Expression of genes related to the mechanism of resistance of *Conyza sumatrensis* to glyphosate

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ABSTRACT. The elucidation of the resistance mechanism of weeds to herbicides is important for management practices. The objective of this work was to investigate the resistance mechanism of glyphosate-resistant *C. sumatrensis* biotypes by determining the expression levels of the constitutive gene *epsps* and two ABC transport protein-coding genes designated *m7* and *m11* with RT-qPCR. Two biotypes of *C. sumatrensis* were evaluated: one resistant and one susceptible to glyphosate. The treatments consisted of the absence or application of two doses of glyphosate (1,080 and 8,640 g a.e. ha^{-1}). Plant leaves were collected at 1 and 4 days after herbicide application. No difference was observed in *epsps* gene expression between the studied biotypes. The expression of the *m7* and *m11* genes revealed that both genes had higher relative expression in the resistant biotype with the application of glyphosate at both doses. The overexpression of the *m*7 and *m11* genes in the resistant biotype treated with glyphosate reveals that these genes play a role in herbicide resistance. These genes may be involved in the sequestration of glyphosate into the vacuole lumen in the resistant *C. sumatrensis* biotype studied.

Keywords: Sumatran fleabane; overexpression; *EPSPs*; ABC transport protein genes.

Introduction

Over the last few years, agriculture has substantially contributed to the evolution of herbicide-resistant weed populations in different continents. Glyphosate is the most used herbicide for weed control for many reasons: high efficacy, broad spectrum of control, low cost and environmental safety. However, the extensive use of this herbicide exerts high selection pressure on various species of weeds, leading to the selection of resistant biotypes.

Conyza sumatrensis is a weed species belonging to the Asteraceae family and, along with *C. canadensis* and *C. bonariensis*, is considered the most important and problematic weed for various agricultural crops (Chachalis & Travlos, 2014). Globally, eleven cases of glyphosate-resistant *C. sumatrensis* have been identified (Heap, 2020).

In *C. canadensis* and *C. bonariensis*, the reported mechanisms of resistance to glyphosate are related to the overexpression of *EPSPs*, vacuole sequestration, differential translocation and metabolization (Feng et al., 2004; Ge, D'Avignon, Ackerman, & Sammons, 2010; Gonzáles-Torralva, Rojano-Delgado, Castro, Mulleder, & De Prado, 2012; Tani, Chachalis, & Travlos, 2015). In *C. sumatrensis*, there is no report of mechanisms of resistance to glyphosate. However, the mechanism of resistance is hypothesized to be similar to that of other *Conyza* species.

Sequestration in the vacuole and the overexpression of *EPSPs* could be probable resistance mechanisms of *C. sumatrensis* to glyphosate. The investigation of the involvement of sequestration in the vacuole as a mechanism of resistance can be performed indirectly through the expression of genes encoding proteins responsible for this sequestration (Tani et al., 2015). The main reason for this is that the genes that regulate ABC transporter proteins, which are able to rapidly transport glyphosate into vacuoles, are overexpressed in resistant biotypes (Ge et al., 2011).

Although there are explanations in the literature, the resistance mechanism of different *Conyza* species to glyphosate has not yet been fully elucidated, and there is a need for specific studies at the molecular level to fill this knowledge gap. The aim of this study was to investigate the mechanism of resistance of one

glyphosate-resistant *C. sumatrensis* biotype by determining the expression of the constitutive genes *epsps, m11,* and *m7.*

Material and methods

Plant materials

To perform this study, a population of *Conyza sumatrensis* resistant to glyphosate and another population susceptible to the same herbicide was used. The populations were collected in southern Brazil by Santos et al. (2014) (Table 1). The biotypes were sown in plastic trays, and after emergence, the seedlings were transplanted into 500 mL-plastic pots. The biotypes were kept in a greenhouse and irrigated daily until reaching the development stage designated to apply the herbicide.

Table 1. Identification of collection sites of the biotypes of *Conyza sumatrensis* susceptible and resistant to the glyphosate herbicide (Santos et al., 2014).

Biotype	City (State)	Geographic coordinates	Altitude	Glyphosate
Biotype 2	Pontão (Rio Grande do Sul State)	Lat: 28°00'20.40'' N Lon: 52°45'12.40'' E	660 m	Susceptible
Biotype 5	Carazinho (Rio Grande do Sul State)	Lat: 28°18'06.51'' N Lon: 52°53'41.31" E	564 m	Resistant

Application of herbicides and collection of plant material

When plants reached the 4-6 leaf stage, the plants were treated with the herbicide glyphosate at two doses, 1x (1,080 g a.e. ha^{-1}) and 8x (8,640 g a.e. ha^{-1}), or were untreated with the herbicide as the control treatment. As a commercial product, Zapp QI 620® was used. The experiment was completely randomized, with three replications for each treatment. To spray the herbicide, a costal spray equipped with fan-type spray tips 110.015 and pressurized with CO_2 was calibrated to provide an application volume equivalent to 150 L ha^{-1}. The intermediate leaves of each plant were collected from each treatment at one and four days after application of treatments (DAT). After the leaves were collected, they were maintained in aluminum foil, properly identified and immediately submerged in liquid nitrogen. Subsequently, the samples were maintained in an ultrafreezer at -80°C until RNA extraction. Three biological replicates were collected from each treatment.

RNA extraction and cDNA synthesis

RNA extraction from leaves was performed with the *NucleoSpin® RNA Plant* extraction kit (Machery-Nagel, Germany) following the manufacturer's instructions. Quantification and determination of sample purity was evaluated using a NanoDrop™2000 nanospectrophotometer (Thermo Scientific, USA), and the samples were measured at the absorbances A260 and A280.

To eliminate contamination with genomic DNA, the samples were treated with RQ1 RNase Free DNase (Promega, USA) according to the manufacturer's instructions. For the construction of cDNA, the *High-Capacity cDNA Reverse Transcription Kit®* (Applied Biosystems, USA) was used following the manufacturer's instructions.

RT-qPCR analysis

The primers used to amplify both reference and target genes are listed in Table 2. The primers of the genes *m11, m7,* and *actin* (reference gene) were selected based on previous work by Peng et al. (2010). Tani et al. (2015) described the primer of the *epsps* gene.

Table 2. Genes and primer sequences of four genes used for RT-qPCR analysis in real time.

Gene	Sequence *forward*	Sequence *reverse*
Actin	5'-gtggttcaactatgttccctg-3'	5'-cttagaagcatttcctgtgg-3'
M11	5'-atgctgtcttcttttacctttgc-3'	5'-cgacttcccactaccagttcttc-3'
M7	5'-tcatgcgtgttggataccgt-3'	5'-accaagaagtgaggcgatgc-3'
epsps	5'-ccaccaccgagaaaccatca-3'	5'-catcctcctcaacgtttagcc-3'

Note: *actin*: reference gene; *m11* and *m7*: ABC transporter proteins; *epsps*: EPSPs enzyme promoter gene.

RT-qPCR was performed using the *Fast SYBR® Green Master Mix* kit (Applied Biosystems). RT-qPCR analysis was performed with Step One Plus equipment® (Applied Biosystems). The concentrations of the primers and cDNA were 5 µM and 20 ng, respectively. RT-PCR was performed in three technical sample replicates.

The reactions were carried out in a final volume of 20 µL, consisting of 2 µL of cDNA sample, 10 µL of *SYBR® Green Master Mix*, 2 µL of the combination of forward and reverse primers, and 6 µL Milli-Q water.

The amplification steps included an initial cycle of 95°C for 20 seconds, followed by a sequence of 40 cycles of 95°C for 3 seconds, 60°C for 30 seconds, 95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds. To verify the specificity of the PCR products, the melting curve was evaluated, which ranged from 60 to 95°C.

Analysis of gene expression

The relative gene expression was calculated using the method $2^{-(\Delta\Delta Ct)}$ (Livak & Schmittgen, 2001), using the reference gene *actin* to normalize the data obtained by RT-qPCR. The means were compared by standard deviation, and the confidence interval for all means was calculated.

Results and Discussion

The results of RT-qPCR revealed that in all treatments (control and glyphosate-treated plants), all genes studied were present in both biotypes.

Analysis of the expression of the *epsps* gene

The relative expression of the *epsps* gene did not change according to the treatments evaluated (Figure 1). In both susceptible and resistant populations, the level of gene expression was similar. Likewise, the relative expression of the *epsps* gene was not affected by the application of glyphosate. These results suggest that the higher expression or number of copies of the *epsps* gene do not act as a resistance mechanism to glyphosate in this population of *C. sumatrensis*.
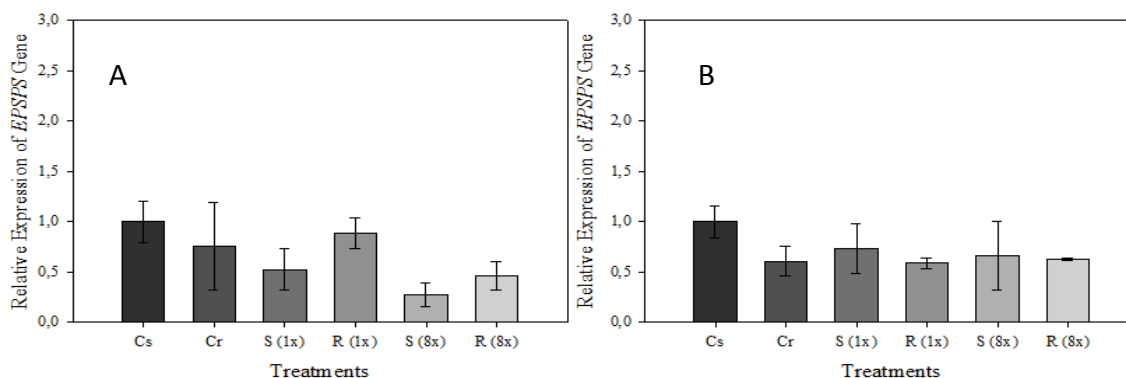

Figure 1. Relative expression of the *epsps* gene of the susceptible (S) and resistant (R) *Conyza sumatrensis* biotypes after glyphosate application. A: relative expression at one day after application of treatments (DAT); and B: relative expression at 4 DAT. C: control without herbicide application; 1x: application of 1x the herbicide dose; 8x: application of 8x the herbicide dose. Vertical bars indicate the confidence interval ($\alpha = 0.05$).

The overexpression of the *epsps* gene was listed as a resistance mechanism to glyphosate in *Amaranthus palmeri* and *Lolium rigidum* (Baerson, Rodrigues, Biest, Tran, & You, 2002; Gaines, Preston, Leach, Chisholm, & Shaner, 2010). The high level of expression of the *epsps* gene is due to the massive amplification of the number of copies of the gene, and commonly, there is an adaptive cost of the species due to its overexpression. For this reason, the competitive ability of the plant and/or survival and dispersion capacity are reduced (Powles & Yu, 2010). The results of the present work differ from those found for *C. canadensis* and *C. bonariensis*, where the overexpression of the *epsps* gene is related to the glyphosate resistance mechanism (Dinelli et al., 2008; Tani et al., 2015). Thus, other mechanisms not related to the herbicide action site are likely associated with the resistance of the *C. sumatrensis* biotype studied. All studies on weeds of the genus *Conyza*, in which the resistance mechanism to glyphosate is related to differential translocation, may act together with other resistance mechanisms (Powles & Yu, 2010).

Analysis of the expression of the genes *m7* and *m11*

Evaluation of the relative expression of the ABC transporter proteins revealed that both genes had higher relative expression in the resistant biotype with the application of glyphosate at both doses in relation to the susceptible biotype.

One day after application of treatments (1 DAT), the relative expression of the *m7* gene was 4.6 times higher in the resistant biotype compared to the susceptible biotype when both were treated with a 1x dose of glyphosate (Figure 2A). With the application of an 8x dose of glyphosate, the relative expression of the *m7* gene was 2.4 times higher than that of the susceptible biotype without herbicide treatment and 4.3 times higher than that of the susceptible biotype treated with an 8x dose of glyphosate.
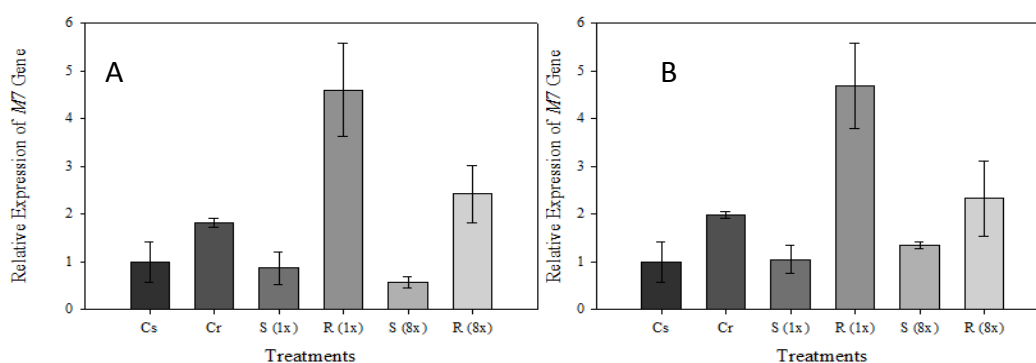

Figure 2. Relative expression of the *m7* gene of susceptible (S) and resistant (R) *Conyza sumatrensis* biotypes after glyphosate application. A: relative expression at one day after application of treatments (DAT); and B: relative expression at 4 DAT. C: control without herbicide application; 1x: application of 1x the herbicide dose; 8x: application of 8x the herbicide dose. Vertical bars indicate the confidence interval (α = 0.05).

When the expression of the *m7* gene was evaluated at 4 DAT (Figure 2B), the gene relative expression pattern remained similar to 1 DAT at both doses of the herbicide. In the resistant biotype, the application of a 1x dose of glyphosate increased the relative expression by 4.7 times in relation to the same treatment for the susceptible biotype. By analyzing the relative expression at 4 DAT with the application of an 8x dose of glyphosate, the resistant biotype showed a relative expression 2.3 times higher than that of the susceptible biotype without herbicide treatment. When compared to the expression of the susceptible biotype treated with an 8x dose of glyphosate, the expression of the resistant biotype treated with the same dose of the herbicide was 1.7 times higher.

In general, the application of glyphosate at both doses promoted the expression of the *m7* gene in the resistant biotype, both at 1 DAT and at 4 DAT. The greatest expression was obtained by applying the 1x dose of glyphosate, which caused the expression level to remain at the same level 4 days after application. With the application of the 8x dose, gene expression was lower; however, it was higher than the level observed in the treatment without herbicide for both biotypes and with the 8x treatment in the susceptible biotype. In both evaluation periods (1 and 4 DAT), in the resistant biotype, the *m7* gene had a relative expression higher than that of the susceptible biotype without herbicide treatment. We observed approximately two times the expression in the resistant biotype, and the same behavior was observed for biotypes of *Conyza canadensis* resistant to glyphosate (Peng et al., 2010; Tani et al., 2015).

In the *Conyza canadensis* biotype, the *m7* gene was found to have higher expression in glyphosate-resistant plants; however, at 1 DAT, the expression was higher with herbicide application. At 8 DAT, the highest level of expression was identified in the resistant biotype without herbicide (Tani et al., 2015). Peng et al. (2010) reported for *C. canadensis* that the expression of the *m7* gene was approximately 8 times higher in the resistant biotype than in the susceptible biotype when both biotypes were treated with glyphosate. *m7* gene expression was higher at 1 DAT than at 4 DAT. This behavior was also observed in similar work carried out with *C. canadensis* (Tani et al., 2015); however, the cause of this result remains unknown

In the evaluation of the gene *m11*, the relative expression was higher in the resistant biotype treated with both doses of herbicides than in the susceptible biotype (Figure 3). However, in the treatments where glyphosate was applied, both biotypes increased the expression of the gene. In contrast to the *m7* gene, the resistant biotype had low expression of the *m11* gene without herbicide treatment. In the evaluation of the relative expression of the *m11* gene at 1 DAT, it was possible to observe that in the resistant biotype treated with a 1x dose of glyphosate, the expression was 8.6 times higher than that in the susceptible biotype without herbicide treatment (Figure 3A). When comparing the 1x treatment between both biotypes, the expression was 3.8 times higher in the resistant biotype, indicating that even in the susceptible biotype, there was an increase in the expression of this gene. When the 8x dose was applied, the relative expression

in the resistant biotype was 7.42 times greater than that of the susceptible biotype without herbicide. The same behavior was observed between the biotypes in the same treatment.
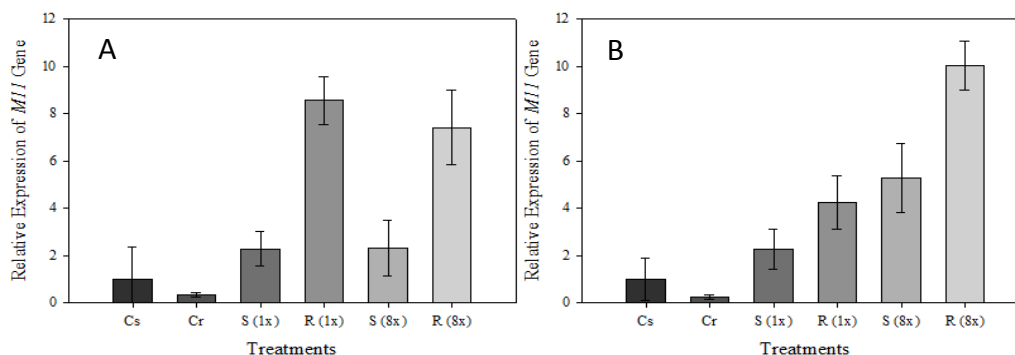

Figure 3. Relative expression of the *m11* gene of *Conyza sumatrensis* susceptible (S) and resistant (R) to glyphosate herbicide after glyphosate application. A: relative expression at 1 day after application of treatments (DAT); and B: relative expression at 4 DAT. C: control without herbicide application; 1x: application of 1x the herbicide dose; 8x: application of 8x the herbicide dose. Vertical bars indicate the confidence interval ($\alpha = 0.05$).

The relative expression of the *m11* gene at 4 DAT presented a different behavior (Figure 3B). In the resistant biotype treated with a 1x dose of glyphosate, the expression of the gene was reduced in relation to 1 DAT and was 4.2 times higher in the resistant biotype. When treated with an 8x dose, the expression increased and was 10 times higher in the resistant biotype than in the susceptible biotype without herbicide treatment. In the susceptible biotype, treatment with an 8x dose increased the expression at 4 DAT compared to treatment without herbicide. Thus, for resistant and susceptible biotypes, the application of glyphosate promoted an increase in the expression of the *m11* gene. Although this increase occurred in both biotypes, in the resistant biotype, the level of expression was higher than that in the susceptible biotype.

The overexpression of the *m11* gene was also related to glyphosate resistance in *Conyza canadensis*, and the application of an 8x dose of glyphosate increased gene expression (Tani et al., 2015). In another study, the *m11* gene was overexpressed in the susceptible and resistant biotypes due to glyphosate application; however, the highest expression was observed for the resistant biotype (Peng et al., 2010). Corroborating the results found in this work, the 8x dose of glyphosate applied to *C. canadensis* biotypes promoted a higher relative expression of the *m11* gene than the 1x dose (Tani et al., 2015).

The evaluation of expression levels of the *epsps*, *m7*, and *m11* genes was performed to relate the overexpression of the genes with a possible resistance mechanism of *C. sumatrensis* to glyphosate. This objective was proposed because of the occurrence of resistance of *C. canadensis* to glyphosate due to herbicide sequestration in the vacuole, which occurs because of the overexpression of the genes encoding the expression of ABC transporter proteins (Ge et al., 2010; Peng et al., 2010; Tani et al., 2015).

Naturally, plants have a limited ability to metabolize toxins; therefore, the development of strategies to detoxify compounds has been part of plant evolution mechanisms (Yuan, Tranel, & Stewart, 2007). ABC transporters are trans-membrane proteins that use energy from ATP hydrolysis to transport numerous substrates through intracellular and extracellular membranes, including lipids, xenobiotics, hormones, metals and secondary metabolism products (Rea, 2007; Verrier et al., 2008). Furthermore, ABC proteins have the ability to carry herbicides and herbicide metabolites (Klein, Martinoia, Hoffmann-Thoma, & Weissenbock, 2000).

The resistance mechanism of *Conyza* spp. to glyphosate has been related to several factors. The overexpression of the gene encoding the *EPSPs* enzyme is one of the mechanisms of resistance related to the site of action of the herbicide (Tani et al., 2015). However, in this work, no differences were identified in *epsps* expression levels in resistant and susceptible biotypes, indicating that this mechanism is not responsible for the resistance of the evaluated population of *C. sumatrensis* to the herbicide glyphosate.

Glyphosate sequestration in the vacuole, differential translocation and metabolization of the herbicide are non-target resistance mechanisms to glyphosate, which have previously been reported in the literature (Feng et al., 2004; Ge et al., 2010; Gonzáles-Torralva et al., 2012). The differential translocation of glyphosate may be directly related to herbicide sequestration in the vacuole because the applied herbicide remains on the leaves and is not distributed to other parts of the plant (Powles & Yu, 2010).

The large family of ABC transport protein genes (*ATP-binding cassette*) is common in the plant genome and requires ATP to function, such as transporting a variety of small molecules across membranes (Rea, 2007; Verrier et al., 2008). Some of the ABC transport proteins perform the sequestration of herbicides from the vacuole, and the investigation of the expression of genes encoding these proteins may be a way to

investigate the resistance mechanism. The identification of the genes related to this process enable the scientific community to identify the mechanism of resistance of species of the genus *Conyza* to the herbicide glyphosate (Peng et al., 2010). These genes may be expressed at higher levels in resistant biotypes or may be overexpressed as a function of herbicide application (Shaner, 2014). This corroborates the results obtained in this work, where the level of expression of the gene *m7* was superior in the resistant biotype even without the application of the herbicide. For the gene *m11*, gene overexpression occurs due to the application of glyphosate, and even in the susceptible biotype, treatment with glyphosate promotes gene expression.

In this work, the *epsps*, *m7*, and *m11* genes were selected for the evaluation because the gene *epsps* encodes the enzyme *EPSPs*, which is the site of action of glyphosate, and because the overexpression of *EPSPs* is a mechanism of resistance of plants in the genus *Conyza* to glyphosate. However, the choice for the genes *m7* and *m11* was based on the close relationship of the overexpression of these genes with resistance to glyphosate in *C. canadensis* (Peng et al., 2010; Tani et al., 2015). The first hypothesis, according to which there would be an overexpression of the *epsps* gene, was not confirmed. However, the hypotheses of the involvement of the ABC transporter protein genes *m7* and *m11* were confirmed. These results are innovative for studies that characterize the mechanisms of resistance to glyphosate in *Conyza sumatrensis*, as works have previously been carried out to identify the differential translocation of glyphosate and the possible relation with the sequestration of the herbicide in the vacuole.

Notably, the number of ABC transporter proteins is large in different species. For this reason, we need more evidence for ABC transporter proteins acting in vacuole sequestration processes and even for the differential translocation of glyphosate in populations of *Conyza* spp. resistant to the herbicide glyphosate. A hypothetical model was created to illustrate the susceptible and resistant cells of *C. sumatrensis*, correlating the overexpression of the *m7* and *m11* genes and a greater number of ABC transporter proteins on the membrane and consequently the glyphosate entry into the vacuole (Figure 4).
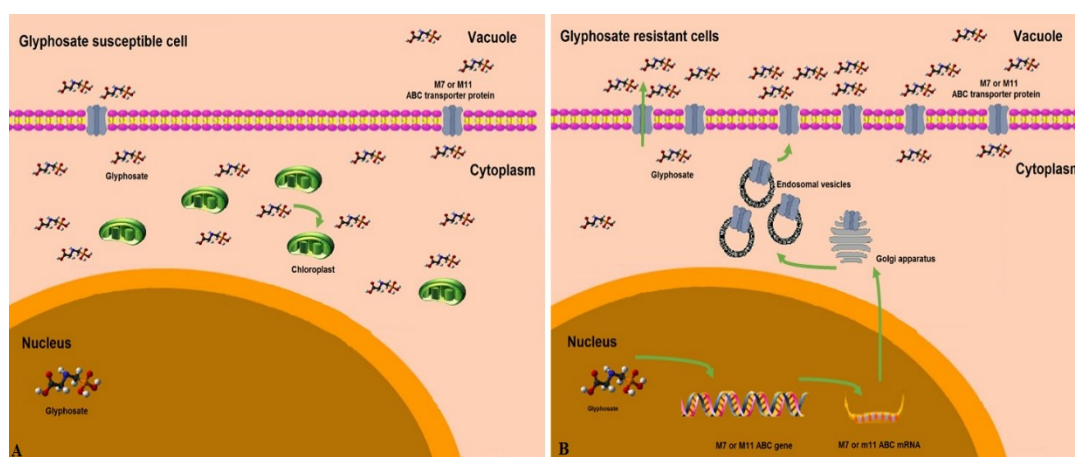

Figure 4. Mechanism of glyphosate interactions with M7 and M11 ABC transporters. In glyphosate-susceptible cells (A), the active glyphosate inhibits 5-enol-pyruvylshikimate-3-phosphate synthase (*EPSPs*) in the chloroplast, resulting in a lack of amino acids from the aromatic amino acid biosynthetic pathway. In glyphosate-resistant cells (B), the resistance mechanism includes regulation of *m7* and *m11* ABC mRNA expression through influences on translation and regulation of protein trafficking, leading to an increase in ABC transporter protein on the membrane and consequently glyphosate entry into the vacuole cell.

However, the results obtained in this work confirm that there is a relationship between the overexpression of the ABC transporter genes *m7* and *m11* and the *C. sumatrensis* biotype studied. This is the first report suggesting a mechanism of resistance in this species. Future studies need to be conducted to investigate the possible involvement of other genes with this relationship and to evaluate the dynamics of glyphosate sequestration in the vacuole in this same population using the ^{31}P-Nuclear Magnetic Resonance method (^{31}P-NMR) (Ge et al., 2014).

Due to the absence of differences in the relative expression of the *epsps* gene in resistant and susceptible populations, the increase in the expression of the *epsps* gene is not the cause of the resistance of *Conyza sumatrensis* biotype evaluated in this study.

The genes *m7* and *m11* show a high relative expression in the resistant biotype treated with glyphosate. Thus, there is a relationship between the overexpression of the genes *m7* and *m11* and the resistance of the analyzed biotype of *Conyza sumatrensis* to the herbicide glyphosate.

Conclusion

No difference was observed in *epsps* gene expression between the studied biotypes. The expression of the *m7* and *m11* genes revealed that both genes had higher relative expression in the resistant biotype with the application of glyphosate at both doses. The overexpression of the *m*7 and *m11* genes in the resistant biotype treated with glyphosate reveals that these genes play a role in herbicide resistance. These genes may be involved in the sequestration of glyphosate into the vacuole lumen in the resistant *C. sumatrensis* biotype studied.

Acknowledgements

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