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The intensity of non-target site mechanisms influences the level of resistance of sourgrass to glyphosate

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ABSTRACT

Non-target site mechanisms are involved in the resistance of sourgrass (*Digitaria insularis*) to glyphosate. Studies on the 14C-glyphosate absorption and translocation as well as the detection of glyphosate and its metabolites in sourgrass plants were carried out under controlled conditions to investigate if the differential response of resistant sourgrass biotypes (R1 and R2) is derived from the intensity of non-target site mechanisms involved in the resistance to glyphosate. Different pattern of absorption was observed between S (susceptible) and R2 from 12 up to 48 hours after treatment with glyphosate (HAT), and between S and R1 just at 12 HAT. The initial difference in glyphosate absorption among the biotypes did not maintained at 96 HAT and afterwards. Smaller amount of herbicide left the treated leaf into the rest of shoot and roots in R2 (25%) than in S (58%) and R1 (52%). In addition, slight difference in glyphosate translocation was observed between S and R1. We found high percentage (81%) of glyphosate in the S biotype up to 168 HAT, while just 44% and 2% of glyphosate was recovered from R1 and R2 plant tissues. In addition, high percentage of glyphosate metabolites was found in R2 (98%) and R1 (56%) biotypes, while a very low percentage (11%) was found in the S biotype. As previous studies indicated resistant factors of 3.5 and 5.6 for R1 and R2, respectively, we conclude that the differential response of sourgrass biotypes is derived from the intensity of the non-target site mechanisms involved in the resistance to glyphosate.

Keywords: *Digitaria insularis*, *N*-phosphonomethylglycine, weed resistance, non-target site resistance.

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INTRODUCTION

Digitaria insularis (L.) Mez ex Ekman/Fedde, commonly known as sourgrass, is a perennial grass weed indigenous to tropical and subtropical America. It is an important weed infesting annual and perennial crops in Brazil. Sourgrass reproduces by seeds and rhizomes (Carvalho et al. 2011). In addition, the small hairy seeds with a high percentage of germination allow sourgrass to rapidly increase in numbers (Correia and Durigan 2009). For those reasons, control sourgrass control is obviously not easy to do in spite of this weed was been killed successfully using glyphosate (*N*-phosphonomethylglycine). However, in the past years, many growers had observed a lack of sourgrass control using glyphosate, referring to the resistance to this herbicide as the most important issue for managing this grass weed in Brazil.

Cases of glyphosate-resistant weeds have increased worldwide in the past years (Heap 2013). Strong selection by herbicides has resulted in the widespread evolution of herbicide resistance in populations of agricultural weeds (Carvalho et al. 2012). Weed populations evolve resistance in response to repeated treatment with herbicides having the same mechanism of action or metabolic degradation pathway (Duke and Powles 2008, Powles and Yu 2010). As glyphosate is the most commercialized herbicide in the world and, as a consequence, it has been used extensively in agriculture for about 30 years (Duke and Powles 2008), an intense pressure of selection on weed populations have been supplied throughout the past two decades, involving into the crescent number of cases of glyphosate resistance around the world.

Biotypes of sourgrass derived from agricultural fields cultivated with annual and perennial crops were already reported in Brazil (Heap 2013) and the glyphosate resistance was confirmed (Carvalho et al. 2011). The main resistance mechanism was detected as being the reduced herbicide translocation and the absorption, metabolism, and gene mutation were also found playing important role (Carvalho et al. 2012). Non-target site mechanisms of resistance refer to any process involved in the herbicide resistance excepting the one related to some alteration in the site of action (e.g. mutation), such as reduced herbicide absorption, reduced herbicide translocation, and enhanced herbicide degradation by plants.

The objective of this research was to investigate if the differential response of some sourgrass biotypes is derived from the intensity of non-target site mechanisms involved in the resistance to glyphosate.

MATERIAL AND METHODS

Plant materials and growing conditions. Three sourgrass biotypes, previously studied by Carvalho et al. (2011) to confirm the resistance to glyphosate, were used in this study. The resistance factor showed different levels of resistance among biotypes, so that R1 and R2 resisted to concentrations of glyphosate 3.5 and 5.6 times higher than the susceptible biotype (S) to reduce the seedling fresh weight by 50% under Petri dish experiments. In addition, the shikimic acid accumulation in leaf tissues of the R1 and R2 biotypes was 3.3 and 5.0 times lower than the S biotype, respectively.

Experiments were carried out under controlled conditions within a growth chamber model AM0705020 (Eldon, The Netherlands) at 28/18 $^{\circ}$C (day/night) in 16 h photoperiod under 850 µmol m^{-2} s^{-1} photosynthetic photon-flux density delivered by a mixture of incandescent and fluorescent lights, with 80% relative humidity. Plants were grown in 300 mL plastic pots containing a 1:2 (v:v) mixture of peat and sandy loam. Water was supplied twice a day and Hoagland nutrient solution was supplied a time a week.

Chemicals and spraying conditions. Commercial herbicide formulation with 45% of isopropylamide salt of glyphosate SL (Monsanto, Belgium) and [^{14}C]-glyphosate-(phosphonomethyl) with specific activity of 52 mc_i $mmol^{-1}$ (American Radiolabel Chemicals, United States of America) were used in this study. All other reagents were obtained at analytical grade. For absorption and translocation studies, glyphosate was applied with a micro-applicator model PB 600 TA (Hamilton, United States of America). For metabolism study, glyphosate was sprayed by using a laboratory tracking sprayer model SBS-060 (DeVries, United States of America) equipped with a flat-fan nozzle model 80.02E VS (TeeJet, United States of America), delivering a spray volume of 200 L ha^{-1} at 200 kPa.

Absorption and translocation studies. For absorption and translocation studies, we used one plant per pot, following the protocol used by González-Torralva et al. (2010). The 14C-glyphosate was mixed with commercially formulated glyphosate to prepare emulsions with a specific activity of approximately 50,000 dpm μL^{-1} (both absorption and translocation studies) and a glyphosate concentration of 3.6 g ae L^{-1} (corresponding to 720 g ae ha^{-1} at 200 L ha^{-1}). The labeled herbicide was applied to the adaxial surface of the second leaf of each plant, at 4-leaf growth stage, in one 1.0 µL droplet. At 12, 24, 48, 72, and 96 hours after treatment (HAT), unabsorbed 14C-glyphosate

was removed from the leaf surface by rinsing the treated area with 3 mL acetone 50% (v/v). Rinses from batches of five replications of plants were pooled and analyzed by liquid scintillation spectrometry (LSS) using a scintillation counter model LS 6500 (Beckman Counter, United States of America).

Plants were also harvested in each batch at the same times and separated into treated leaf, root and rest of shoot. The plant tissue was dried at 55 ºC for 72 h and combusted in a Packard Tri Carb 307 Sample Oxidizer (Perkin-Elmer, United States of America). The $14CO_2$ evolved was trapped and counted in a 10 mL mixture of Carbo-Sorb E and Permafluor E+ (3:7, v/v) (Perkin-Elmer, United States of America). The radioactivity was quantified by LSS, and percentage of herbicide absorbed (PHA) was expressed as:

$$PHA = \frac{dpm\ in\ combusted\ tissue}{dpm\ in\ combusted\ tissue + dpm\ in\ leaf\ washes} \times 100$$

The data of oxidized samples of each plant part were expressed as a percentage of the total 14C-glyphosate absorbed (treated leaf + rest of shoot + roots) for translocation studies. The experiments were arranged in a completely randomized design with five replicates and repeated twice.

Metabolism study. The protocol described by Rojano-Delgado et al. (2010) was followed for the metabolism study. The concentration of glyphosate and its metabolites was determined by reversed-polarity capillarity electrophoresis. Glyphosate, aminomethylphosphonic acid (AMPA), glyoxylate, sarcosine, and formaldehyde were detected in leaf tissues of plants of all biotypes at 48, 96, and 168 HAT. Leaf tissues of treated and untreated plants were cut and frozen. Then, samples were washed, ground, mixed with acetone solution, submitted to ultrasound, centrifuged, dried under nitrogen flow, mixed with potassium phthalate, cetyl trimethylammonium bromide, and acrylonitrile, filtered, and then submitted to electrophoresis on equipment model G1600A (Agilent). Electropherograms were obtained, and the concentrations of glyphosate and its metabolites were determined on the basis of standard equations estimated by Rojano-Delgado et al. (2010). The experiments were arranged in a completely randomized design with three replicates.

Statistical analysis. All data were submitted to ANOVA (F-test), using the Statistix statistical software version 8.0 (Analytical Software, United States of America), to analyze the interaction between biotypes and times of evaluation.

RESULTS AND DISCUSSION

ANOVA test for absorption studies indicated significant interaction between biotypes and times of evaluation ($P < 0.05$). We observed strong differences between S and R2 from 12 up to 48 HAT, and between S and R1 just at 12 HAT (Figure 1). The initial difference in glyphosate absorption among the biotypes did not maintained at 96 HAT and afterwards, as verified by Carvalho et al. (2012). At 12 HAT, 36%, 26%, and 12% of the 14C-glyphosate was recovered from S, R1, and R2, while 49%, 46%, and 45% of the 14C-glyphosate was recovered from those biotypes at 96 HAT, respectively. So, there was no difference in the glyphosate absorption among sourgrass biotypes after 96 HAT, but the initial different pattern of absorption could influence the plant response if considering the herbicide metabolism (we have discussed below).

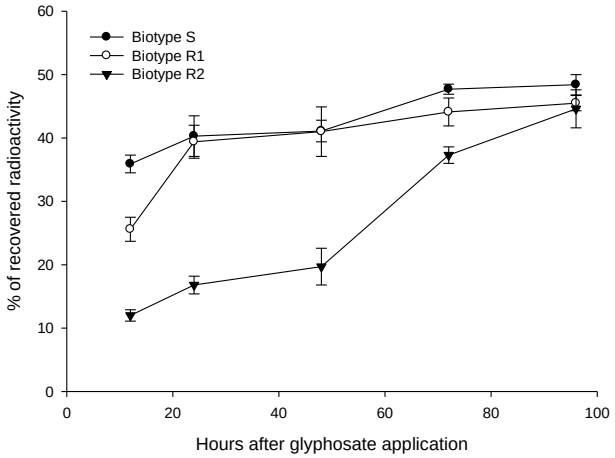

Figure 1. Absorption (% of recovered radioactivity) of 14C-glyphosate by susceptible (S) and resistant (R1 and R2) sourgrass (*Digitaria insularis*) biotypes at different times after treatment.

ANOVA test for translocation studies indicated significant interaction between biotypes and times of evaluation ($P < 0.05$). We observed strong differences between S and R2 from 12 up to 96 HAT, and between S and R1 just at 12 HAT (Figure 2). The strong difference in glyphosate absorption between the S and R2 biotypes maintained at the full time evaluation, but it was too weak between the S and R1 biotypes. At 12 HAT, 70%, 75%, and 83% of the absorbed 14C-glyphosate remained in the treated leaf of S, R1, and R2, while 42%, 48%, and 75% of the 14C-glyphosate remained in those biotypes at 96 HAT, respectively (Figure 1 – up). That result indicated that smaller amount of herbicide left the treated leaf into the rest of shoot and roots in the R2 biotype than in the S and R1 biotypes. In addition, slight differences were observed between S and R1 biotypes. So, there was significant

difference in the glyphosate translocation among sourgrass biotypes, so that much more glyphosate remained in the treated leaf of the R2 biotype, comparing with the other biotypes, as verified by Carvalho et al. (2012). Moreover, the slight different pattern of translocation between S and R1 could influence the plant response if considering the herbicide metabolism (we have discussed below).

ANOVA test for metabolism studies indicated significant interaction between biotypes and times of evaluation ($P < 0.05$). We found high percentage (81%) of glyphosate in the S biotype up to 168 HAT, while just 44% and 2% of glyphosate was recovered from R1 and R2 plant tissues (Figure 3). In addition, high percentage of glyphosate metabolites was found in R2 (98%) and R1 (56%) biotypes, while a very low percentage (11%) was found in the S biotype. So, glyphosate was degraded much faster in the resistant biotypes, as verified by Carvalho et al. 2012, 2013), than the susceptible biotype and R2 showed higher degradation than R1. The different metabolism between R1 and S, associated to slight differences in the initial herbicide absorption and translocation, could also contribute to the distinct plant response to glyphosate, since less glyphosate was within the plant being degraded rapider by the R1 biotype. Thus, less amount of glyphosate attained the site of action in glyphosate-resistant sourgrass plants, so that lower growth reduction and percentage of plant death was observed for the resistant biotypes, comparing with the susceptible biotype.

Glyphosate is an herbicide with a molecular target site related to the inhibition of the shikimate pathway (Duke et al. 2003). The inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) results in strong reductions of EPSP and ensuing metabolic products, such as the aromatic acids phenylalanine, tyrosine, and tryptophan that are required for protein synthesis (Siehl 1997; Herrman and Weaver 1999). The inhibition of the shikimate pathway causes its deregulation, resulting in carbon flow from other pathways, leading to disruption of other metabolic pathways (Duke et al. 2003). Thus, the intensity of EPSPS inhibition affects the plant growth dynamics. One of the reasons to explain a lesser inhibition of EPSPS in glyphosate-resistant plants is related to a limitation of glyphosate attaining the site of action in resistant plants at high levels. That limitation can occurs due to a reduced herbicide absorption and/or translocation, as observed by Carvalho et al. (2012) in a study to found resistance mechanisms to glyphosate in other sourgrass biotypes. Limited herbicide absorption and/or translocation is common to be found in other glyphosate-resistant species, such as *Conyza* spp. (Feng et al. 2004, Koger et al. 2005, Dinelli et al. 2008, González-Torralva et al. 2012a) and *Lolium* spp. (Perez et al. 2004, Wakelin et al. 2004, Perez-Jones et al. 2005, González-Torralva et al. 2012b).

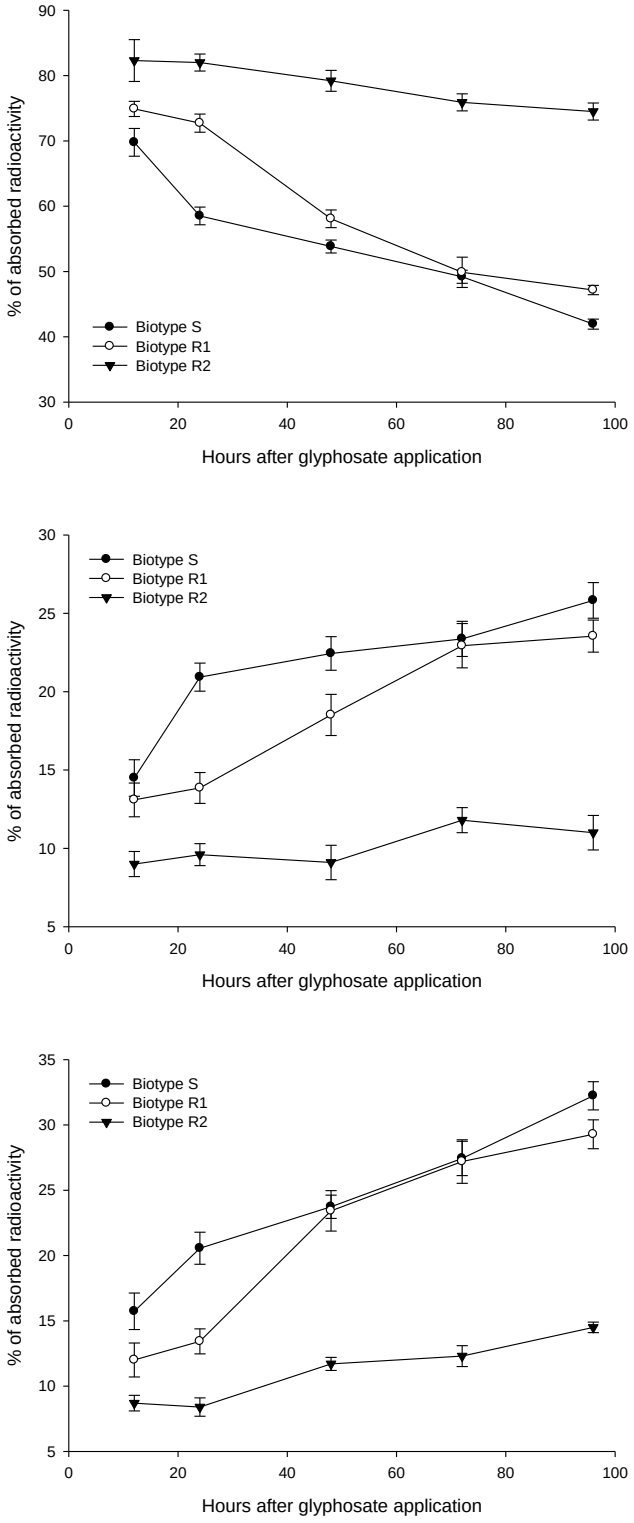

Figure 2. Translocation (% of absorbed radioactivity) of 14C-glyphosate, in the treated leaf (up), rest of shoot (middle), and roots (down), by susceptible (S) and resistant (R1 and R2) sourgrass (*Digitaria insularis*) biotypes at different times after treatment.

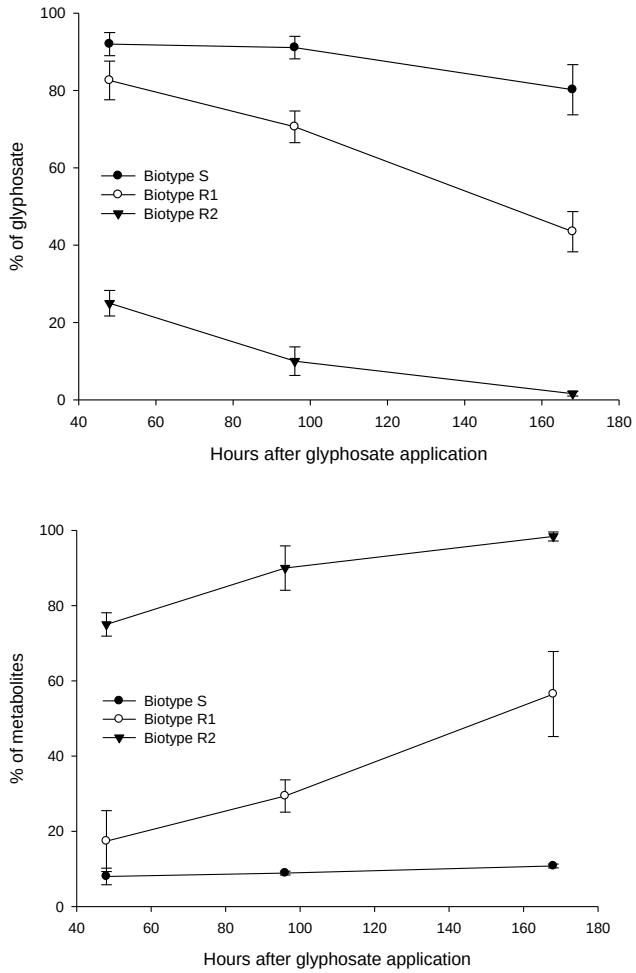

Figure 3. Relative percentage of glyphosate (up) and its metabolites (down) – AMPA + sarcosine + formaldehyde – in susceptible (S) and resistant (R1 and R2) sourgrass (*Digitaria insularis*) biotypes at different times after treatment.

Michitte et al. (2004) examined the involvement of the cuticle properties on the resistance of *Lolium multiflorum* Lam. and did not find evidence of wax crystallization in both susceptible and resistant biotypes, although certain zones on the surface of resistant biotype had wrinkles and the leaf cuticle was thicker than the susceptible one. Differences in herbicide absorption between leaves with thicker and thinner cuticles were recently observed in *Abutilon theophrasti* Medik., so that lower absorption of acifluorfen, a moderately polar herbicide, was observed in leaves of thicker epicuticular wax (Hatterman-Valenti et al. 2011). Thus, since glyphosate is a polar herbicide, lower absorption may occur in plants with thicker cuticles (Guimarães et al. 2009). Differences in glyphosate absorption may be also related to distinct leaf epicuticular wax composition (Nandula et al. 2008), as observed between glyphosate-resistant and -susceptible biotypes of *L. multiflorum* (Guimarães et al. 2009). Authors found up to 5% more alcohols and aldehydes (polar components) in resistant biotype, increasing slightly the leaf polarity. Contact angle of droplets and foliar herbicide retention can also play an important role in glyphosate absorption (Michitte et al. 2007).

The main process involved in the limitation of herbicide translocation is probably the vacuolar glyphosate sequestration, as described to *Conyza canadensis* (L.) Cronq. (Ge et al. 2010, 2011, 2012). As observed by Carvalho et al. (2012) for other sourgrass glyphosate-resistant biotypes, an acropetal translocation occurred in resistant biotypes in addition to the basipetal herbicide translocation, suggesting that glyphosate is ambimobile in the plant (Franz et al. 1997), showing both xylem and phloem translocation (Perez-Jones et al. 2007). This suggests that an altered symplast transport is responsible for a differential glyphosate translocation between susceptible and resistant biotypes (Feng et al. 2004, Wakelin et al. 2004, Lorraine-Colwill et al. 2003). These authors discussed the existence of an altered cellular transport that would retain glyphosate in the apoplastic space, thus preventing its entry into the phloem tissues. Altered glyphosate symplast transport has been associated with lack of translocation in weeds resistant to glyphosate, such as *Conyza* spp. (Feng et al. 2004), *L. multiflorum* (Michitte et al. 2007), and *Lolium rigidum* Gaud. (Lorraine-Colwill et al. 2003, Powles and Preston 2006).

An explanation for differences in glyphosate translocation was provided by Ge et al. (2010, 2011, 2012), studying *C. canadensis* resistant to glyphosate. Glyphosate enters the cytoplasm of both resistant and susceptible plant variants at the same dose, but begins to occupy the vacuole in the resistant but not the susceptible biotype. Glyphosate in the cytoplasmic pool is available for translocation to sink tissues. However, glyphosate sequestered within the vacuole is effectively removed from the phloem-accessible pool of glyphosate. The resistance mechanism for resistant plants reflects an inherent ability (Zelaya et al. 2004) to sequester glyphosate in the vacuole (Ge et al. 2010, 2011, 2012), where, presumably, it stays indefinitely or is released slowly at a sublethal dose.

Other reasons to explain a lesser inhibition of EPSPS in glyphosate-resistant can be related to gene mutation, gene amplification, and/or herbicide degradation. Some mutations on the *EPSPS* gene have been reported to confer resistance in sourgrass (Carvalho et al. 2012), *Eleusine indica* (L.) Gaertn. (Baerson et al. 2002, Ng et al. 2003), *L. rigidum* (Wakelin et al. 2006), and *L. multiflorum* (Perez-Jones et al. 2005, González-Torralva et al. 2012b). We also investigated *EPSPS* gene mutation in this study but no amino acid changes were observed (data not shown).

EPSPS gene amplification was found conferring glyphosate resistance in *Amaranthus palmeri* S.Wats. (Gaines et al. 2010) and *Lolium perenne* ssp. *multiflorum* (Lam.) Husnot. (Salas et al. 2012). Degradation of glyphosate was reported in playing an important role as a mechanism of glyphosate resistance in other previously studied resistant sourgrass biotypes (Carvalho et al. 2012, 2013) and *C. canadensis* (González-Torralva et al. 2012a).

Thus, the non-target site mechanisms are involved in the resistance of sourgrass to glyphosate. In addition, the association between the reduced translocation and the enhanced metabolism was the main mechanism responsible for high levels of resistance, since it was responsible to the strong differences between the S and the R2 biotypes. Moreover, the enhanced metabolism was also an important mechanism for determination of low levels of resistance of the R1 biotype, since strong difference was observed between S and R1.

CONCLUSION

Taken together, our results allow us to conclude that the differential response of sourgrass biotypes is derived from the intensity of the non-target site mechanisms involved in the resistance to glyphosate.

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