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Effect of glyphosate on the physiological parameters of horseweed

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ABSTRACT

The aim of this study was to determine changes in the photosynthetic process and the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) by shikimic acid accumulation, after application of the herbicide glyphosate in four biotypes of *C. sumatrensis* collected in the state of Rio Grande do Sul. Therefore, the experiments were conducted under greenhouse and laboratory, assessing plant biomass, photosynthesis rate, transpiration and water use efficiency; the amount of shikimic acid was calculated for each treatment. Glyphosate application reduced growth and affected the photosynthesis rate and water use efficiency of the biotypes differentially, and the resistant biotype had small interference from glyphosate, while physiological parameters of the susceptible biotype were deeply affected by the herbicide. Susceptible biotypes had higher shikimic acid accumulation than the resistant biotype, indicating differential sensitivity of EPSPs to glyphosate between resistant and sensitive biotypes.

Key words: *Conyza sumatrensis*, EPSPs, resistance, shikimic acid

Efeito do glyphosate sobre parâmetros fisiológicos de buva

RESUMO

O objetivo do trabalho foi determinar alterações no processo fotossintético e na inibição da 5-enolpiruvilchiquimato-3-fosfato sintase (EPSPs), por meio do acúmulo de ácido chiquímico, após a aplicação do herbicida glyphosate, em quatro biótipos de *C. sumatrensis* coletados no Estado do Rio Grande do Sul. Para isto foram conduzidos experimentos em casa-de-vegetação e em laboratório avaliando fitomassa seca da parte aérea, taxa de fotossíntese, transpiração e eficiência do uso da água; a quantidade de ácido chiquímico foi calculada para cada tratamento. A aplicação do glyphosate reduziu o crescimento e afetou a taxa de fotossíntese a eficiência do uso da água dos biótipos de forma diferencial, sendo que o biótipo resistente teve pequena interferência do glyphosate enquanto os parâmetros fisiológicos do biótipo suscetível foram profundamente afetados pelo herbicida. Nos biótipos suscetíveis o acúmulo de ácido chiquímico foi maior que no biótipo resistente evidenciando sensibilidade diferencial da EPSPs dos biótipos resistente e sensíveis ao glyphosate.

Palavras-chave: *Conyza sumatrensis*, EPSPs, resistência, ácido chiquímico

Introduction

Currently, one of the main discussions on weed management in agriculture is associated with the selection of herbicide-resistant biotypes. It is a natural phenomenon that occurs spontaneously in populations; therefore, the herbicide is not the causative agent, but it selects resistant specimens which are at low initial frequency (Christoffoleti et al., 1994).

The emergence of resistant weeds occurs more frequently in areas where there is repeated use of herbicides from the same chemical group or belonging to different groups, but with the same mechanism of action (Gressel & Segel, 1990). Thus, the constant use of technologies involving Genetically Modified Organisms (GMOs), associated with the use of glyphosate, have contributed to the increased selection pressure and the appearance of biotypes which are resistant to glyphosate.

In southern Brazil, horseweed (*Conyza* spp.) is one of the major glyphosate-resistant weeds. Horseweed has a high competitive potential for essential growth factors and high seed dispersibility; thus, it may cause direct and indirect damage to crops. In Brazil, glyphosate resistance has already been found in biotypes of the horseweed species *C. bonariensis, C. canadensis* and *C. sumatrensis* in areas of glyphosate-resistant transgenic soybean (Heap, 2013).

The mechanism of action of glyphosate includes the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs), responsible for the condensation reaction of shikimate-3-phosphate and phosphoenolpyruvate into EPSP and inorganic phosphate in the shikimic acid pathway (Geiger & Fuchs, 2002). Inhibition of EPSPs results in shikimic acid accumulation in plants and reduced biosynthesis of essential aromatic amino acids, such as tryptophan, tyrosine and phenylalanine. (Zablotowicz & Reddy, 2004). Thus, shikimic acid accumulation in plants can be used to determine if plants are resistant to glyphosate when the resistance mechanism is insensitivity of EPSPs (Carvalho et al. 2012). Another way to evaluate resistance is by means of the inhibitory effect of photosynthesis in plants after glyphosate application (Duke et al. 2003). Thus, the aim of this study was to determine changes in the photosynthetic process and inhibition of EPSPs, through shikimic acid accumulation, after glyphosate application in four biotypes of *C. sumatrensis* collected in the state of Rio Grande do Sul, southern Brazil.

Material and Methods

The experiments were conducted in a greenhouse in Passo Fundo, Rio Grande do Sul (S 28º15'46" and W 52º24'24", at 684 m of altitude). The first stage of the study was carried out with the aim of selecting four populations - one glyphosate-resistant and three glyphosate-susceptible populations - out of 25 populations collected in the field. For this purpose, a discriminatory dose of glyphosate (720 g a.i. ha^{-1}) was applied. Seeds of susceptible biotypes were collected from the pots where the herbicide had not been applied, so that they could be used in the next stages of the study. The seeds of the resistant biotype were collected from plants that had survived glyphosate application. The selected biotypes were identified as *Conyza sumatrensis* (Retz.) E. Walker, and deposited in the herbarium of the Federal University of Santa Maria, under numbers SMDM 13950, SMDM 13951, SMDM 13952 and SMDM 13953.

In the second stage, an experiment was conducted in a completely randomized 2x2 factorial design with three replications. Four biotypes of *C. sumatrensis* were evaluated, selected in the first stage (termed 2, 5, 17 and 20), considering two doses of glyphosate (0 and 1440 g a.i. ha^{-1}). The biotypes were sown on 27 April 2012 in 500 mL plastic cups (experimental units), containing Garden Plus Turfa Fértil® substrate, turf and limestone, and supplemented with minerals (N = 0.02%, P_2O_5 = 0.08% and K_2O = 0.04%). The technical characteristics of the substrate were: pH = 5.8 ± 0.5; electrical conductivity = 1.5 ± 0.3; maximum humidity (weight / weight) = 55%, dry basis density = 290 kg m^{-3}, water holding capacity (WHC) = 60% of dry weight. After emergence on May 6, 2012, the plants were thinned, and three plants were left in each plastic cup. The herbicide was applied on 23 July 2012 with a CO_2 pressurized backpack sprayer outfitted with TeeJet XR 115.02 flat fan tips, spaced at 0.5 m, spray volume of 150 L ha^{-1}, at pressure of 1.62 kgf cm^{-2}.

The variables evaluated were photosynthesis rate (A), transpiration (E), water use efficiency (WUE), and concentrations of shikimic acid in the leaves at five evaluation times: 0 (before herbicide application), 3, 7, 10 and 14 days after treatment (DAT). The plants were harvested at 28 DAT to determine the production of dry biomass in the shoots. For this purpose, the plant material was dried in a forced air circulation oven at 60 ° C until constant weight, and then weighed. The weight values were transformed into percentage values, comparing the plant biomass obtained in the treatments with herbicide with the average plant biomass obtained in the control, considered as 100%.

The photosynthesis rate (A) and transpiration (E) were measured using a LI-COR infrared gas analyzer (IRGA) (LI-6400XT), outfitted with a LI-COR light chamber (LI-6400-2B) and an automatic CO_2 injection system. During the measurements, the following were established: photon flux density of 1500 µmol m^{-2} s^{-1}, CO_2 injection into the chamber of 400 µmol mol^{-1} and air flow of 500 µmol s^{-1}. Water use efficiency (WUE) was calculated as the ratio between variables A and E. The evaluations were made between 9 a.m. and 11 a.m., using the leaf with further development of all three plants in each plastic cup. For shikimic acid determination, the plants were cut off at the soil surface and oven dried at a temperature of 60 ° C for 16 hours. After the samples reached constant weight, they were ground in a milling machine (2500 rpm) and stored under refrigeration (-10ºC) until the time of extraction and shikimic acid determination by the method described by Matallo et al. (2009).

The data were checked for homogeneity of variance and then subjected to ANOVA ($p \leq 0.05$), using the software "ASSISTAT 7.6 BETA". For the variables dry plant biomass of the shoots, photosynthesis rate (A) and water use efficiency (WUE), the means were compared by the Scott-Knott test for grouping means ($p < 0.05$). The values for shikimic acid concentration in the leaves, when statistical significance was

found by the F-test ($p < 0.05$), were subjected to regression analysis for the factor evaluation times after treatment with glyphosate in each biotype evaluated. Regression analysis was performed using the software SigmaPlot 10.0, adjusting the data to the regression equation of the nonlinear cubic polynomial model, as follows:

$$y = y_0 + ax + bx^2 + cx^3$$

where: y = shikimic acid accumulation; x = days after glyphosate application, and y_0, a, b and c are the four coefficients of the cubic model. The mean accumulation of shikimic acid in the equations were represented by the confidence interval at 95%, so the overlap of the confidence interval indicates no significant difference among biotypes at evaluation times after treatment with glyphosate.

Results and Discussion

The four biotypes selected in the first stage, and used in the second stage, were from the species *C. sumatrensis*. Biotype 2 was collected in the municipality of Pontão (Rio Grande do Sul, Brazil) (Lat: 28°00'20.40" N and Lon: 52°45'12.40" E), and it was considered to be the most susceptible to glyphosate (data not shown); Biotype 5 was collected in the municipality of Carazinho (Rio Grande do Sul, Brazil) (Lat: 28°18'06.51" N and Lon: 52°53'41.31" E), and it was considered to be resistant to glyphosate (data not shown); Biotype 17 was collected in the city of Coqueiros do Sul (Rio Grande do Sul, Brazil) (Lat: 28°07'28.00" N and Lon: 52°42'47.90" E), and it was considered to be less susceptible to glyphosate (data not shown); Biotype 20 was collected in the municipality of Tio Hugo (Rio Grande do Sul, Brazil) (Lat: 28°18'06.51" N and Lon: 52°53'41.31" E), and it was also considered to be less susceptible to glyphosate (data not shown). These biotypes were used in the second stage of the study.

The result of the analysis of variance of the four biotypes in the second stage indicated that there was interaction between biotype and herbicide doses in all variables. All biotypes had reduced plant biomass accumulation, compared with their respective control (Table 1). However, there was variation in the degree of reduction when the biotypes were compared. Biotypes 2, 17 and 20 were controlled by glyphosate and had reductions in dry weight of 67%, 55% and 52%, respectively, compared to their untreated controls without glyphosate application (Table 1). In contrast, Biotype 5 showed the smallest reduction in DM (18%) compared to its control without herbicide application. However, the reduction of DM of Biotype 5 was not expected, because it was classified as resistant to glyphosate in stage 1 of this research. Thus, a possible explanation for the reduction of DM in Biotype 5 is phytotoxicity symptoms that were observed at 28 DAT. These symptoms may have been caused by the presence of aminomethylphosphonic acid (AMPA). AMPA is the product of glyphosate degradation in plants, and it may cause phytotoxicity even in plants whose EPSPS are insensitive to glyphosate (Reddy et al. 2004).

Table 1. Percentage of plant dry weight of the shoots of Biotypes 2, 5, 17 and 20 of C. sumatrensis at 28 days after application of 1440 g a.i. ha^{-1} of glyphosate, and the control without herbicide application. Passo Fundo, RS, 2012

Biotypes	Doses (g a.i. ha^{-1})	
	0	1440
	%	
2	100 aA[1]	67 aB
5	100 Aa	18 cB
17	100 aA	55 bB
20	100 aA	52 bB
Mean	100	48
CV[2] (%)	3.38	

[1] Means followed by the same lowercase letters in the columns and uppercase letters in the lines are not statistically different from each other by the Scott-Knott test (p<0.05); [2] Coefficient of Variation Percentage.

A similar result was observed by Trezzi et al. (2011), when glyphosate-resistant biotypes of *Conyza* spp. showed a reduction in dry weight after glyphosate application. A study conducted by Zobiole et al. (2012) compared the production of DM between glyphosate-resistant soybeans and conventional soybeans, and also observed lower production of DM in resistant soybeans compared to conventional soybeans. The smaller DM in resistant soybeans has been attributed to the effects of glyphosate on the amount of chlorophyll or the immobilization of Mg^{++} and Mn^{++}, which are essential to the function and production of chlorophyll in plants (Zobiole et al. 2010).

In evaluations of the photosynthesis rate, a continuous reduction was observed in the photosynthesis rate (A) in all biotypes treated with glyphosate and in the evaluations. However, Biotypes 5 and 20 showed recovery of A at 14 DAT, and Biotype 5 had the highest A in this evaluation, showing resistance to glyphosate (Table 2). Although glyphosate has specific action on EPSPs, it can directly affect plant

Table 2. The photosynthesis rate, transpiration and water use efficiency of C. sumatrensis according to days after treatment (DAT) with glyphosate (1440 g a.i. ha^{-1}), in biotypes 2, 5, 17 and 20. Passo Fundo, RS, 2012

DAT	Photosynthesis rate				Transpiration				Water use efficiency			
	Biotypes				Biotypes				Biotypes			
	2	5	17	20	2	5	17	20	2	5	17	20
	µmol CO_2 m^{-2} s^{-1}				mmol H_2O m^{-2} s^{-1}				(µmolCO_2m^{-2}s^{-1}) / (mmolH_2Om^{-2}s^{-1})			
0	18.2 aA[1]	15.4 aB	17.1 aA	14.5 aB	6.9 aA	4.9 aB	4.8 aB	5.3 aB	2.6 bA	3.2 aA	3.6 bA	2.7 cA
3	7.8 bB	10.2 bA	7.1 bB	10.1bA	4.5 bB	5.1 aA	4.1 aB	4.3 bB	1.7 bA	2.0 aA	1.8 cA	2.4 cA
7	1.7 cC	8.4 cA	2.0 eC	3.5 dB	0.2 cB	3.2 bA	0.3 bB	0.4 cB	8.5 aA	2.6 aC	6.4 aB	8.6 aB
10	2.4 cB	5.4 dA	5.4 cA	3.2 dB	0.6 cB	1.8 cA	1.4 bB	0.6 cB	3.9 bA	3.0 aA	3.8 bA	5.1 bA
14	2.3 cC	7.4 cA	3.6 dC	4.9 cB	0.8 cB	1.8 cA	0.9 bB	1.1 cB	2.9 bA	4.1 aA	3.9 bA	4.6 bA
Média	6.5	9.4	7.0	7.2	2.6	3.4	2.3	2.3	3.9	3.0	3.9	4.7
CV[2] (%)	11.52				16.91				24.87			

[1] Means followed by the same lowercase letters in the columns and uppercase letters in the lines are not statistically different from each other by the Scott-Knott test (p<0.05); [2] Coefficient of Variation Percentage.

photosynthesis, reducing the activity of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) and the synthesis of 3-phosphogliceric acid, decreasing the synthesis of chlorophyll and interfering in the organization of the photosynthetic apparatus (Ahsan et al. 2008). It also increases the cellular respiration rate according to the stress exerted (Flexas et al. 2005). Furthermore, the inhibition of EPSPs affects carbon flux in the shikimate pathway, with the consequent reduction in metabolites occurring in the photochemical step of photosynthesis, and photosynthesis is inhibited by this change in carbon metabolism in the leaves (Geiger et al. 1999).

The practical implication of the reduction in the photosynthesis rate (A) of Biotype 5 (glyphosate-resistant), in response to treatment with glyphosate, is that the competitive ability of this biotype with the crop is affected. Thus, under field conditions, it is observed that, after glyphosate application, resistant biotypes of horseweed show signs of phytotoxicity, and have lower growth than the cultivated species. Thus, the crop will have greater development, shadowing the resistant biotype and limiting its development by the lack of light. However, as can be observed in Table 2, between 10 and 14 DAA there was a reduced effect of glyphosate on A; thus, at the end of the crop cycle, when the crop loses its leaves, it is observed that weed growth resumes normally, and completes the cycle. Hence, a management practice should be adopted to control the resistant biotype at the end of the crop cycle, avoiding the dispersion and the increase in the seed bank of the resistant biotype.

Glyphosate application decreased transpiration in each biotype evaluated (Table 2). However, glyphosate-resistant Biotype 5 showed greater transpiration at 3, 7, 10 and 14 DAT (Table 2). For water use efficiency (WUE), which is the ratio between the photosynthesis rate and the transpiration rate, there was an increase at 7 DAT in Biotypes 2, 17 and 20, which were considered to be glyphosate-susceptible (Table 2). Increased WUE in susceptible biotypes can be explained by stomatal closure and consequent reduction in transpiration. However, for Biotype 5, considered to be resistant, there was no influence of glyphosate in WUE (Table 2). According to Reddy et al (2004), side effects of glyphosate, such as the production of metabolites (AMPA), may contribute to the action of the herbicide, causing phytotoxic effects affecting photosynthesis, transpiration and WUE (Zobiole et al., 2010).

There was no difference in the concentration of endogenous shikimic acid in biotypes of *C. sumatrensis* 2, 5, 17 and 20 prior to glyphosate application (0 DAT), and the mean concentrations were 188, 201, 174 and 144 µg g^{-1}, respectively (Figure 1). The concentrations were similar to the one found in *C. canadensis* by Reddy et al. (2008).

Figure 1 shows that, after glyphosate application, there was an increase in shikimic acid concentration until 7 DAT in all biotypes. However, this increase was higher at all evaluation times in biotypes 2, 17 and 20, considered to be susceptible to glyphosate, compared to Biotype 5, which is glyphosite-resistant. Shikimic acid accumulation in tissues occurs by the competitive inhibition of EPSPs by glyphosate (Bresnahan et al., 2003). Figure 2 shows the chromatograms at 7 DAT, with the absorption peaks of endogenous shikimic acid, with

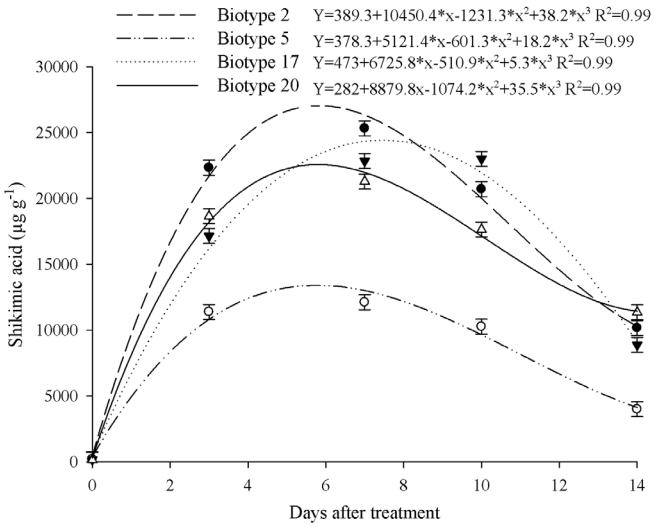

Figure 1. Shikimic acid accumulation in biotypes of *C. sumatrensis* (2, 5, 17 and 20) according to days after treatment with glyphosate (1440 g a.i. ha^{-1}). The vertical bars represent 95% confidence interval. Passo Fundo, RS, 2012

retention time of 5.0 minutes for the standard shikimic acid (A) and Biotypes 2 (B), 5 (C), 17 (A) and 20 (E) in response to treatment with glyphosate.

In Biotype 2, the peak concentration of endogenous shikimic acid was 25000 µg g^{-1}, representing a 133-fold increase over the endogenous concentration before glyphosate application (DAT 0) (Figure 1), while the increase in the endogenous concentration was 114-fold and 122-fold for biotypes 17 and 20, respectively (Figure 1). Reports in the literature show that the faster and drastic effect of glyphosate application in sensitive plants is shikimic acid accumulation (Bresnahan et al., 2003), and this has been used as a marker for the sensitivity of EPSPs to glyphosate in plants (Gonzalez-Torralva et al., 2010). Therefore, considering shikimic acid accumulation, Biotypes 2, 17 and 20 are sensitive to glyphosate.

In Biotype 2, the peak concentration of endogenous shikimic acid was 25000 µg g^{-1}, representing a 133-fold increase over the endogenous concentration before glyphosate application (DAT 0) (Figure 1), while the increase in the endogenous concentration was 114-fold and 122-fold for biotypes 17 and 20, respectively (Figure 1). Reports in the literature show that the faster and drastic effect of glyphosate application in sensitive plants is shikimic acid accumulation (Bresnahan et al., 2003), and this has been used as a marker for the sensitivity of EPSPs to glyphosate in plants (Torralva-Gonzalez et al. 2010). Therefore, considering shikimic acid accumulation, Biotypes 2, 17 and 20 are sensitive to glyphosate.

For glyphosate-resistant Biotype 5, the concentration of endogenous shikimic acid at 7 DAT with glyphosate application was 12000 µg g^{-1} (Figure 1). This result represents a 60-fold increase over the endogenous concentration without herbicide application (DAT 0) (Figure 1). Shikimic acid accumulation in the tissues of Biotype 5 (glyphosate-resistant) was unexpected, but the accumulated concentration was 2.2 times lower than the one in Biotype 2, which clearly indicates that Biotype 5 of *C. sumatrensis* has a lower level of inhibition of EPSPs and, therefore, can be considered resistant to glyphosate. There are similar results in the literature on shikimic acid accumulation

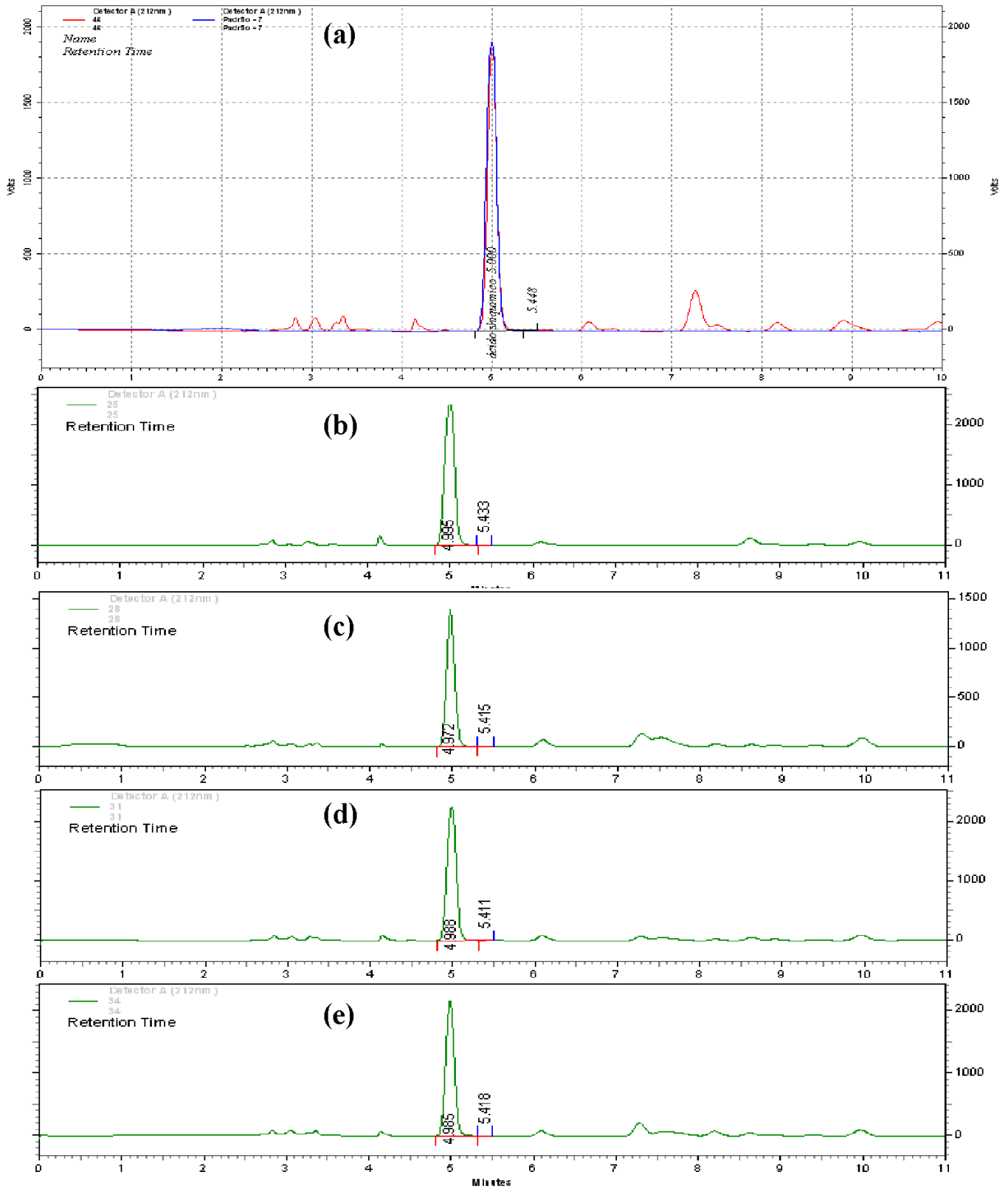

Figure 2. Chromatogram with absorption peaks of endogenous shikimic acid in the standard sample of shikimic acid (a) and in biotypes of *C. sumatrensis* 2 (b), 5 (c), 17 (b) and 20 (e), at 7 days after treatment with 1440 g a.i. ha^{-1} of glyphosate. Campinas SP, 2012

in different standards, both in glyphosate-susceptible and glyphosate-resistant biotypes (Dinelli et al., 2008; Feng et al., 2004).

As there was shikimic acid accumulation in Biotype 5, it can be inferred that the mechanism of resistance is not the result of total insensitivity of EPSPs to glyphosate. If there were total insensitivity of EPSPs to glyphosate, there would be shikimic acid accumulation, thus discarding the possibility of change in the site of action of the herbicide in Biotype 5. Among the mechanisms of weed resistance to glyphosate

reported in the literature, the study of Feng et al. (2004) can be highlighted. It suggests that this resistance is probably due to an altered cellular distribution of glyphosate, which prevents the herbicide from being loaded into the phloem and imported into the plastids, thereby resulting in a decreased translocation of the herbicide into the plant. Thus, if this mechanism of resistance is the same of Biotype 5, it can account for shikimic acid accumulation.

Another mechanism of resistance, offered by Ge et al. (2010) in *C. canadensis* and possible for Biotype 5, is reduced translocation of glyphosate, which is associated with the sequestration of the herbicide into the vacuole of the cell. The authors reported that when glyphosate reaches the cytoplasm of susceptible plants, it is directed to the phloem, and consequently distributed to the sites of action. In resistant plants, glyphosate that reaches the cytoplasm of the cells is sequestrated and taken to the vacuole, becoming unavailable for transportation by the phloem, and therefore it does not reach the target for its action. The reduced translocation was also cited as a mechanism of resistance in *C. bonariensis* by Dinelli et al. (2008) and in *C. canadensis* by Feng et al. (2004).

The mechanism of resistance of Biotype 5 may also be associated with the expression and amplification of the gene EPSPs in multiple chromosomes. Gaines et al. (2010) observed in biotypes of *Amaranthus* that the ratio of the number of copies of the EPSPs was higher in the resistant biotype compared to the susceptible one. The authors also reported that in this type of resistance, the EPSPS enzyme remains sensitive to glyphosate; what changes is that the amount of herbicide required to reduce the activity of EPSPs increases with the number of copies of gene EPSPs. Baerson et al. (2002) found evidence of gene amplification or co-segregation of the specific gene of the varying enzyme in the resistant biotype of *Lolium multiflorum*.

Finally, the metabolism of glyphosate is also a possibility. González-Torralva et al. (2012) observed differential metabolism among glyphosate-resistant and glyphosate-susceptible biotypes of *Conyza canadensis*. In these biotypes, glyphosate metabolism was faster in the resistant biotype compared to the susceptible biotype, and the herbicide was transformed into metabolites (AMPA), glyoxylate and sarcosine. This differential metabolism of glyphosate could account for the resistance of Biotype 5, but additional studies should be conducted to clarify this issue.

Conclusions

Glyphosate application reduces the growth of the biotypes evaluated. The greatest reduction was observed in Biotype 2, and the lowest, in Biotype 5. Glyphosate inhibits the photosynthesis rate an interferes in the water use efficiency of susceptible biotypes. However, glyphosate partially inhibits the photosynthesis rate and does not affect the water use efficiency of Biotype 5. However, shikimic acid accumulation in Biotype 5 indicates that the resistance mechanism is not associated with total insensitivity of EPSPs to glyphosate and/or other resistance mechanisms may be involved.

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