS enzyme. This study aimed to assess glyphosate resistance of *E. indica* from several oil palm plantations in West Java using bioassays and molecular studies. Ecotypes exhibiting altered sensitivity were subjected to DNA isolation using the CTAB method to analyze genetic variation in the *EiEPSPS* gene to determine whether resistance was conferred via a target-site resistance (TSR) mechanism. Bioassays indicated the Cicareuh ecotype (resistance-to-susceptible ratio, R/S 2.6) is classified as decreasing sensitivity, while Mekarjaya (R/S 3.6), Sukasirna (R/S 3.3), Prabugantungan (R/S 3.9), and East Rangkasbitung (R/S 4.7) are classified as low-resistance ecotypes. Molecular analysis revealed region-specific nucleotide variations in both introns and exons. Four nucleotide substitutions were identified in the non-coding regions at positions 1,781, 1,810, 1,843, and 2,844. Within the coding regions, two homozygous non-synonymous mutations were detected, resulting in amino acid substitutions at position 1,375 (Pro-106-Ser) and 3,422 (Pro-381-Leu). Our study indicates that previously reported homozygous nucleotide variations at substitution sites are insufficient to confirm glyphosate resistance, as the same alleles were detected in both resistant and sensitive genotypes. Combined bioassay and molecular analyses suggest that reduced glyphosate sensitivity in *E. indica* populations sampled from Bogor, Sukabumi, and Lebak has not yet been consistently associated with known *EiEPSPS* target-site mutations.

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is one of the mainstay plantation commodities in Indonesia, especially for food and biodiesel (Farobie and Hartulistiyoso 2021). The area of oil palm plantations in Indonesia is increasing in line with the world's demand for vegetable oil, reaching 16.83 million hectares with a production of 47.47 million tons in 2024, and making Indonesia one of the major global producers of oil palm (Ministry of Agriculture 2024, 2025). Increasing palm oil yields is key to ensuring the industry's long-term sustainability and meeting higher demand for crude palm oil (CPO). Therefore, agronomic aspects such as environment, plant materials, and technical cultural measures are essential to ensure optimum productivity (Sugianto *et al.* 2025).

Weeds are a problem in plant cultivation and may disrupt harvesting and fertilization activities (Iddris *et al.* 2023). Unmanaged weed populations can reduce palm oil production by 20 to 80% (Nugraha and Guntoro 2022). *Eleusine indica* (L.) Gaertn. or goosegrass is the most dominant grass weed during early oil palm growth, competing strongly with the crop for water, nutrients, light, and growing space (Kurniadie *et al.* 2023). This weed is considered one of the most problematic because

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it grows rapidly, thrives in various environments, and competes with cultivated plants (Zhang *et al.* 2019).

Glyphosate is currently the most extensively utilized herbicide active ingredient worldwide due to its systemic action against weeds, low toxicity profile, and low soil residual activity (Martins-Gomes *et al.* 2022), including in oil palm plantations (Purba and Sipayung 2022). Its mode of action involves the inhibition of enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme of the shikimate pathway. EPSPS converts the glycolytic intermediates phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) into essential precursors for the synthesis of aromatic amino acids, including phenylalanine, tyrosine, and tryptophan, as well as the vitamins folate and menaquinone (Hertel *et al.* 2021). However, intensive glyphosate use with minimal diversification of strategies has contributed to the emergence of resistant weeds (Duke 2017).

Glyphosate resistance mechanisms are classified into target-site resistance (TSR) and non-target-site resistance (NTSR). The TSR mechanism involves genetic mutations that alter the herbicide's binding site, reducing its effectiveness and allowing the weed to survive (Gaines *et al.* 2020). Several non-synonymous mutations in the *EiEPSPS* genes of *E. indica* were suspected to cause glyphosate resistance, including Pro-106-Ser (Baerson *et al.* 2002; Ng *et al.* 2004), Pro-106-Ala (Zhang *et al.* 2021), Pro-106-Leu (Chen *et al.* 2023), Pro-381-Leu (Zhang *et al.* 2015), and a double amino acid mutation Thr-102-Ile + Pro-106-Ser (Zhang *et al.* 2021). *E. indica* resistance to glyphosate herbicide in Indonesia is increasingly reported in North Sumatra, Lampung, Bangka Belitung, Riau, and West Kalimantan (Umiyati *et al.* 2023; Kurniadie *et al.* 2023). However, reports on glyphosate resistance of *E. indica* in West Java remain limited. Therefore, this experiment aimed to (1) classify the resistance level of several *E. indica* ecotypes to glyphosate herbicide from West Java, and (2) identify the nucleotide variation on the *EiEPSPS* gene from *E. indica* ecotypes differing in their resistance level that may contribute to the TSR mechanism.

2. Materials and Methods

2.1. Plant Materials and Experimental Conditions

E. indica samples were collected within a ±10 m radius of a fixed coordinate. This study investigated three ecotypes of *E. indica* originating from Bogor, Sukabumi, and Lebak (Table 1).

The Bogor ecotype (Babakan, Cigudeg, and Mekarjaya) is located at an elevation of approximately 200 m a.s.l. (meters above sea level), with a mean temperature of 26.7°C, relative humidity of 82.6%, and annual rainfall of 453.8 mm (BMKG 2025). The Sukabumi ecotype (Cicareuh, Cikidang, and Sukasirna) occurs at an elevation of about 500 m a.s.l., with a mean temperature of 26.9°C, a relative humidity of 83.2%, and an annual rainfall of 262.8 mm (Statistics Indonesia 2025). Meanwhile, the Lebak ecotype (Cikareo, Prabugantungan, and East Rangkasbitung) is situated at an elevation of approximately 100 m a.s.l., with a mean temperature of 28.2°C, relative humidity of 81.1%, and annual rainfall of 1209.9 mm (BMKG 2025). The

Table 1. Sampling sites of *E. indica* from West Java and Banten for glyphosate resistance

	Ecotypes	Coordinate	Planting time	Herbicide use history	Application intensity per year
	Babakan (susceptible)	6°33'36.7"S 106°43'51.4"E	-	-	-
Bogor	Cigudeg	6°32'47.6"S 106°31'52.5"E	2001-2004	Glyphosate and 2,4-D dimethyl amine	2x-3x
	Mekarjaya	6°31'04.1"S 106°30'11.2"E	2002-2004	Glyphosate and methyl metsulfuron	2x-3x
Sukabumi	Cicareuh	6°53'50.9"S 106°41'58.9"E	2018	Glyphosate and paraquat	4x
	Cikidang	6°54'14.4"S 106°38'10.7"E	2008	Glyphosate and paraquat	2x-3x
	Sukasirna	6°52'41.6"S 106°43'50.3"E	2005-2007	Glyphosate and paraquat dichloride	2x-3x
Lebak	Cikareo	6°29'19.0"S 106°04'20.8"E	2018-2019	Glyphosate and ammonium gluphosynate	2x-3x
	Prabugantungan	6°27'04.3"S 106°03'20.8"E	2003-2005	Glyphosate and ammonium gluphosynate	2x-4x
	East Rangkasbitung	6°22'22.3"S 106°17'07.2"E	2003-2005	Glyphosate	2x-3x

Sampling site of susceptible ecotype based on Ferdinans *et al.* (2023); putative resistant ecotypes inferred from farmer interviews

variations in altitude and climatic conditions reflect distinct environmental differences among the ecotypes. Plants were cultivated in plastic pots (15 cm in diameter), with 20 plants per pot. The experimental design was a randomized complete block design (RCBD) consisting of two factors with three replications. The first factor was the origin of weed seeds (nine locations), and the second was the glyphosate dose (seven levels).

2.2. Dose-Response Experiment

Glyphosate was applied when the plants were two weeks old using Roundup herbicide, a YOTO electric knapsack sprayer (16 L), and a spare-part polijet nozzle sprayer (1.4 min^{-1}). Glyphosate was applied at 0, 243, 486, 972, 1,944, 3,888, and 7,776 g a.i. ha^{-1}. These rates are equivalent to 0, 0.25, 0.5, 1, 2, 4, and 8 times the recommended field rate of glyphosate (972 g a.i. ha^{-1}), with application volumes of 0, 1.25, 2.5, 5, 10, 20, and 40 ml L^{-1}. The plants were harvested 8 days after treatment (DAT), when one of the treatment combinations exhibited 95% damage relative to the total population. Samples were dried in an oven at 80°C for 48 hours.

2.3. Data Analysis

Data were analyzed using RStudio with ANOVA at the 5% level, followed by Duncan's Multiple Range Test (DMRT). GR_{50} was analyzed using OriginPro 2024 software with a non-linear regression test of the log-logistic model. The weed percentage of damage and GR_{50} (y) are obtained using the formula (Kurniadie *et al.* 2023):

$$\text{Percentage of damage (\%)} = (1 - (T / C) \times 100)$$

Where:
T: dry weight of herbicide-treated weeds
C: dry weight of herbicide-untreated weeds

$$y = C + \frac{D - C}{1 + (\chi / I_{50})^b}$$

Where:
C: lower limit of data range
D: upper limit of data range
B: slope
I_{50} = 50% dose response

The R/S value was calculated by comparing the GR_{50} value of the test ecotype (R) to the GR_{50} value of the sensitive ecotype (S). According to Stankiewicz-Kosyl *et al.* (2022), weed resistance levels were categorized based on resistance-to-susceptible ratio (R/S) values: R/S<2 = sensitive, R/S 2-2.9 = decreased sensitivity, R/S 3.0-4.9 = low resistance, R/S 5.0-9.9 = moderate resistance, R/S 10.0-68.1 = high resistance, R/S >68.2 = very high resistance.

2.4. DNA Isolation, *EiEPSPS* Gene Amplification, DNA Sequencing, and Identification of Target Site Resistance on the *EiEPSPS* Gene

Total genomic DNA was isolated from the leaves of *E. indica* ecotypes with altered glyphosate sensitivity relative to the sensitive ecotypes, following the CTAB method (Aboul-Maaty and Oraby 2019) with slight modifications. The quantity and purity of the isolated genomic DNA were estimated with a MaestroNano™ Micro-volume Spectrophotometer, and DNA integrity was assessed by electrophoresis. Primers were designed based on the *EiEPSPS* gene sequence (GenBank accession number: AY157642.1) and the draft genome of *E. indica* (GenBank accession number: QEPD01001275.1) as shown in Table 2.

DNA amplification was performed in a total volume of 50 µL, consisting of 25 µL 2x MyTaq HS Red Mix, 2 µL each of forward and reverse primers with 10 µM concentration, 17.5 µL *E. indica* genomic DNA with 12 ng μL^{-1} concentration, and 3.5 µL of nuclease-free water (NFW). The PCR profile used was initial denaturation temperature for 1 minute at 95°C, continued with 32 cycles (fragment 1), 35 cycles (fragment 2 and 4), 35

Table 2. Forward (Fw) and reverse (Rv) primer pairs used to amplify the *EiEPSPS* gene fragments

Primers name	Nucleotide base sequence (5' – 3')	Tm (°C)	Ta (°C)	Target region (bp)	Amplicon size (bp)
EiEPSPS-Fw1[1]	CAACCCACCACCACCATCA	57.8	63-65	266-1,408	1,143
EiEPSPS-Rv1[1]	TTCCTCCAGCAGCAGTTACG	57.2			
EiEPSPS-Fw2[2]	ACTGTGGTGGATAACCTTTT	51.6	50	1,177-2,160	984
EiEPSPS-Rv2[2]	TTGACCTCCCTTGATGTAGA	52.6			
EiEPSPS-Fw3[3]	CTGGTTCCATCAGCAGTCAG	55.6	53	1,964-2,962	999
EiEPSPS-Rv3[3]	TCGCTCCCATCATCTCGA	55.5			
EiEPSPS-Fw4[4]	GTCCCCTAAAAATGCCTACG	53.3	55	2,591-3,581	991
EiEPSPS-Rv4[4]	TAGTCTGGGAAGGTCTTGC	53.8			

[1]fragment 1, [2]fragment 2, [3]fragment 3, [4]fragment 4, Tm: melting temperature, Ta: annealing temperature, bp: base pairs

or 40 cycles (fragment 3), denaturation for 15 seconds at 95°C, annealing for 15 seconds at the Ta according to Table 2, and extension for 10 seconds at 72°C. PCR was terminated with a final extension for 10 minutes at 72°C. PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gel in 1x TAE buffer (Tris-acetate-EDTA) at 100 V for 35 minutes. Gels were further stained by ethidium bromide staining, and DNA fragments were visualized by UV transilluminator (Gel Doc EZTM, Bio-Rad, United States). PCR samples with the expected amplicon size were sent to PT. Genetika Science Indonesia for sequencing using the Sanger method. Nucleotide sequences from four overlapping PCR fragments, amplified using the primer pairs listed in Table 2, were assembled into contigs and subsequently aligned with the MUSCLE algorithm in BioEdit version 7.2.

3. Results

3.1. Toxicity Indication

E. indica ecotypes from Babakan, Cigudeg, Cikidang, and Cikareo exhibited stunting, chlorosis, necrosis, and death at 8 DAT from 0.5× to 8× recommended dose of glyphosate. On the other hand, *E. indica* ecotypes from Mekarjaya, Cicareuh, Sukasirna, Prabugantungan, and East Rangkasbitung survived and exhibited only chlorosis at 8× recommended dose (7,776 g a.i. ha^{-1}) (Figure 1).

The herbicide application affected the dry weight and percentage of damage to *E. indica*. Cigudeg ecotype showed the highest dry weight, while Cikareo ecotype had the lowest dry weight. Cikareo ecotype showed the highest percentage of damage, while East

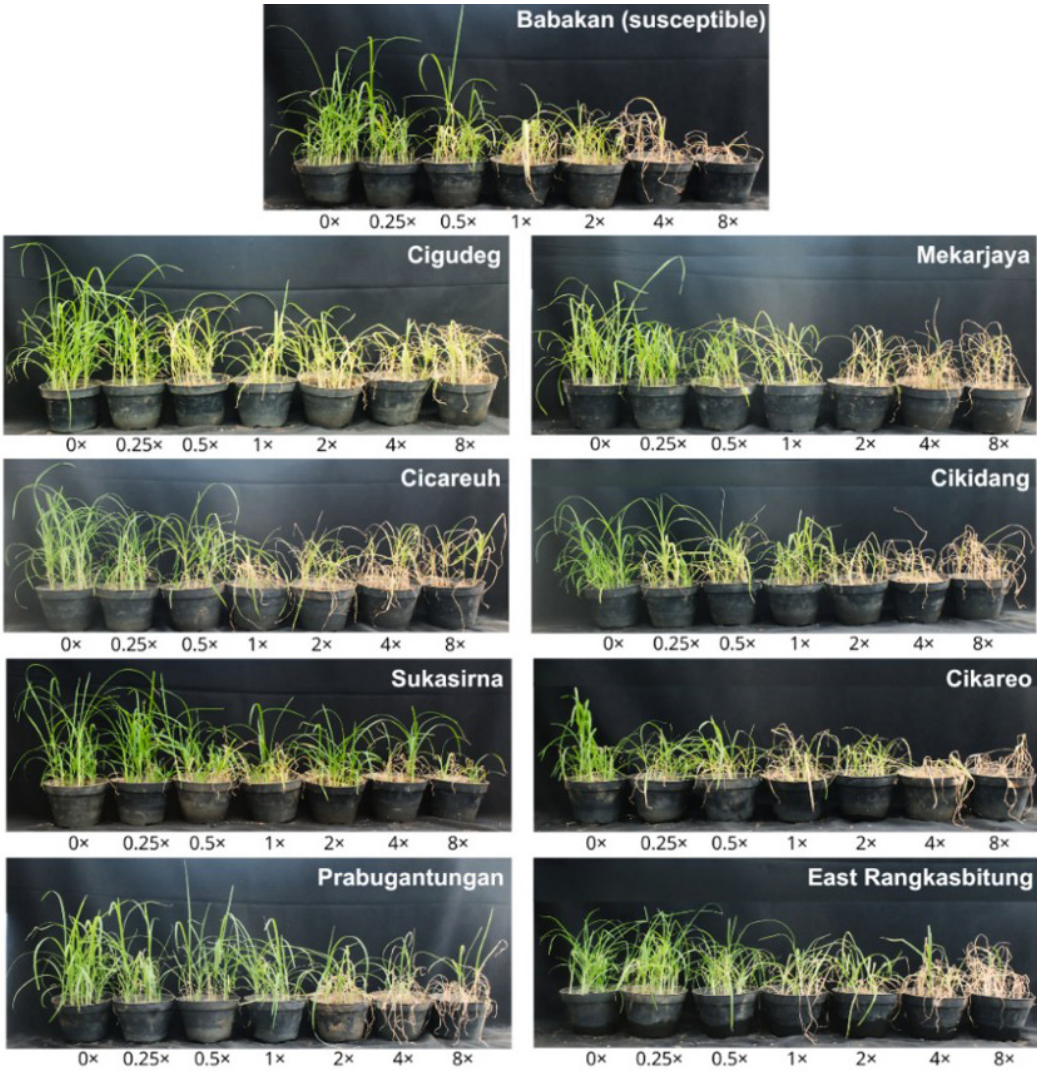

Figure 1. Visual comparison of ecotype responses to increasing glyphosate doses (0× – 8× of the recommended dose), observed at 8 DAT

Rangkasbitung ecotype had the lowest percentage of damage (Table 3).

3.2. Resistance Level

The dose-response curve was used to estimate the GR_{50} values for each *E. indica* ecotype following glyphosate application (Figure 2). Dose-response analysis revealed that no *E. indica* ecotype reached 50% injury at 0.25× recommended dose. At 0.5×, Babakan (susceptible) and Cikidang ecotypes exhibited 50% injury, whereas at 1× - 8× doses, all ecotypes were affected, with Mekarjaya, Prabugantungan, and East Rangkasbitung showing the lowest injury. The susceptible Babakan ecotype exhibited the lowest GR_{50} value (507.97 g a.i. ha^{-1}), confirming its sensitivity to glyphosate. In contrast, five of the eight resistant ecotypes showed elevated GR_{50} values, indicating reduced susceptibility (Table 4). The highest GR_{50} value was observed in the East Rangkasbitung ecotype (2,404.38 g a.i. ha^{-1}), which required a glyphosate dose 2.47 times higher than the recommended field rate to achieve 50% growth reduction. Similarly, the Prabugantungan (GR_{50} 1,978.64 g a.i. ha^{-1}), Mekarjaya (GR_{50} 1,843.06 g a.i. ha^{-1}), Sukasirna (GR_{50} 1,665.79 g a.i. ha^{-1}), and Cicareuh (GR_{50} 1,303.49 g a.i. ha^{-1}) ecotypes exhibited dosage 2.04×, 1.90×, 1.71×, and 1.34× higher than the recommended dose, respectively. The survival of resistant ecotypes occurred at glyphosate doses exceeding those required for the susceptible population.

The R/S classification indicated that four *E. indica* ecotypes were glyphosate-sensitive and five were glyphosate-resistant (Table 4). Ecotypes with R/S values <2 were categorized as sensitive, whereas those with R/S values >2 were classified as resistant. The R/S ratio indicates greater herbicide tolerance in the East Rangkasbitung ecotype than in the sensitive ecotype.

Table 3. Average dry weights and percentages of damage of nine *E. indica* ecotypes at several doses of glyphosate herbicide

Treatment *E. indica* ecotypes	Dry weight (g pot^{-1})	Percentage of damage (%)
Babakan	0.82^{b}	46.45^{c}
Cigudeg	1.00^{a}	55.56^{ab}
Mekarjaya	0.62^{cd}	47.95^{c}
Cicareuh	0.80^{b}	48.87^{bc}
Cikidang	0.70^{bc}	51.73^{abc}
Sukasirna	0.68^{bcd}	49.46^{abc}
Cikareo	0.52^{d}	56.66^{a}
Prabugantungan	0.77^{bc}	45.44^{c}
East Rangkasbitung	0.59^{cd}	44.71^{c}
F-Test Herbicide dosage (g a.i. ha^{-1})	**	**
0 (0×)	1.50^{a}	0.00^{f}
243 (0.25×)	1.10^{b}	23.00^{e}
486 (0.5×)	0.86^{c}	38.56^{d}
972 (1× recommended dose)	0.57^{d}	58.17^{c}
1,944 (2×)	0.46^{d}	66.93^{b}
3,888 (4×)	0.31^{e}	78.04^{a}
7,776 (8×)	0.24^{e}	82.84^{a}
F-Test	**	**
CV%	19.32%	19.83%
Interaction	n	n

** significant, n: non-significant, the same letter indicates the treatment is not significantly different with DMRT at 5% level

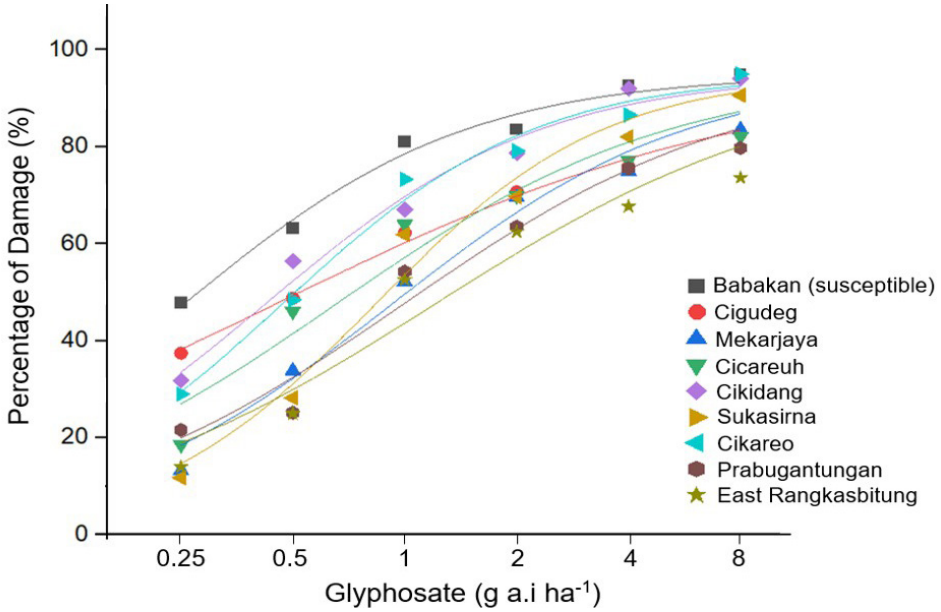

Figure 2. Inhibition curves by glyphosate from nine populations of *E. indica*. The lines are the reaction bends predicted by non-linear regression; symbols represent shares of average survival, held constant for the untreated controls

3.3. Nucleotide Base Variation in the *EiEPSPS* Gene in Four Ecotypes of *E. indica*

The *EiEPSPS* genes were successfully amplified from five *E. indica* ecotypes that exhibit decreased glyphosate sensitivity relative to Babakan, the sensitive ecotype (Figure 3). Although the first exon and intron were not appropriately amplified, this study successfully amplified major regions of the *EiEPSPS* gene, as illustrated by the blue lines in Figure 3. Fragments 2, 3, and 4 from part of exon 2 to part of exon 8 were obtained from the Babakan, Sukasirna, Prabugantungan, and East Rangkasbitung ecotypes. In the Mekarjaya and Cicareuh ecotypes, intron four of the *EiEPSPS* gene was not successfully amplified. Therefore, only the *EiEPSPS* sequences from the Babakan, Sukasirna, Prabugantungan, and East Rangkasbitung ecotypes were further analyzed and have been deposited in GenBank (NCBI) under accession numbers PV704970.1, PV704971.1, PV704972.1, and PV704973.1, respectively.

The *EiEPSPS* sequences from the four ecotypes were aligned with previously reported *EiEPSPS* genes available in the database to identify potential base variations responsible for the altered glyphosate sensitivity. The analysis revealed region-specific nucleotide variations across both intronic and exonic regions (Figure 4). In the non-coding regions, three homozygous nucleotide substitutions were identified in intron 3 at positions 1,781, 1,810, and 1,843, along with a single homozygous variation in intron 5 at position 2,844. Sequencing chromatograms revealed clear single-base calls at these positions, confirming the homozygosity of all detected intronic substitutions.

Within the coding regions, two non-synonymous mutations resulting in amino acid substitutions were detected: one at position 1,375 in exon 2 and another at position 3,422 in exon 8. Compared to the sensitive reference (S-AY157642.1), the Prabugantungan ecotype exhibited a nucleotide substitution at position 1,375 (CCA to TCA), resulting in a Pro-106-Ser amino acid change. In contrast, no variation was detected at position 3,422 (CCG) in either the sensitive reference (S-AY157642.1) or the Prabugantungan ecotype, which was categorized as having low resistance (LR) based

Table 4. GR_{50} value and resistance level of *E. indica* to glyphosate herbicide dose treatment

Ecotypes	GR_{50} (g a.i. ha^{-1})	R^2	R/S	Resistance level[1]
Babakan (susceptible)	507.97	0.98	-	-
Cigudeg	894.76	0.99	1.8	Sensitive
Mekarjaya	1,843.06	0.98	3.6	Low resistance
Cicareuh	1,303.49	0.94	2.6	Decreased sensitivity
Cikidang	840.68	0.98	1.7	Sensitive
Sukasirna	1,665.79	0.98	3.3	Low resistance
Cikareo	933.76	0.99	1.8	Sensitive
Prabugantungan	1,978.64	0.96	3.9	Low resistance
East Rangkasbitung	2,404.38	0.93	4.7	Low resistance

GR_{50} refers to the measurements of herbicide success in reducing 50% dry weight and is expressed as active ingredient grams per hectare (g a.i. ha^{-1}); R/S is the index of resistance, which was computed as the ratio of GR_{50} R to GR_{50} S; [1]Based on Stankiewicz-Kosyl *et al.* (2022)

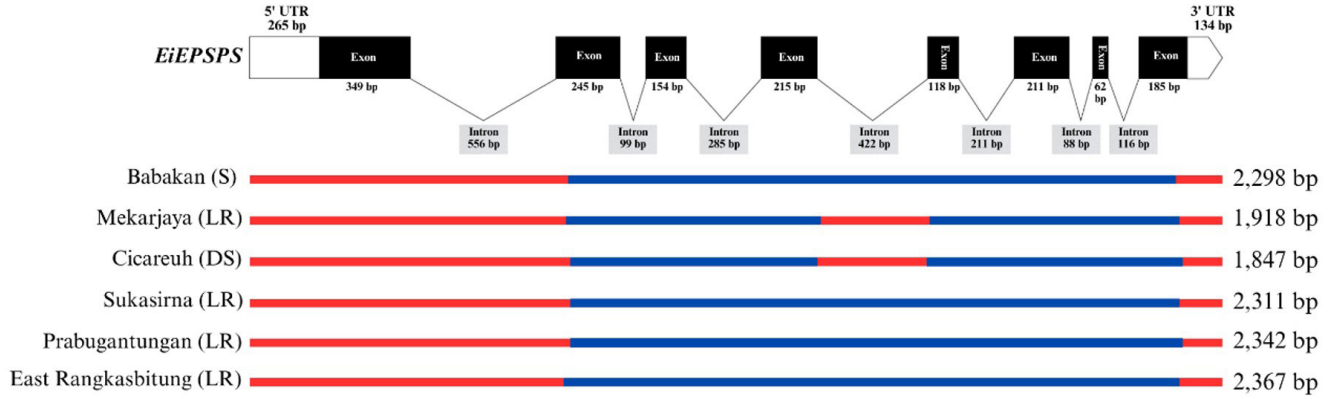

Figure 3. Scheme of successful reading of nucleotide base sequences in the *EiEPSPS* gene from six ecotypes of *E. indica*. The well-amplified region is marked in blue, while the poorly amplified region is marked in red; S: sensitive, DS: decreased sensitivity, LR: low resistance, bp: base pairs, UTR: untranslated region

EXON

Ecotypes	Base Position 1,281	Base Position 1,375	Base Position 2,091	Base Position 3,422
Reference (S-AY157642.1)[1]	GCA (Ala_{74})	CCA (Pro_{106})	TTA (Leu_{216})	CCG (Pro_{381})
Reference (R-AY157643.1)[1]	GCG (Ala_{74})	TCA (Ser_{106})	TTG (Leu_{216})	CTG (Leu_{381})
S-JN004268.1[2]	GCA	CCA	TTA	CCG
R-JN004269.1[2]	GCG	TCA	TTG	CCG
S-AJ417034.1[3]	GCA	CCA	TTA	CCG
R-AJ417033.1[3]	GCG	TCA	TTG	CTG
S-AY395700.1[4a]	GCG	CCA	TTG	CCG
R-AY395699.1[4b]	GCG	GCA (Ala_{106})	TTG	CCG
S-HQ403647.1[5]	GCA	CCA	TTA	CCG
R-HQ403648.1[5]	GCG	CCA	TTG	CTG
S-KM387415.1[6]	GCG	CCA	TTG	CCG
R-KM387414.1[6]	GCA	CCA	TTA	CCG
R-KM387413.1[6]	GCG	CCA	TTG	CTG
R-KM387412.1[6]	GCA	TCA	TTA	CCG
Babakan (S-PV704970.1)	GCG	CCA	TTG	CTG
Sukasirna (LR-PV704971.1)	GCG	CCA	TTG	CTG
Prabugantungan (LR-PV704972.1)	GCA	TCA	TTA	CCG
East Rangkasbitung (LR-PV704973.1)	GCG	CCA	TTG	CTG

INTRON

Ecotypes	Base Position 1,781	Base Position 1,810	Base Position 1,834	Base Position 2,844
Reference (S-AY157642.1)[1]	T	T	A	A
Reference (R-AY157643.1)[1]	C	C	G	G
S-JN004268.1[2]	T	T	A	A
R-JN004269.1[2]	C	C	G	A
Babakan (S-PV704970.1)	C	C	G	G
Sukasirna (LR-PV704971.1)	C	C	G	G
Prabugantungan (LR-PV704972.1)	T	T	A	A
East Rangkasbitung (LR-PV704973.1)	C	C	G	G

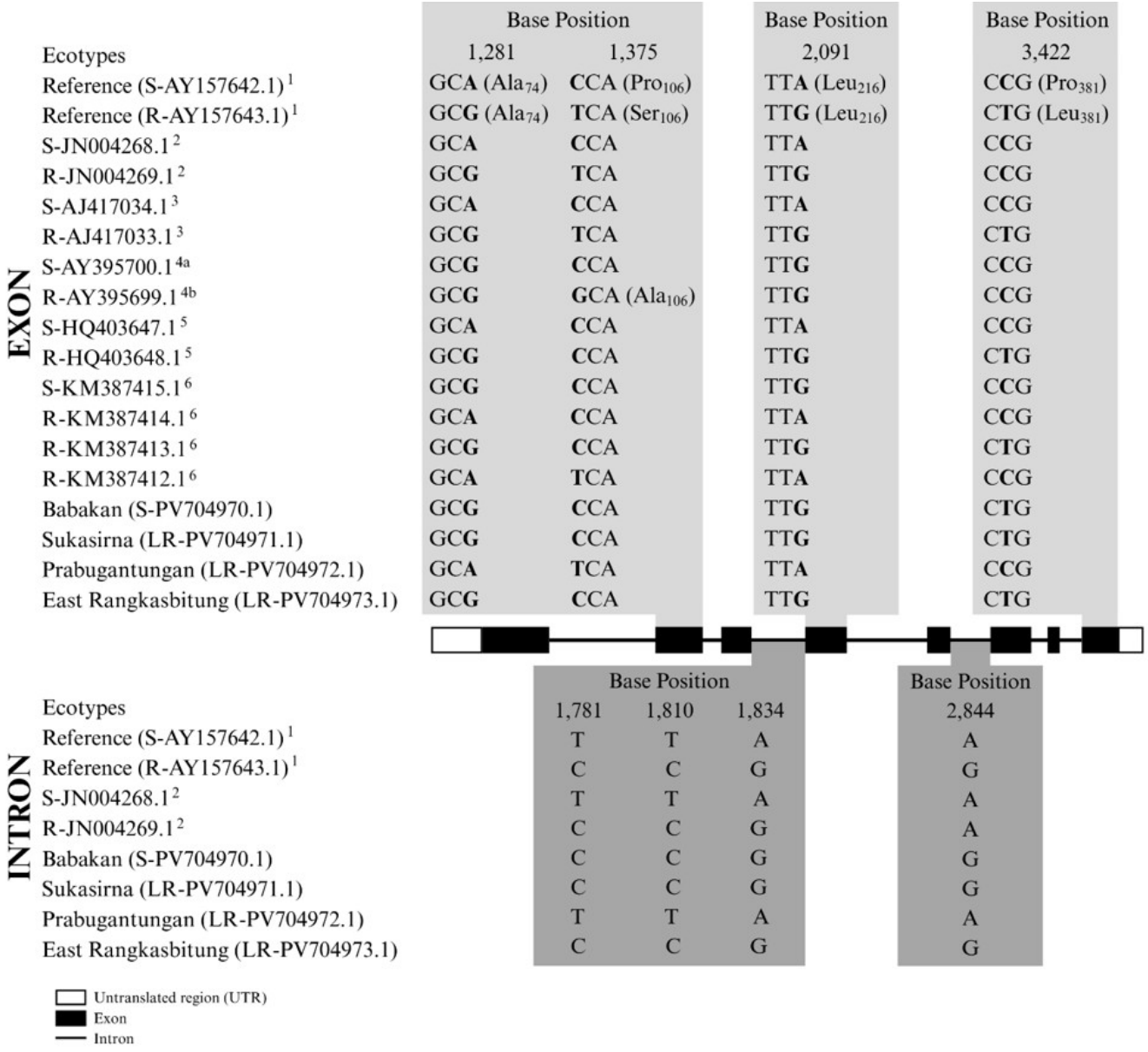

Figure 4. Nucleotide base variations of the *EiEPSPS* gene from four ecotypes of *E. indica*; [1]Ng *et al.* 2004; [2]Molin *et al.* (2013); [3]Baerson *et al.* (2002); [4a]Yuan and Chiang (2003a); [4b]Yuan and Chiang (2003b); [5]Zhang *et al.* (2015); [6]Chen *et al.* (2015); S: susceptible; R: resistance; LR: low resistance

on bioassay results. Notably, the resistant reference (R-AY157643.1) retained the CTG codon at position 3,422, identical to that observed in the Babakan ecotype (sensitive reference in this study), as well as in the Sukasirna and East Rangkasbitung ecotypes, both classified under the LR category. This base variation at position 3,422 led to amino acid variation Pro-381-Leu.

4. Discussion

A study of *E. indica* resistance to glyphosate warrants critical investigation, as this species is a dominant weed in tropical and subtropical regions and exhibits high adaptability to herbicide selection pressure (Li *et al.* 2022). In the bioassay experiment, 20 *E. indica* sample

plants within a single treatment combination were used to evaluate resistance levels. A large sample size (20-40 plants) is necessary to enhance the power to detect rare resistance alleles (Burgos 2015). This sampling design improves the accuracy and representativeness of detection results, enabling reliable identification of resistance levels across populations.

The GR_{50} value is a critical parameter in weed resistance assays, as it represents the herbicide dose required to reduce weed growth by 50% relative to the sensitive control ecotype (Escorial *et al.* 2019). It can also be used to estimate weed injury at untested doses, making GR_{50} essential for early resistance diagnosis, herbicide efficacy evaluation, and the development of sustainable weed management strategies (Wang *et al.* 2022; Mahajan and Chauhan 2024). Based on the GR_{50} values (Table 4), glyphosate application at up to 8× the recommended dose revealed that certain individuals within the resistant ecotype possess resistance mechanisms that reduce glyphosate efficacy.

E. indica samples were collected from multiple oil palm plantations based on consistently applied glyphosate over extended periods (Table 1), although often under various commercial formulations. As a result, intensive glyphosate application has led to the development of resistance in several ecotypes, including Mekarjaya (R/S 3.6), Cicareuh (R/S 2.6), Sukasirna (R/S 3.3), Prabugantungan (R/S 3.9), and East Rangkasbitung (R/S 4.7) ecotypes. Continuous glyphosate exposure in agricultural systems imposes strong selective pressure, thereby reducing herbicidal efficacy and adversely impacting crop productivity (Deng *et al.* 2022). Therefore, early detection of resistance is crucial for developing sustainable and effective weed management strategies.

Bioassay and molecular analysis are complementary methods for identifying herbicide resistance in weed populations (Deng *et al.* 2022), enabling GR_{50} values and R/S ratios to classify resistance levels (Kurniadie *et al.* 2023). However, the bioassay approach cannot elucidate the underlying mechanisms of resistance. Molecular analysis is necessary to identify amino acid changes in the *EiEPSPS* gene, which serve as indicators of target-site-based resistance. TSR analysis supports sustainable production by preserving weed-control efficacy, reducing reliance on single-herbicide actives, and minimizing environmental impact (Liu *et al.* 2020). The integration of both approaches supports the development of herbicide rotation and mixture strategies to mitigate the evolution of resistance.

A Pro-106-Ser amino acid substitution was identified in the Prabugantungan ecotype relative to the susceptible reference sequence S-AY157642.1 (Ng *et al.* 2004). The same substitution has also been reported in other resistant ecotypes, such as R-JN004269.1 and R-AJ417033.1 (Baerson *et al.* 2002; Molin *et al.* 2013). In contrast, the nucleotide at this position remained unchanged in resistant ecotypes R-HQ403648.1, R-KM387414.1, and R-KM387413.1 (Zhang *et al.* 2015; Chen *et al.* 2015), indicating a consistent sequence pattern among resistant populations. Additionally, the R-AY395699.1 sequence exhibits a different nucleotide substitution (CCA to GCA), resulting in a Pro-106-Ala change (Yuan and Chiang 2003b). Taken together, these comparisons across multiple studies suggest that the Pro-106-Ser substitution, although present in some susceptible ecotypes, is not consistently associated with glyphosate resistance and therefore may not serve as a definitive marker of resistance mechanisms.

Sequence alignment of the Babakan (susceptible), Sukasirna, and East Rangkasbitung ecotypes compared to the S-AY157642.1 reference sequence (Ng *et al.* 2004) revealed a Pro-381-Leu amino acid substitution. A similar substitution has also been previously reported in other ecotypes, including R-AJ417033.1 and R-HQ403648.1 (Baerson *et al.* 2002; Zhang *et al.* 2015; Chen *et al.* 2015). However, the nucleotide base at this position remained conserved across the resistant ecotypes, including R-JN004269.1, R-AY395699.1, R-KM387414.1, and R-KM387412.1 (Yuan and Chiang 2003b; Molin *et al.* 2013; Chen *et al.* 2015). This indicates that the site is conserved among resistant populations. The presence of the Pro-381-Leu substitution in the Babakan ecotype, which is phenotypically sensitive, suggests that the substitution remains unconfirmed as a contributor to glyphosate resistance in the Sukasirna and East Rangkasbitung ecotypes.

The observed homozygous nucleotide substitutions are consistent with the frequent homozygosity reported in glyphosate-resistant *E. indica* populations (Vila-Aiub *et al.* 2021). Specifically, nucleotide substitutions at positions 1,781, 1,810, 1,843, and 2,844 within the intronic regions of the *EiEPSPS* gene may be attributed to several underlying mechanisms. These include environmental adaptation, selective pressure, and alterations in copy number variation (CNV) of the *EPSPS* gene (Zhang *et al.* 2023). Intronic substitution can influence splice site recognition and splicing efficiency, thereby affecting gene expression and functional outcomes (Cheng *et al.* 2023). Moreover,

structural rearrangements and genetic diversity within introns are known to contribute to the development of glyphosate resistance, particularly through gene duplication events involving the *EPSPS* gene (Zhang *et al.* 2023).

The previously reported Pro-106-Ser and Pro-381-Leu amino acid substitutions in *EiEPSPS* genes might not be the primary determinants of glyphosate resistance (Franci *et al.* 2020; Vila-Aiub *et al.* 2021). This is likely due to the multifactorial nature of herbicide resistance in weeds, which may involve altered gene expression and NTSR mechanisms associated with herbicide metabolism and detoxification pathways (Deng *et al.* 2020; Araújo *et al.* 2023). NTSR mechanisms identified in *E. indica* include overexpression of a glutathione S-transferase gene (GSTU3) (He *et al.* 2023), enhanced activity of cytochrome P450 enzymes, specifically CYP94s and CYP71 (He *et al.* 2023), CYP88 (Deng *et al.* 2022), and CYP71AK44 (He *et al.* 2024), as well as increased activity of ATP-binding cassette transporters (ABCC4-mediated uptake) and aldo-keto reductases (AKR4C10-mediated detoxification) (Deng *et al.* 2022). Subsequent studies are needed to complete the sequences that have not yet been successfully sequenced, with the potential to discover new amino acid substitutions across numerous *E. indica* ecotypes.

Future research on glyphosate resistance in *E. indica* could be enhanced by the integration of genome editing and genome-wide association studies (GWAS). Genome editing is a form of genetic engineering that involves the deliberate insertion, deletion, or modification of DNA sequences (Asmamaw and Zawdie 2021), enabling precise single-nucleotide substitutions and facilitating the validation of specific base changes associated with herbicide resistance (Bhuyan *et al.* 2023). GWAS is a genetic tool used to examine numerous genetic variants across the genome to identify associations with specific traits (Uffelmann *et al.* 2021). Genome editing and GWAS provide a complementary framework for elucidating the genetic basis of herbicide resistance in *E. indica*, offering valuable insights to guide future research and management strategies.

These findings highlight the need for integrated management strategies, including the use of diverse herbicide active ingredients, rotation and combination of active compounds, and mechanical control in oil palm plantations in Bogor, Sukabumi, and Lebak. In addition, preventive measures such as regular monitoring and education for field personnel are essential to prevent the emergence of resistant weeds.

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