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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (3): 476-479, 2008
ISSN 1028-8880
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Nucleotide Variability in the 5-Enolpyruvylshikimate-3-Phosphate Synthase Gene from *Eleusine indica* (L.) Gaertn

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Abstract: This study reports the results of the partial DNA sequence analysis of the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) gene in glyphosate-resistant (R) and glyphosate-susceptible (S) biotypes of *Eleusine indica* (L.) Gaertn from Peninsular Malaysia. Sequencing results revealed point mutation at nucleotide position 875 in the R biotypes of Bidor, Chaah and Temerloh. In the Chaah R population, substitution of cytosine (C) to adenine (A) resulted in the change of threonine (Thr_{106}) to proline (Pro_{106}) and from C to thymidine (T) in the Bidor R population, leading to serine (Ser_{106}) from Pro_{106}. As for the Temerloh R, C was substituted by T resulting in the change of Pro_{106} to Ser_{106}. A new mutation previously undetected in the Temerloh R was revealed with C being substituted with A, resulting in the change of Pro_{106} to Thr_{106} indicating multiple founding events rather than to the spread of a single resistant allele. There was no point mutation recorded at nucleotide position 875 previously demonstrated to play a pivotal role in conferring glyphosate resistance to *E. indica* for the Lenggeng, Kuala Selangor, Melaka R populations. Thus, there may be another resistance mechanism yet undiscovered in the resistant Lenggeng, Kuala Selangor and Melaka populations.

Key words: Goosegrass, DNA sequence analysis, glyphosate-resistance, glyphosate-susceptible, Malaysia

INTRODUCTION

The management of herbicide resistant weeds is one of the most significant problems facing growers and researchers today (Dekker, 1997). The development of herbicide-resistant weed species is brought about through selection pressure imposed by repeated, often nearly continuous use of a herbicide in agriculture. In Malaysia, glyphosate-resistant *E. indica* populations have been reported in several areas after years of repeated usage of the herbicide glyphosate (Tran *et al.*, 1999).

Glyphosate, a widely used herbicide, acts by competitively inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), an enzyme in the shikimate pathway leading to the formation of the aromatic amino acids Tyr, Phe and Trp (Haslam, 1993; Franz *et al.*, 1997). According to Baerson *et al.* (2002), glyphosate resistance in *E. indica* was ascribed to a polymorphic, resistant 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) gene. The resistant biotype was attributed to a less sensitive (5-fold) EPSPS due to the substitution of proline (Pro) at position 106 for either serine (Ser) or threonine (Thr) (Baerson *et al.*, 2002; Ng *et al.*, 2003).

Thus, this study aims to gain more insight into the EPSPS gene polymorphism by sequencing 100 samples from the different R and S populations for a 202 bp section in the EPSPS region encompassing the mutation site of Pro_{106} to Ser_{106} previously demonstrated to play a pivotal role in conferring glyphosate resistance to *E. indica.* With the identification of the resistance gene and targeted site in glyphosate-resistant *E. indica*, it would further facilitate the monitoring of distribution and abundance of the R weeds in a particular geographical area and for planning and implementation of integrated control strategies.

MATERIALS AND METHODS

Seeds from mature *E. indica* of putative S and R plants in four areas in Peninsular Malaysia reported as glyphosate-resistant and glyphosate-susceptible namely Bidor, Chaah, Lenggeng and Temerloh were collected in February, March and April 2000. In addition, seeds from

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mature *E. indica* were also collected from putative R plants from Kuala Selangor (April 2002) and Melaka (December 2002) prior to conducting this study in June 2004.

Before germination, the seeds were scarified to break dormancy before placed according to blocks representing the S and R biotype from each area. To avoid contamination of outcrossing, the blocks were isolated by a boundary of about 1 meter from each other. Screening of the R and S biotypes were conducted by applying glyphosate at the recommended dosage of 1.08 kg ae ha^{-1} (Ng *et al.*, 2003) on the seedlings. For R biotypes, survived seedlings were allowed to reach maturity and inflorescence of each mature individual were collected and kept separately. After 1 week, the seedlings were transferred to polybags containing a peat-based compost (Right Grow commercial potting mix distributed by Kosas Profil Sdn. Bhd.) and grown in a greenhouse at 29± 4°C, with the light intensity of 800 μE m^{-2} sec^{-1} and a 12 h photoperiod. The plants were watered twice daily.

A modified CTAB method (Doyle and Doyle, 1990) was used to isolate total genomic DNA from fresh leaves of ten samples each of both S and R biotypes from Bidor, Chaah, Lenggeng and Temerloh and 10 samples each from the R populations from Kuala Selangor and Melaka. To amplify a region containing the Pro_{106} to Ser_{106} mutation site (Tran *et al.*, 1999), a pair of primers designed by Ng *et al.* (2003) (forward primer 5' GCGGTAGTTGTTGGCTGTGGTG, reverse primer 5' TCAATCCGACAACCAAGTCGC) based on the EPSPS sequence from the GenBank (accession number AJ417034) was used.

The PCR was conducted using 50 μL reactions containing 500 ng of genomic DNA, 1x PCR buffer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer and 1U of Taq polymerase (Promega ®). The cycling program consisted of one denaturing step at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 1 min of annealing at 58°C and extension for 2 min at 72°C, with a final extension step of 10 min at 72°C.

The amplified products were cleaned using QIAquick PCR purification Kit (QIAGEN) and sequenced using ABI3100 Genetic Analyzer (Applied Biosystems). Only the reverse strand was sequenced. All the sequences were aligned and compared using Clustal W (EBI 2005) with the EPSPS sequence obtained from the GenBank (accession number AY157643) namely a 3079 bp sequence from Bidor R.

RESULTS AND DISCUSSION

In this study, the edited sequences were compared to those obtained by Ng *et al.* (2003) for the *E. indica* 5-enolpyruvyshikimate-3-phosphate synthase (EPSPS-R) gene (Table 1). The nucleotide position of all the four S biotypes was conserved with proline as the amino acid at position 106. In the Chaah R, the samples was noted for the Pro_{106} to Thr_{106} change reported earlier by Ng *et al.* (2003) which was due to C being substituted by A at the nucleotide position 875. As for the Bidor resistant population, the samples showed the Pro_{106} to Ser_{106} change (Tran *et al.*, 1999; Ng *et al.*, 2003). Akin to Chaah R, a point mutation occurred at the same nucleotide position but C was substituted by T and thus causing a change of Pro_{106} to Ser_{106} in Bidor R and Temerloh R.

Comparisons of the sequences further revealed a new mutation undetected in other previous studies (Tran *et al.*, 1999; Ng *et al.*, 2003). In addition to the Pro_{106} to Ser_{106} mutation recorded earlier by Ng *et al.* (2003), three samples from Temerloh R population showed a new mutation. These samples exhibited the Pro_{106} to Thr_{106} change, which was originally reported in the Chaah R biotype by Ng *et al.* (2003). The existence of different amino acid changes in the EPSPS gene recorded in the Temerloh resistant population could be due to multiple founding events rather than to the spread of a single resistant allele. Another possibility of the resistant alleles is due to human intervention through agricultural practices via seedlots, compost and agricultural machines.

Table 1: Partial DNA sequences and inferred amino acid sequence of the EPSPS gene from the 100 samples of *E. indica*

Population	Biotype	No. of samples	DNA nucleotide position 875	Inferred amino acid at position 106	Consensus sequence
Bidor	R	10	T	Ser	TCA
	S	10	C	Pro	CCA
Chaah	R	10	A	Thr	ACA
	S	10	C	Pro	CCA
Lenggeng	R	10	C	Pro	CCA
	S	10	C	Pro	CCA
Temerloh +	R	3	A	Thr	ACA
		7	T	Ser	TCA
	S	10	C	Pro	CCA
Kuala selangor	R	10	C	Pro	CCA
Melaka	R	10	C	Pro	CCA

+: Two types of point mutations (C→A and C→T) detected in Temerloh R

At the same time, the amino acid changes recorded may imply the flexibility of the EPSPS enzyme in different populations as it evolves resistance to glyphosate exposure in the fields (Ng *et al.*, 2004).

As for the Kuala Selangor R, Melaka R and Lenggeng R populations, no mutation was detected in the EPSPS region sequenced encompassing the mutation site of Pro_{106} to Ser_{106} previously demonstrated to play a pivotal role in conferring glyphosate resistance to *E. indica*. The possible cause of resistance in these populations could be due to sequestration (Foley, 1987), cellular compartmentation (Hetherington *et al.*, 1998), differential translocation (Tucker *et al.*, 1994), enhanced metabolism (Komoßa *et al.*, 1992), increased transcription, or extended $t_{1/2}$ of the peptide encoded by EPSPS (Holländer-Czytko *et al.*, 1992; Zelaya *et al.*, 2004).

Furthermore, it has been recorded that a single species can develop different resistance mechanisms in different populations. For example, Gressel *et al.* (1983) reported that resistance to triazines in *Brachypodium distachyon* (L.) Beauv. biotypes from Israel was achieved by a combination of two mechanisms, namely enhanced metabolism and by alteration of the target site at the plastid level (Fraga and Tasende 2003). Besides, another glyphosate-resistant weed, *Lolium rigidum* was recorded as the first in the world to show a diversity of resistance mechanisms and pattern of resistance in the same populations (Preston *et al.*, 1996).

It has been documented that a much lower rate of the herbicide glyphosate is required for the evolution of the Lenggeng resistant population (Ng *et al.*, 2004). At the lower glyphosate dosage as exhibited by the Lenggeng resistant population, it is possible that another mechanism is conferring resistance. Thus, detailed studies on the glyphosate usage and mechanism of resistance are proposed for the better understanding of the development of herbicide resistance in *E. indica*. As the altered site of action was not the only mechanism in conferring resistance to glyphosate resistance in *E. indica*, studies on gene expression and biochemical aspects need to be conducted.

ACKNOWLEDGMENTS

This research was funded by the Monsanto Grant D/8/2000. In addition we thank the Malaysian Ministry of Science, Technology and Innovation (MOSTI) for the National Science Fellowship scholarship awarded to the first author.

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