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Article

Sourgrass Resistance Mechanism to the Herbicide Glyphosate

Mecanismo de Resistência do Capim-Amargoso ao Herbicida Glyphosate

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ABSTRACT - The knowledge on the mechanism that gives a weed resistance to a particular herbicide is essential regarding scientific, academic, and practical aspects because it determines the recommendations for prevention and management of resistance in the field. Studies on the sourgrass (*Digitaria insularis*) glyphosate resistance mechanism in the literature have not been conclusive. Thus, the aim of this research was to study and evaluate the putative resistance mechanisms whichgives sourgrass biotypes, the ability to survive after glyphosate application. For this, ^{14}C-glyphosate leaf absorption and translocation were compared in the biotypes Matão (R), Campo Florido (MG), Diamantino (MT), and Iracemápolis (S) as a function of the time after its application. In addition, the possibility that the mechanism of resistance results from a mutation in the EPSPs-encoding gene was also studied. The biotypes S, R, MG, and MT absorbed similar amounts of ^{14}C-glyphosate. The biotypes R, MG, and MT did not present differences in ^{14}C-glyphosate translocation when compared to the biotype S. The sequencing of the EPSPs-encoding gene showed no mutation in the regions 106 and 182, which normally give resistance to glyphosate in the case of other species. No mutation in the EPSPs-encoding gene was observed. Therefore, they are not glyphosate resistance mechanisms for the evaluated biotypes.

Keywords: absorption, translocation, mutation, ^{14}C-glyphosate, *Digitaria insularis*.

*RESUMO - O conhecimento do mecanismo que confere a uma planta daninha resistência a um determinado herbicida é de fundamental importância tanto sob o aspecto científico e acadêmico, como prático, pois determina as recomendações de prevenção e manejo da resistência no campo. Os estudos até o momento sobre os mecanismos de resistência do capim-amargoso (**Digitaria insularis**) ao glyphosate existentes na literatura não são conclusivos. Assim, o intuito desta pesquisa foi estudar e avaliar os possíveis mecanismos que conferem resistência aos biótipos de capim-amargoso para sobreviver ao controle pelo glyphosate. Para isso, foi comparada a absorção foliar e translocação do ^{14}C-glyphosate nos biótipos de Matão (R), Campo Florido (MG), Diamantino (MT) e Iracemápolis (S), em função do tempo após sua aplicação. Além disso, também foi estudada a possibilidade de o mecanismo de resistência ser resultante de mutação no gene que codifica a EPSPs. Os biótipos S, R, MG e MT absorveram quantidades similares de ^{14}C-glyphosate. Os biótipos R, MG e MT não apresentaram diferenças de translocação de ^{14}C-glyphosate quando comparados com o biótipo S. O sequenciamento do gene que codifica a EPSPs mostrou não haver mutação nas regiões 106 e 182, as quais normalmente conferem resistência em outras espécies ao glyphosate. Nenhuma mutação no gene que codifica a EPSPs foi observada. Conclui-se que esses não são os mecanismos de resistência ao glyphosate para os biótipos avaliados.*

Palavras-chave: absorção, translocação, mutação, 14C-glyphosate, Digitaria insularis.

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INTRODUCTION

Chemical control of weeds through herbicide application is a frequent and constant practice in agricultural areas due to its high effectiveness. However, farmers do not always use this tool properly, the use of herbicides associated with other control methods and the use of molecules with different modes of action, which are concepts of integrated weed management (Lopez-Ovejero et al., 2006; Powles, 2008). Glyphosate is the most used herbicide in agricultural crops and has a diversified use so that it has been used indiscriminately for more than thirty years (Galli and Montezuma, 2005). Its intensive use caused weed communities of agroecosystems to create response mechanisms in the specific flora or selection of certain resistant biotypes in weed populations (Lopez-Ovejero et al., 2006).

Glyphosate has been widely adopted in agriculture due to several factors, such as its broad spectrum of control, ease of application, and efficient control in the late weed stages. It has a high translocation capacity in plants as it affects meristems, roots, and organs of vegetative propagation, inhibiting the action of EPSPs, an essential enzyme in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, tryptophan, and several other secondary compounds (Amrhein et al., 1980).

The mechanisms that confer weed resistance to glyphosate can be divided into two classes: (a) related to the site of action (target site resistance) and (b) not related to the site of action (non-target site resistance). In the first case, the herbicide reaches the site of action but cannot inhibit the enzyme of the resistant biotype, as with glyphosate-resistant biotypes that have a mutation at position 106 of the EPSPs, where the amino acid proline may be replaced by serine, threonine, alanine or leucine, changing the way glyphosate couples to EPSPs (Perez-Jones et al., 2007; Yu et al., 2007; Wakelin and Preston, 2006; Kaundun et al., 2008; Preston et al., 2009; Funke et al., 2009). In some cases, the herbicide is able to perform its function, inhibit the action of EPSPs, but resistant biotypes have the ability to produce more EPSPs than glyphosate is able to inhibit it, which is known as gene overexpression (Gaines et al., 2010).

Regarding the resistance not related to the site of action, there are several mechanisms that give the resistant biotype the ability to survive after glyphosate application: low absorption, low translocation, vacuolar compartmentalization, and metabolization (Délye et al., 2002; Yuan et al., 2006).

Carvalho et al. (2012) studied the resistance mechanisms of sourgrass to glyphosate and concluded that a pool of mechanisms, including absorption, translocation, metabolization, and genetic mutation, conferred resistance to the herbicide simultaneously, not specifically pointing to an isolated mechanism as the main one. However, the study presented here was carried out with different populations from those investigated by these authors and with the hypothesis that there is a predominance of a mechanism that confers resistance since even populations at early growth stages have a high resistance, which usually does not happen when translocation is the resistance mechanism. There is also evidence that shikimate tests did not demonstrate insensitivity of the enzyme to glyphosate, which would impair the hypothesis of change in the site of action.

Therefore, this study was developed with the objective of studying three possible glyphosate resistance mechanisms of sourgrass to glyphosate: absorption, differential translocation, and alteration of the site of action of this herbicide, using sourgrass biotypes demonstrably resistant to glyphosate.

MATERIAL AND METHODS

The experiments were conducted in parallel in Brazil and the United States. In order for the research to be carried out, the seeds of the biotypes were exported from Brazil to the United States through the US Department of Agriculture (USDA) and its agency specialized in the supervision of plants and animals, which regulates the entry and exit of material (APHIS – Animal and Plant Health Inspection Service).

Absorption and translocation of glyphosate in resistant and susceptible biotypes of *Digitaria insularis*

The experiment was developed from August to November 2014. The biotypes of sourgrass were sown in trays with a commercial substrate, being immediately placed in a greenhouse with an average temperature of 28 °C. When the seedlings were established, they were transplanted into individual pots and maintained in a growth chamber until they reached the stage 23 on the BBCH scale (Hess et al., 1997), at which time the treatment with the labeled glyphosate (^{14}C-glyphosate) was applied. The resistant biotypes, which survived the application of the glyphosate dose of 1,440 g a.i. ha^{-1}, originated from Diamantino, MT (MT), Campo Florido, MG (MG), and Matão, PR (R), whose geographical coordinates are 14°25'53.57" S and 56°13'41.31" W, 19°45'48.80" S and 48°34'19.47" W, and 21°36'44.4" S and 48°27'24.4" W, respectively. The susceptible biotype was originated from an area with no glyphosate application history in the municipality of Iracemápolis, SP (S), whose geographical coordinates are 22°39'17.1" S and 47°29'32.39"W. All the biotypes had their characteristic of resistance or susceptibility to glyphosate confirmed by Melo (2015).

First, the glyphosate treatment at a dose of 960 g a.e. ha^{-1} was applied using a CO_2-pressurized backpack sprayer calibrated to a spray solution volume proportional to 200 L ha^{-1}. The plants were then taken to the laboratory and received an application of radiolabeled glyphosate so that each plant received 40,000 disintegrations per minute (DPM). Treatments were performed on the youngest fully expanded leaf, in five replications of each biotype, in which one replication was destined to autoradiographs and the others were analyzed statistically. The application was carried out with a precision pipette (Gilson Pipetman®), used to apply ten drops of 0.5 µL each. The plants remained in the laboratory for approximately one hour to allow the drying and stabilization of the applied treatments to be transported to the greenhouse, where they remained until the time of sample collection.

After intervals of 6, 12, 24, 48, and 96 hours after treatment application (HAT), each plant had its applied leaf sampled with scissors, close to the ligule, and immediately washed inside flasks with 10 mL of a solution containing 10% methanol, 89.75% deionized water, and 0.25% nonionic surfactant (NIS) so that the active ingredient remaining on the leaf surface was removed and calculated thereafter. Another 10 mL of a liquid scintillation cocktail (LSC) was added to the 10 mL in which the leaves were washed in order to read the beta radiation emitted by the radiolabeled atoms in the glyphosate molecule, called Ultima Gold™, which does not need correction for the Quench fluorescence (Gibson, 1980). Blank samples, without the addition of any product other than the cocktail, were also made with the addition of ^{14}C-glyphosate directly in the cocktail to allow the amount of glyphosate applied in each plant to be measured with higher precision. Plant parts were separated into treated leaves, central stem, tillers, and root, being taken to dry in a forced air circulation oven at 60 °C for 72 hours. After drying, the material was incinerated in an oxidizer, causing the ^{14}C in the samples to be transformed into $^{14}CO_2$ and trapped in a vial containing 5 mL LSC. Plants not used for the oxidation and quantification of ^{14}C-glyphosate in their parts were submitted to autoradiography.

An individual from each biotype per collection was pressed for one week, as adopted for voucher specimen, and then transferred to a cassette (GE Health Care, USA), where they remained for 48 hours so that the beta particles emitted by the radiolabeled glyphosate were captured. After this period, the cassettes were read in a Phosphorus Imaging (Molecular Dynamics, Storm 860, USA). Lastly, the flasks were placed in a liquid scintillation analyzer and the results were submitted to the model proposed by Kniss et al. (2011), using the following equation to obtain the amount of absorbed glyphosate:

$$Absorption = Amax\ x < 1 - \exp\{\log[(100 - \theta)100]x(t/t\theta)]\}$$

where A_{max} represents the maximum absorption or percentage amount of labeled glyphosate, θ is a determined percentage of A_{max}, t is the time, and $t\theta$ is the time required to reach the determined percentage.

The data were analyzed for the homogeneity of variance and residuals. Subsequently, the Tukey's test for mean comparison was performed in a factorial scheme with two factors, in

which the biotype factor had four levels (biotypes S, R, MG, and MT) and time factor had six levels (0, 6, 12, 24, 48, and 96 hours after treatment), with p>0.05. The regression curves for the parameters absorption and translocation, comparing the biotypes as a function of time, were performed with software R.

Mutation in the EPSPs-encoding gene in resistant and susceptible biotypes of *Digitaria insularis*

The biotypes used in this experiment were those of Iracemápolis, SP (S) and Matão, SP (R). Samples of S and R biotypes were collected from plants grown in a greenhouse, being chosen the youngest plant tissue, with a total of four replications per biotype. The plant tissue was stored in an Eppendorf® tube with a volume of 1.5 mL, which was immediately dipped in liquid nitrogen (around -210 °C) until the end of the collection and then stored in a freezer at -80 °C for later extraction of the genetic material. The plant tissue was immersed immediately after collection in order to prevent the molecular components from degrading (Jones et al., 2012).

The frozen samples were macerated with a mortar and liquid nitrogen, always keeping the samples cooled to avoid the action of nucleases and destruction of the genetic material. At the end of maceration, the RNeasy® Plant Mini Kit was used to extract the messenger RNA (mRNA) from the samples, according to the manufacturer's instructions. After extraction, a spectrophotometer (NanoDrop® 2000c) was used to quantify the concentration of nucleic acid and its purity.

After RNA extraction with the abovementioned kit, the DNase I, Amplification Grade® was used for the digestion of DNA with one and two strands into smaller forms, eliminating the DNA present in the samples, which caused them to have only mRNA, according to the manufacturer's specifications.

After obtaining from samples only single-stranded RNA, the kit qScript™ cDNA SuperMix, was used to obtain complementary DNA (cDNA).

With the synthesized cDNA, specific primers were made to amplify only the nucleic acid sequence related to the EPSP-encoding gene. For this, bibliographic references (Perez-Jones et al., 2007; Kaundun et al., 2008; Carvalho et al., 2012; Gaines et al., 2010) and online libraries (NCBI – National Center for Biotechnology Information) were used to obtain the sequence of EPSPs- encoding nucleotides and amino acids in species of evolutionary proximity to sourgrass, such as *Lolium multiflorum* and *Digitaria sanguinalis*. By means of several trials and errors, the primer that best amplified the desired sequence was as follows: 5'-AGCTGTAGTCGTTGGCTGTG-3', representing the forward primer, and 5'-GCCAACAAATAGCTCGCACT-'3, representing the reverse primer. The traditional PCR (MyCycler™, Bio-Rad, USA) was then performed, being used the following reagents: 25 uL EconoTaq® PLUS Green Master Mix, 2 µL forward and reverse primers (20 mM), 1 µL cDNA (50 ng μL^{-1}), and pure water to complete 50 µL per reaction. The cycle that obtained the best performance of primers was as follows: 5 minutes at 95 °C, 1 minute at 95 °C, 30 seconds at 60 °C, 1 minute at 72 °C, and 3 minutes at 72 °C.

The products were submitted to electrophoresis with TAE buffer solution (Tris-Acetate-EDTA, pH 8.0) and 1.5% agarose gel for the separation of cDNA according to its size in relation to DNA fragments of known size (GeneRuler™ 1kb DNA Ladder). The bands obtained by agarose gel electrophoresis were trimmed and purified with the QIAquick® Gel Extraction kit, following the manufacturer's guidelines, and then sent for sequencing in the Colorado State University (PMF – Proteomics and Metabolomics Facility). Sequencing results were analyzed with the software CHROMAS® by comparing susceptible and resistant biotypes to possible mutations in this gene. The online software ClustalW2 (Larkin et al., 2007) was used to align the obtained sequences with those found in the literature.

From searches in the literature regarding the nucleotide and amino acid sequence that makes up the EPSPs, the size of bands that the designed primers were supposed to have when performing the product electrophoresis of the traditional PCR was estimated. In addition to the electrophoresis technique, the samples were also submitted to the equipment Fragment Analyzer™ – Advanced Analytical, which shows the exact size of DNA fragments amplified by PCR through

the same principle of electrophoresis. Reference primers related to CCR, ALS, and HDI enzyme-encoding genes were used at each step, as well as primers and cDNA of *Amaranthus palmeri* used by Gaines et al. (2010).

The bands obtained by agarose gel electrophoresis were trimmed and purified through the QIAquick® Gel Extraction kit following the manufacturer's instructions and sent for sequencing in the Colorado State University (PMF – Proteomics and Metabolomics Facility). The results of sequencing were analyzed with the software CHROMAS® by comparing susceptible and resistant biotypes, generating the conclusions on the mutations in this gene. The online software ClustalW2 (Larkin et al., 2007) was used to align the obtained sequences with those found in the literature.

RESULTS AND DISCUSSION

Absorption and translocation of glyphosate in resistant and susceptible biotypes of *Digitaria insularis*

The average percentages for the absorbed and translocated amounts of ^{14}C-glyphosate in the different evaluation periods are shown in Table 1. The results show an increase in the absorption of ^{14}C-glyphosate in the evaluations carried out at 48 and 96 hours after treatment application (HAT), which also happens for the translocation to roots and stems.

Table 1 - ^{14}C-glyphosate absorbed and translocated in different evaluation periods, related to the biotypes S, R, MG, and MT of *Digitaria insularis*. Piracicaba, 2015

Biotype	Hours after treatment (HAT)	Radioactivity (%)[1]		
		Absorption	Leaf retention	Translocation*
S	6	42.7	88.5	11.5
	12	37.8	92.9	7.1
	24	32.6	81.5	18.5
	48	63.4	78.1	21.9
	96	80.6	63.0	37.0
R	6	58.0	93.4	6.6
	12	36.2	90.5	9.5
	24	37.4	86.9	13.1
	48	72.7	78.8	21.2
	96	89.5	52.6	47.4
MG	6	33.9	93.7	6.3
	12	33.2	85.9	14.1
	24	27.7	77.9	22.1
	48	79.7	72.0	28.0
	96	89.1	40.6	59.4
MT	6	44.7	88.2	11.8
	12	21.8	91.0	9.0
	24	28.0	88.5	11.5
	48	60.4	77.4	22.6
	96	87.1	57.0	43.0

[1] Average percentage of data obtained from radioactivity; * Translocation equals the sum of the percentage amounts of ^{14}C-glyphosate translocated for roots and stems.

The biotype factor for the maximum amount of glyphosate absorption and the time required to absorb 90% of the maximum value (t90) showed no statistical differences between the biotypes R, MG, and MT when compared to the biotype S by using the model proposed by Kniss et al. (2011) (Table 2).

Table 2 - Parameters estimated by the software R using the model proposed by Kniss et al. (2011), related to the biotypes S, R, MG, and MT of *Digitaria insularis*, which describe, at p<0.05 level, the maximum leaf absorption (Amax), the time required to obtain 90% of the maximum leaf absorption (t90), the maximum translocation (Tmax), and the time required to obtain 90% of the maximum total translocation (t90*) of ^{14}C-glyphosate. Piracicaba, 2015

Biotype	Estimated value	Standard error	P-value of significance
Amax: S	74.6	11.2	<0.01
Amax: R	82.1	11.1	<0.01
Amax: MG	96.7	14.6	<0.01
Amax: MT	96.2	21.5	<0.01
t90: S	48.7	22.2	0.0309
t90: R	44.5	19.3	0.0238
t90: MG	82.3	27.8	0.0040
t90: MT	104.8	47.9	0.0314
Tmax: S	54.2	10.8	<0.001
Tmax: R	99.9	38.9	0.012
Tmax: MG	78.7	35.4	0.029
Tmax: MT	100	24.4	<0.001
t90*: S	146.7	51.3	0.005
t90*: R	368.8	182.2	0.046
t90*: MG	294.2	179.4	0.105
t90*: MT	270.1	91.6	0.004

* Time required to obtain 90% of the maximum translocation of ^{14}C-glyphosate.

Experiments that evaluate the movement of a particular herbicide in the plant use destructive techniques for each evaluation at different times after application, which requires independent experimental units for each evaluation. Kniss et al. (2011) observed that each collection in a given period (hours after treatment) has a punctual value, which is important for the characterization of the experiment as a whole. According to these authors, it is common to find in the literature studies that ignore their structure as a whole since they use statistical comparisons within each treatment, such as multiple comparison tests. Cousens (1985) pointed out that the experiment on herbicide absorption should be analyzed as a whole since its objective is to evaluate, in a destructive way, the plants, creating regressions that represent the behavior of the data during the whole period in which the study was conducted, making feasible the verification of some data at a particular point of interest and not just at the collection dates.

The use of asymptotic regression functions to describe herbicide absorption in plants is a common practice for better visualization of the herbicide behavior over time (Grangeot et al., 2006; Bukun et al., 2009). Thus, a graph illustrating the ^{14}C-glyphosate absorption in the biotypes submitted to this experiment was constructed, as shown in Figure 1.

Figure 1 shows the ^{14}C-glyphosate absorption from its application on the leaf surface of the studied biotypes up to 96 hours after treatment. Considering Table 1, the biotype S had a maximum absorption of 80.6% of the total applied, while the biotype R had a maximum absorption of 89.5%. The biotypes MG and MT reached maximum absorptions of 89.1 and 87.1%, respectively. Koger and Reddy (2005) studied both ^{14}C-glyphosate absorption and translocation in *Conyza canadensis* and concluded that the susceptible biotype absorbed statistically similar amounts of the active ingredient applied when compared to the resistant biotype. Perez-Jones et al. (2007) also found similar ^{14}C-glyphosate absorption by resistant and susceptible biotypes of *Lolium multiflorum*. Brunharo (2014) found a maximum absorption 17% lower of the resistant biotype when compared to that susceptible for the species *Chloris elata*.

Carvalho et al. (2012) observed that the susceptible biotype of *Digitaria insularis* absorbed at least 12% more of ^{14}C-glyphosate than the resistant biotype in the evaluation carried out at 48 hours after treatment (HAT). However, the biotypes achieved similar maximum absorptions in the evaluations at 96 HAT, which corresponds to the results obtained in this research.

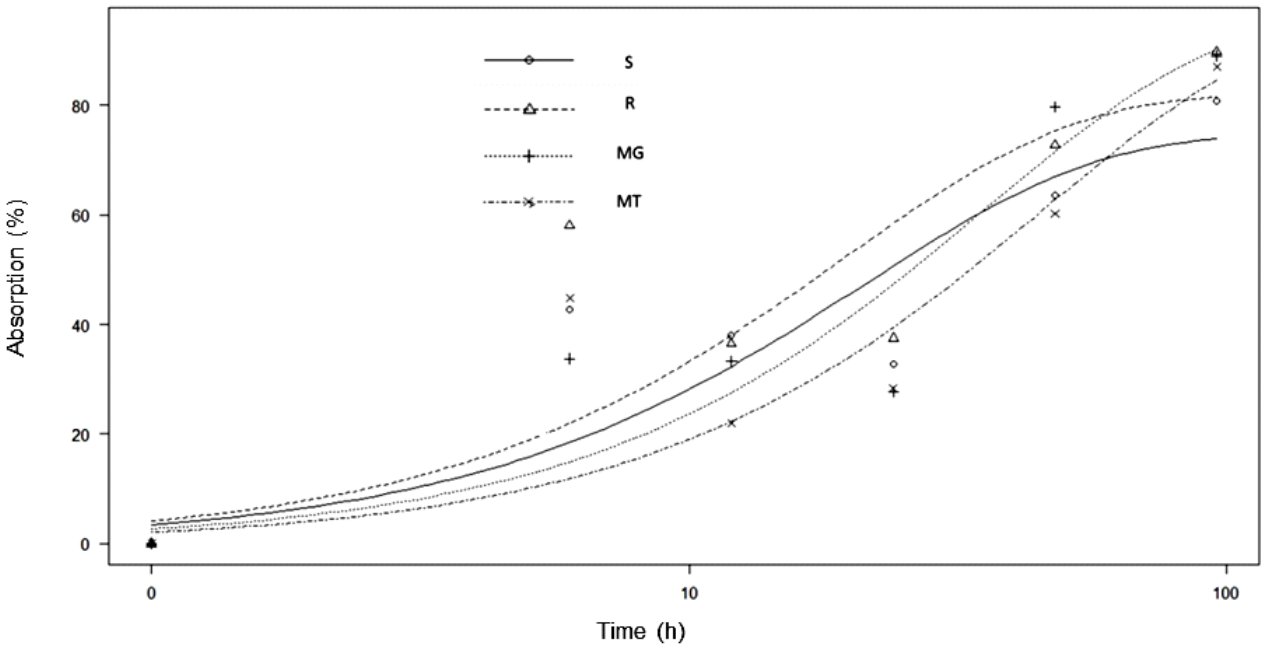

Figure 1 - ^{14}C-glyphosate absorption in the biotypes S, R, MG and MT as a function of time. Piracicaba, 2015.

Considering the t90 values of biotypes shown in Table 2, the resistant biotypes MG and MT needed a longer time to absorb 90% of the total ^{14}C-glyphosate when compared to the biotypes S and R. However, the comparison between biotypes shows no statistical difference between the t90 values, as shown in Table 3.

Adu-Yeboah et al. (2014) analyzed resistant and susceptible biotypes of *Lolium rigidum* and found that at 48 hours after treatment application the resistant biotypes retained twice as many ^{14}C-glyphosate in the treated leaves in relation to susceptible biotypes, translocating less herbicide to the rest of the plant, concluding that this is the mechanism of resistance. Another experiment with resistant biotypes of *Sorghum halepense* showed higher leaf retention of glyphosate in the resistant biotype, with a percentage difference of 28% when compared to the susceptible biotype (Riar et al., 2011). According to the data found at 96 HAT (Table 1), the susceptible biotype S translocated 37% of ^{14}C-glyphosate, while the resistant biotypes R, MG, and MT translocated 47.4, 57.4, and 43%, respectively. After analyzing these data using the model proposed by Kniss et al. (2011), the test of comparison between biotypes showed no statistical differences between them (Table 3), indicating that this is not the mechanism of resistance related to populations evaluated in this study.

These results differ from those observed by Carvalho et al. (2012), who found 70% of the herbicide applied in the treated leaf and 17.78% in the roots of the resistant biotype, while the susceptible biotype presented 42.94% in the treated leaf and 32.23% in the roots. Brunharo (2014) studied the ^{14}C-glyphosate translocation in resistant and susceptible biotypes of *Chloris elata* and noticed higher herbicide retention in the treated leaf of the resistant biotype and, consequently, a lower translocation.

Figure 2 shows the amount of ^{14}C-glyphosate translocated in the plant from the treated leaves to stems and roots in the studied biotypes during all evaluation periods after treatment. All curves show an increase over time, which indicates an increased translocation as a function of the hours after treatment. Figure 3, on the other hand, shows the presence of ^{14}C-glyphosate in the plant at each evaluation period of the biotypes S, R, MG, and MT. The autoradiographs also show that the herbicide translocated to all plant parts in the last evaluation period, indicating no difference between biotypes.

Table 3 - Parameters estimated by the software R using the model proposed by Kniss et al. (2011), related to the biotypes S, R, MG, and MT of *Digitaria insularis*, which describe, at $p<0.05$ level, the maximum leaf absorption (Amax), the time required to obtain 90% of the maximum leaf absorption (t90), the maximum translocation (Tmax), and the time required to obtain 90% of the maximum total translocation (t90*) of ^{14}C-glyphosate. Piracicaba, 2015

Compared biotypes	Estimated value	Standard error	P-value of significance
Amax: S – R	-7.4	15.7	0.6384
Amax: S – MG	-22.1	18.4	0.2331
Amax: S – MT	-21.5	24.2	0.3764
Amax: R – MG	-14.6	18.3	0.4258
Amax: R – MT	-14.1	24.2	0.5606
Amax: MG – MT	0.5	25.9	0.9838
t90: S – R	4.2	15.7	0.8874
t90: S – MG	-33.6	18.4	0.3477
t90: S – MT	-56.1	24.2	0.2908
t90: R – MG	-37.8	18.3	0.2680
t90: R – MT	-60.3	24.2	0.2464
t90: MG – MT	-22.5	25.9	0.6854
Tmax: S – R	-45.0	40.4	0.2609
Tmax: S – MG	-24.4	37.0	0.5108
Tmax: S – MT	-457.0	26.7	0.0903
Tmax: R – MG	-21.2	52.6	0.6870
Tmax: R – MT	-0.03	45.9	0.9995
Tmax: MG – MT	-21.3	42.9	0.6216
t90*: S – R	-222.8	189.3	0.2437
t90*: S – MG	-147.5	186.6	0.4316
t90*: S – MT	-123.4	105.1	0.2433
t90*: R – MG	74.7	255.7	0.7709
t90*: R – MT	98.8	203.9	0.6294
t90*: MG – MT	24.1	201.5	0.9052

* Time required to obtain 90% of the maximum translocation of ^{14}C-glyphosate.

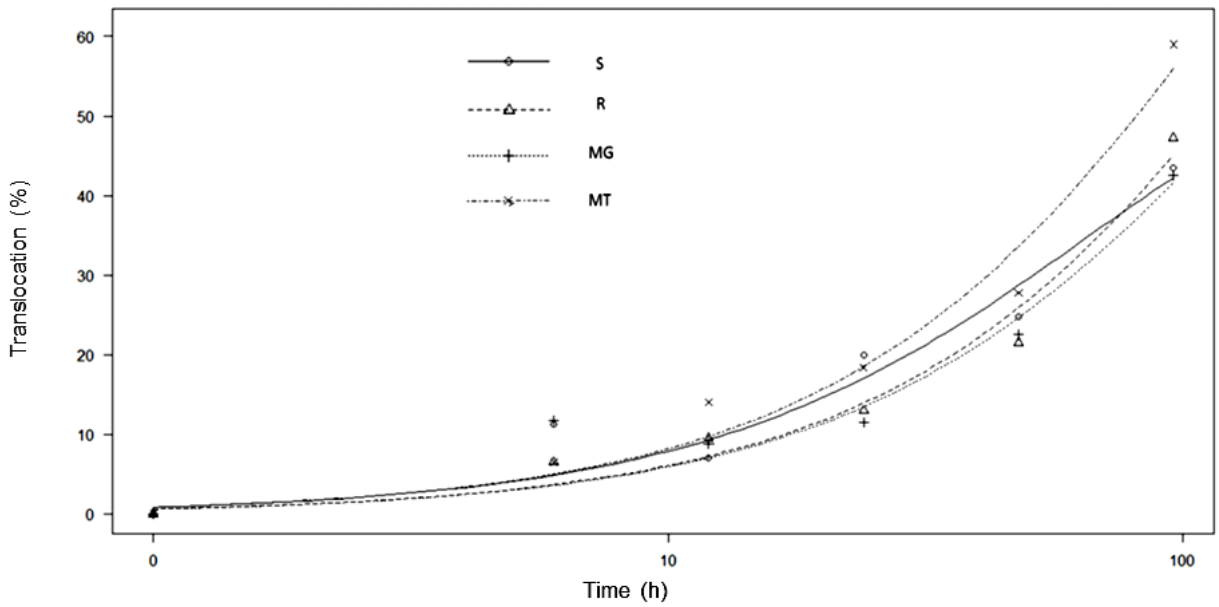

Figure 2 - ^{14}C-glyphosate translocation in the biotypes S, R, MG, and MT as a function of time. Piracicaba, 2015.

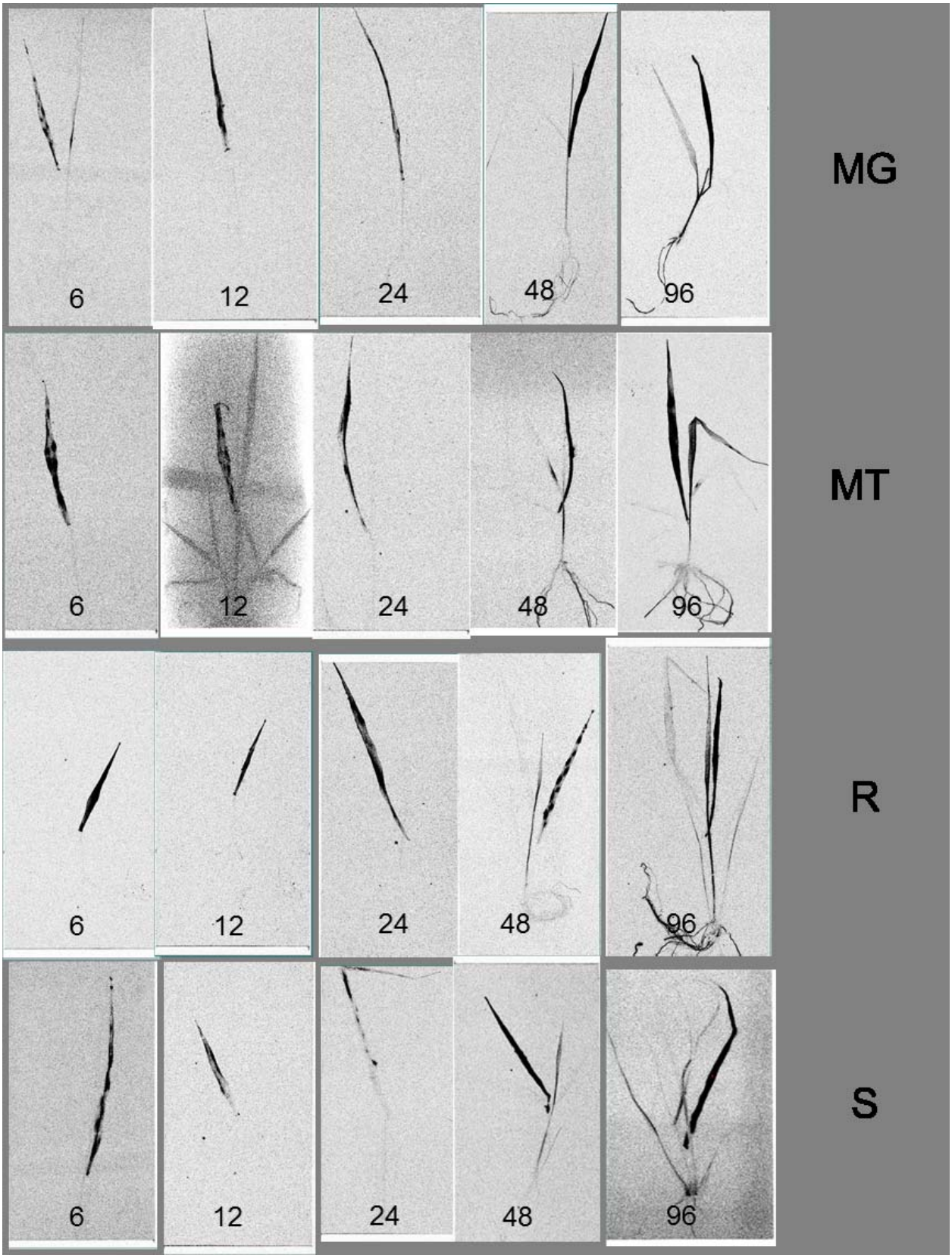

Figure 3 - Autoradiography of ^{14}C-glyphosate present in the biotypes S, R, MG, and MT at 6, 12, 24, 48, and 96 hours after treatment. Piracicaba, 2015.

Mutation in the EPSPs-encoding gene in resistant and susceptible biotypes of *Digitaria insularis*

A PCR was performed with cDNA of five replications of the biotypes S and R, being later sequenced. As expected, based on the designed primers, the fragment of the EPSPs-encoding gene should have about 200 base pairs. The term DIGIN_S represents the susceptible biotype and DIGIN_R represents the resistant biotype, in addition to a sequencing found in the literature, which is represented by the weed *Lolium multiflorum* (LOLMU) (Perez-Jones et al., 2007). The square-shaped marking at the top position of Figure 4 highlights position 106, commonly documented as the position where the substitution of the amino acid proline occurs with another amino acid and confers weed resistance to glyphosate (Wakelin and Preston, 2006; Perez-Jones et al., 2007; Kaundun et al., 2008).

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DIGIN_S         GTGCAGCTCTTCTTGGGGAATGCTGGAACGGCAATGCGGCCATTGACAGCAGCCGTAACT 60
DIGIN_R         GTGCAGCTCTTCTTGGGGAATGCTGGAACGGCAATGCGGCCATTGACAGCAGCCGTAACT 60
LOLMU           GTCAAGCTCTTCTTGGGCAACGCTGGAACTGCAATGCGGCCATTGACGGCTGCTGTAGTA 60
                **..*************.**.********.*****************.**.**.***...

DIGIN_S         GCTGCTGGAGGAAATGCAACTTATGTGCTTGATGGAGTGCCAAGAATGCGGGAGAGACCC 120
DIGIN_R         GCTGCTGGAGGAAATGCAACTTATGTGCTTGATGGAGTGCCAAGAATGCGGGAGAGACCC 120
LOLMU           GCTGCTGGTGGAAATGCGACTTATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCT 120
                ********.********.********.***********.*********.*****.****.

DIGIN_S         ATTGGCGACTTGGTTGTCGGATTGAAACAGCTCGGTGCGGATGTTGATTGCTTCCTTGGC 180
DIGIN_R         ATTGGCGACTTGGTTGTCGGATTGAAACAGCTCGGTGCGGATGTTGATTGCTTCCTTGGC 180
LOLMU           ACCGGTGACTTAGTTGTCGGTTTGAAACAGCTAGGTGCGAATGTTGATTGTTTCCTTGGC 180
                *..**.*****.********.***********.******.**********.*********

DIGIN_S         ACTGACTGCCCACCTGTTCGTGTCAAGGGAATTGGAGGGCTACCTGGTGGCAAGGTTAAG 240
DIGIN_R         ACTGACTGCCCACCTGTTCGTGTCAAGGGAATTGGAGGGCTACCTGGTGGCAAGGTTAAG 240
LOLMU           ACTGACTGCCCACCTGTTCGGATCAACGGCATTGGAGGGCTACCTGGTGGCAAGGTTAAG 240
                ********************..**** **.******************************

DIGIN_S         CTATCTGGCTCTATCAGCAGTCAGTACTTGAGTGCCTTGC 280
DIGIN_R         CTATCTGGCTCTATCAGCAGTCAGTACTTGAGTGCCTTGC 280
LOLMU           CTGTCTGGTTCCATCAGCAGCCAATACTTGAGTTCCTTGC 280
                **.*****.**.********.**.*********.******
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Figure 4 **-** Nucleotide sequence of the EPSPs-encoding biotypes S and R, with the positions 106 and 182 highlighted. Piracicaba, 2015.

The square at the bottom of the image illustrates position 182, which documented a mutation related to the resistance of *Digitaria insularis* to glyphosate in biotypes of the State of São Paulo (Carvalho et al., 2012).

In Figure 4, with the alignment of a segment of the EPSPs-encoding gene, it is possible to realize that there is no mutation at position 106, which is occupied by the amino acid proline, nor in other positions of that sequence, such as position 102, which may also lead to lower susceptibility of the individual to glyphosate. The differences are represented by the absence of an asterisk in the lower part of the sequence and show the mutations that occur between the ryegrass (*Lolium multiflorum*) and biotypes of *D. insularis*, but it is not possible to visualize differences between sequences within the biotypes S and R.

Two types of documented EPSPs can be found in the literature, both represented according to their sensitivity to glyphosate. Class I EPSPs is found in all plant species and in bacteria such as *Escherichia coli* and *Salmonella typhimurium*, and their activity is inhibited by low glyphosate concentrations (Franz et al., 1997). Class II EPSPs, found in some species of bacteria, even in *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Agrobacterium* sp. strain CP4, is endowed with the ability to maintain its catalytic activity even in the presence of high concentrations of this herbicide (Priestman et al., 2005; Funke et al., 2006; Dill et al., 2008).

Insensitivity to glyphosate by some plants with class I enzyme can be achieved artificially or through natural selection. Assuming that glyphosate and phosphoenolpyruvate (PEP) bind at the same site in EPSPs, it is expected that mutants having the mutation at that position have both lower affinity for PEP and glyphosate (Franz et al., 1997). Some point mutations, such as the substitution of threonine by methionine at position 47 (He et al., 2003), glycine by alanine at position 101 (Padgette et al., 1991), and proline by serine at position 106 (Stalker et al., 1985), may result in lower affinity to glyphosate, but not sufficient to withstand high dosages of this herbicide. On the other hand, the mutation of threonine by isoleucine at position 102, together with the mutation of proline by serine at position 106, also known as a TRIS mutation, can enable individuals to receive high amounts of glyphosate, which has led to its use artificially in genetically modified crops. Another combination of mutations known as TIPS, which consists of a mutation at position 102 along with a mutation at position 106 (T102I+P106S), was found in biotypes of *Eleusine indica*, which presented a resistance factor of the order of 180 times when compared to the susceptible biotype and of 32 times when compared to the resistant biotype that had only the proline mutation at position 106 (Yu et al., 2015).

Therefore, the biotypes R, MG, and MT had no differences in the ^{14}C-glyphosate absorption and translocation when compared to the biotype S. In addition, according to the EPSPs-encoding gene, there is no mutation in the region 106 or 182 or even another type of mutation in the analyzed sequence of that gene. Thus, these mechanisms do not confer resistance of sourgrass to glyphosate. A possible mechanism of resistance to be evaluated in future researches would be the sequestration of the herbicide to the vacuole of cells of the resistant biotype (Ge et al., 2010).

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