EPSPs expression and enzymatic activity in glyphosate resistant D*igitaria insularis*

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ABSTRACT: Weed resistance to herbicides is a natural phenomenon which selects individuals inside a population. In Brazil, it was recently detected glyphosate resistance in sourgrass (*Digitaria insularis*) plants. There are several mechanisms of weed resistance, mostly regulated by genetic alterations in the herbicide action site. In this study, *EPSPS* expression and EPSPS enzymatic activity were measured in two sourgrass biotypes, one resistant and one susceptible to glyphosate. Enzymatic activity was slightly higher in resistant plants without glyphosate application, but almost same *EPSPS* gene expression in both biotypes, which suggests a feedback regulation.

Key words: Herbicide resistance, Enzyme activity, Over-expression.

INTRODUCTION

Herbicide usage is the most used for controlling weeds in agriculture fields. Glyphosate is a non-selective herbicide which inhibits *EPSPS* gene (5-enolpyruvylshikimate 3-phosphate synthase). In Brazil, the glyphosate resistance was recently described in sourgrass (*D. insularis*) plants (CARVALHO et al., 2012). Several mechanisms are known to provide resistance to herbicides. Among them, the genomic *EPSPS* copy number increase and *EPSPS* overexpression. The resistant plants can also present more than one mechanism. In sourgrass, *EPSPS* mutations were found in 182 and 310 positions, changing a proline for a threonine and a tyrosine for a cysteine, respectively (CARVALHO et al., 2012). According to Gaines et al. (2013), the presence of double mutations in *EPSPS* gene can be related to its overexpression. The aim of this study is analyze gene expression and enzymatic activity of resistant and susceptible plants contributing to elucidate mechanisms of resistance in this specie.

MATERIALS AND METHODS

Plant material

Seeds from glyphosate-resistant *D. insularis* plants (“R”) were collected from citrus culture located in the city of “Matão”. Also, seeds of *D. insularis* were harvested from natural

areas untreated with herbicides (“S”) from lettuce areas located in the city of “Mogi das Cruzes”, São Paulo, Brazil. Plants originated from these seeds were submitted to a whole-plant dose response curve. For genetic analysis, four plants of these two biotypes were used.

Total RNA extraction and cDNA synthesis

Frozen tissue samples of 1.0 g were weighed and ground to fine powder in liquid nitrogen using a sterilized mortar and pestle in four plants per bioype. Total RNA extraction was performed in frozen tissue samples of 1.0 g following TRIzol® protocol (Life Technologies, USA). 1 µl of each extraction was analyzed in a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA) and only RNA samples with 260/280 ratio close to 2.0 were used for subsequent analyses. 4 µg of total RNA from each sample was treated with DNAse I (Promega, USA). 0.5 µl of each treated sample was analyzed in agarose gels, all displaying clear bands corresponding to rRNA, absence of DNA and no degradation. cDNA samples were synthesized from 1.0 µg of the treated RNA using the SuperScript^{TM} III First-Strand Synthesis System for RT-PCR (Life Technologies, USA) according to the manufacturer´s instructions.

PCR, qRT-PCR primer design and sequence analysis

PCR primers for *ACTIN (ACTAct)* were manually designed flanking the conserved domains after doing Clustal alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2) of several orthologous plant sequences (obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) to amplify by PCR this gene from *D. insularis* leaf cDNA. *ACT* was used as internal control gene. Primers for *EPSPS* were taken from Perez-Jones et al. (2007) to perform PCR using cDNA samples. The primers for qRT-PCR were designed flanking the *EPSPS* and *ACT* sequences with OligoPerfectTM Designer (Life technologies, USA) with default parameters. Confirmation of primer specificity was based on the dissociation curve at the end of each run. To determine the amplification efficiencies of the *EPSPS* and *ACT* genes, it was used cDNA samples from leaf with five dilutions to obtain the standard curve, and then the PCR efficiency for each gene was calculated according to the equation $(1+E)=10^{slope}$. The correlation coefficient (R^2) and slope values were obtained from the standard curve.

***Digitaria insularis* quantitative real-time reverse transcription PCR**

The qRT-PCR mixture contained 100 ng of the six synthesized cDNAs from each tissue or organ, primers to a final concentration of 50 µM each, 12.5 µl of the SYBR Green PCR Master Mix (Applied Biosystems, USA) and PCR-grade water up to a total volume of 25

µl. Each gene reaction was performed in technical replicate. PCR reactions without template were also done as negative controls for each primer pair. The quantitative PCRs were performed employing the StepOnePlus™ System (Applied Biosystems, USA). All PCR reactions were performed under the following conditions: 2 min at 50˚C, 2 min at 95˚C, and 45 cycles of 15 s at 95˚C and 1 min at 65˚C in 96-well optical reaction plates (Applied Biosystems, USA). Actin was used as internal control gene and leaf samples were used as calibrator to normalize the values between different plates. Statistical analyses were performed using SAS ®Statistical Software.

EPSPS enzymatic activity in Digitaria insularis

Total protein was extracted following protocol suggested by Umesha (2006). Extracts were prepared from 1 g of leaf tissue pulverized using a mortar and pestle in liquid nitrogen. The powdered tissues were situated in 2 ml (1:2 w/v) of the buffer extraction (25 mM Tris-HCl pH 8.8, 32 mM ß-Mercaptoethanol). Each sample was homogenized for 1 min in vortex and centrifuged for 30 min at 10.000 X g at 4°C. Supernatant was used immediately.

Protein concentrations were determined following Bradford (1976) with BSA as the standard. The EPSPS activity was assessed by the release of inorganic phosphate when transferring the enolpyruvyl group of the phosphoenolpyruvate (PEP) to the shikimate-3-phosphate (S3P) using the malachite green dye assay proposed by Lanzetta et al. (1979). The reactions were measured in a final volume of a 0.1 mL mixture containing 200 mM Hepes-NaOH pH 7.0, 100 mM shikimate-3-phosphate (S3P), 10 mM phosphoenolpyryvate (PEP), 5 mM (NH4)6Mo7O24·4H2O and crude extracts.

After incubation for 20 min at 35°C, 1 mL of colorimetric solution (9.2 mM green malachite, 8.5 mM (NH4)6Mo7O24·4H2O in 1 M HCl, 2 g L^{-1} CHAPS) was added followed by an addition of 0.1 mL of a 34% sodium citrate solution for 1 min. After 15 min incubation period at room temperature, reactions were centrifuged for 1 min at 2000 X g in room temperature and the supernatants were filtered with PDVF syringe filter 0.45µm to remove solids. Finally, the absorbance was measured at 660 nm. Statistical analyses were performed using SAS ®Statistical Software.

RESULTS AND DISCUSSION

***Epsps* gene expression and enzymatic activity**

The resistance factor value between biotypes was 2.36. The comparison of *EPSPS* gene expression without glyphosate application (using *Actin* as control gene and sample “Resistant1” as reference) between the two biotypes (resistant and susceptible) had

statistical difference and indicated that resistant plants appeared 1.1-fold higher in susceptible together compared to resistant plants with 95% of confidence interval (Figure 1).

Overexpression of *EPSPS* gene without glyphosate exposure was found in resistant other plants (WANG et al., 2014).

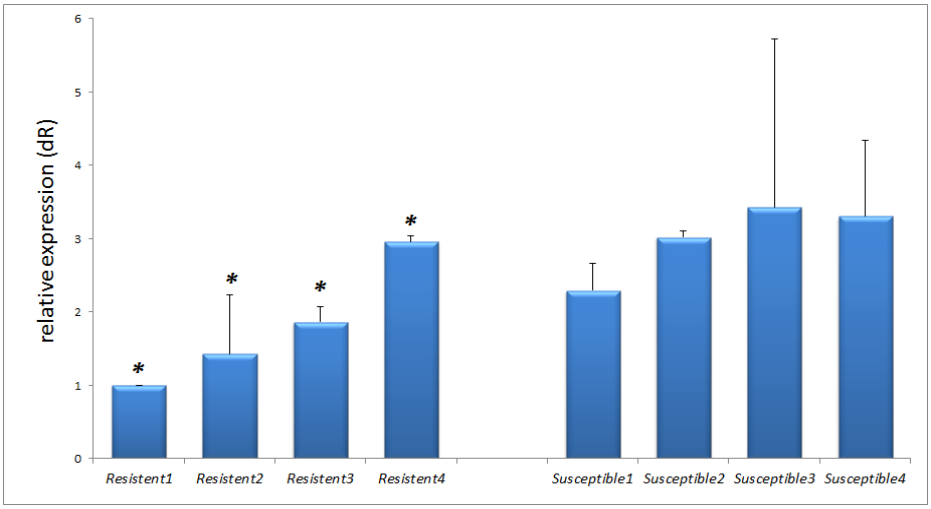

Figure 1. Expression levels of the *EPSPS* gene in resistant and susceptible plants (4 replicates) of *D. insularis*, using *Act* gene for normalization. Bars are mean standard deviation calculated from three technical replicates. Asterisk indicates statistical difference with 95% confidence.

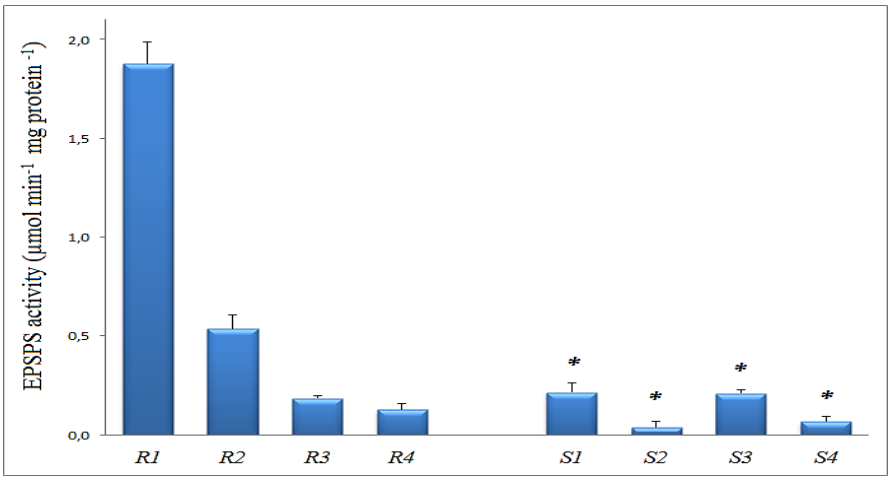

Figure 2. *D. insularis* EPSPS activity without glyphosate application of resistant and susceptible plants (4 replication). Bars are mean standard deviation calculated from three technical replicates. Asterisk indicates statistical difference with 95% confidence.

EPSPS enzyme activity levels between resistant and susceptible plants showed 5.1-fold more activity in resistant plants compared to susceptible with 95% of confidence interval (Figure 2). *Eleusine indica* glyphosate-resistants plants presented 2 to 4-fold greater *EPSPS* expression and 5-fold higher *EPSPS* activity than susceptible ones (BAERSON et al., 2002). Studies have shown that without glyphosate treatment resistant plants grow faster and have stronger competitive ability (SHRESTHA et al., 2010) as sourgrass plants.

CONCLUSIONS

The results suggest that *D. insularis* resistance is probably associated with a feedback regulation, with increased levels of EPSPS enzymatic activity but not gene expression. It was elucidated more about the mechanisms of glyphosate resistance for *D. insularis*.

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