ANSWER OF WEED POPULATIONS AND CROPS TO GLYPHOSATE

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REZIME

Measuring amount of shikimate and chlorophyll content of hybrids of maIze, line of soybean, *C. canadensis* and *L. rigidum* populations were examined after application of 1 kg a.e. ha⁻¹ of the herbicide product TOUCHDOWN® Šactive ingredient: glyphosate trimesium salt (syn. sulfosate), 500 g L⁻¹Ć. Samples collected 2, 4 and 6 day after treatment. Changes in amount of shikimate in treated plants vs control were significant for S plants and nosignificant for R plants. Content of chlorophyll in tretaed plants were statisticaly lower vs nontreated plant in every tested poulations/lines/hybrids, except in hybrids of maIze (differences were not significant).

Key word: weeds, crops, shikimate, chlorophyll

INTRODUCTION

Glyphosate is used mainly for postemer- herbicide control of monocot and dicot weeds in glyphosate resistant crops (GR), in ruderal habitat and stubbles. After expiry of patents in the 90s, glyphosate became a common herbicide manufactured and distributed by several companies (Marsh et al., 2006). Glyphosate causes relatively few ecological and toxicological side effects (Giesy et al., 2000). It binds to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which consequently inhibits the biosynthesis of the aromatic amino acids – tryptophan, tyrosine and phenylalanine (Siehl, 1997, Feng et al., 2004; Dill et al., 2010). Therefore, by inhibiting shikimate pathway, synthesis of aromatic amino acids is interrupted. Applied in dosages of about 1 kg a.e. ha⁻¹ or more, glyphosate is a non-selective herbicide. It is taken up by leaves of target plants and is also translocated to plant roots, which enables a systemic activity. Herbicide resistant weeds are having a major impact on world agriculture. Alternatively, ineffectiveness of glyphosate may be due to natural tolerance of larger plants (Wu et al., 2008) and/or environmental conditions (Somervaille, 2004).

This paper reports on the evaluation of resistance of Soybean lines, Maize hybrids, *Conyza canadensis* and *Lolium rigidum* populations by shikimate and chlorophyll content methods.

MATERIAL AND METHODS

Experiment was conducted under controlled environmental conditions (temp. 22.8/10.5°C day/night, 54.6% RH, 12:12h period) at the University of Pretoria. Seeds of soybean (GRS, SS), maize (GRM, SM), *C. canadensis* (CC) and *L. rigidum* (LR, LPR, LS) were planted in 1 L pots and placed in greenhouse. Watered every second day and fertilized as needed. Resistant seed of *L. rigidum* (LR) was provided by the Institute for Plant Production, Western Cape, SA. Susceptible (LS) and presumably resistant (LPR) seeds were collected in cereals near the Western Cape. Plants were treated with 1 kg a.e ha^{-1} TOUCHDOWN® Šactive substance: glyphosate trimesium salt, 500 g L^{-1}Ć. Treatments were performed 4 weeks after planting using the indor hand sprayer equipped with an RS-MM 110°/04 nozzles and applying 300 L water per ha at 276 kPa. The experimental design was a full randomization. Samples were collected from sprayed and unsprayed plants, in both varieties 2, 4 and 6 DAT (days after treatment).

Extraction of Shikimate: Plant material was ground using mortar and pestle in liquid nitrogen. About 1.5 g of grounded material was mixed with 10 ml 1M HCl and shaked for 24 h. pH was adjusted with 1M NaOH and 0.1M NaOH to pH = 3.0 - 3.5. Analysis of Shikimate (HPLC) was performed using a method of Mueller et al. (2003). Material extracted as described above was then centrifuged at 15.000 g for 5 min to remove any particulate matter. An aliquot (20 µl) of the supernatant was injected into Water HPLC (Hewlett Packard Agilent 1100 series, DAD (Diode Array Detector), Lune-NH$_2$, column diameter 5 µl, flow 1 ml min^{-1}).

Extraction of Chlorophyll: Plant material was ground using mortar and pestle in liquid nitrogen. About 0.5 g of grounded material was mixed with 5 ml methanol (in the dark) and analyzed on spectrophotometer (Beckman Coulter, DU 530, Life Science UV/VIS Spectrofotometer). Before the reading of chlorophyll content samples were centrifuged at 1500 rpm. Chlorophyll absorption was measured at wavelenghts of λ=653 i λ=666.

Statistical analysis was performed with the SigmaPlot 4.0 software.

RESULTS

A significant amount of shikimate was detected in soybean plants of SS line: on the second day 19,6x, on the fourth day 28,4x and on the sixth day 30,7x more after applying herbicide when compared to the values measured before treatment (Fig. 1). In the GRS line the amount of shikimate was close to the values measured in the control: on the second day 2,3x, on the fourth day 3,9x and on the sixth day 1,9x more than in the control. Based on this, it can be concluded that the GRS line of soybean has reacted mildly to the presence of herbicide, and that on the 6th DAT it has already recovered from the stress recorded on the second and fourth DAT (Fig. 1).

The results recorded for the treated SM maize hybrid plants show an increase in the shikimate amount: 3,2x on the second day, 3,1x on the fourth day and 1,6x on the sixth DAT. Unilke the sensitive hybrid type, glyphosate resistant maize hybrid didn't react to the 1 kg a.e. ha^{-1} herbicide application (Fig. 2).

With all tested *L. rigidum* populations (LS, LR and LPR), an increase in the amount of the shikimate two days after herbicide treatment was registered. On the fourth and sixth day of the analysis this tendency of increase continued with LS (4,8x and 5,2x) and LPR (2,4x and 2,9x) populations, while with LR population a decrease in the shikimate amount was detected (Fig. 3).

Analysis of the shikimate amount in *C.canadensis* plants showed a tendency for accumulation of shikimate in treated plants, when compared to the values measured before herbicide application (Fig. 4). However, if the 2-6 DAT trend is analysed, a tendency for a decrease in shikimate content can be registered, which could imply a tendency of plants to overcome the stress caused by herbicide treatment by methabolic degradation of shikimate, although the amount measured was still greater than the amount measured in the non-treated plants (3,8x on the second day, 7,7x on the fourth day and 2,5x more on the sixth day; Fig. 4).
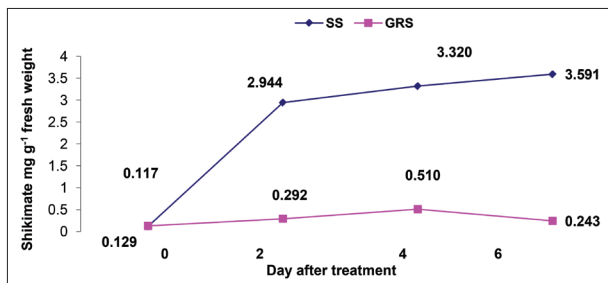
Figure 1. Shikimate content of SS and GRS lines of soybean vs day of sampling after treatment with 1 kg a.e. ha-1 glyphosate
Slika 1. Sadržaj šikiminske kiseline kod SS i GRS linija soje u odnosu na dan uzorkovanja posle primene 1 kg a.m. ha-1 glifosata
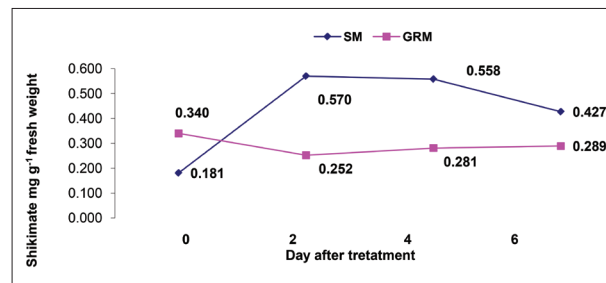

Figure 2. Shikimate content of SM and GRM hybrids of maize vs day of sampling after treatment with 1 kg a.e. ha-1 glyphosate
Slika 2. Sadržaj šikiminske kiseline kod SM i GRM hibrida kukuruza u odnosu na dan uzorkovanja posle primene 1 kg a.m. ha-1 glifosata
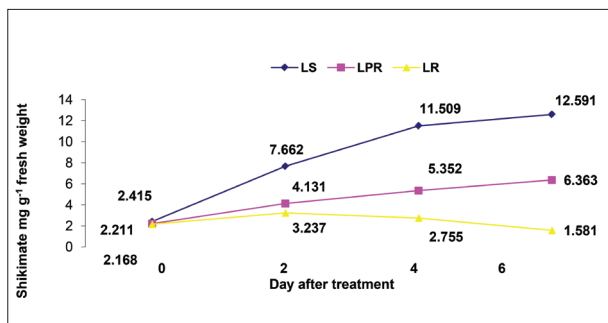

Figure 3. Shikimate content of LS, LPR and LR pop. Of L. rigidum vs day of sampling after treatment with 1 kg a.e. ha-1 glyphosate
Slika 3. Sadržaj šikiminske kiseline kod LS, LPR i LR pop. *L. rigidum* u odnosu na dan uzorkovanja posle primene 1 kg a.m. ha-1 glifosata
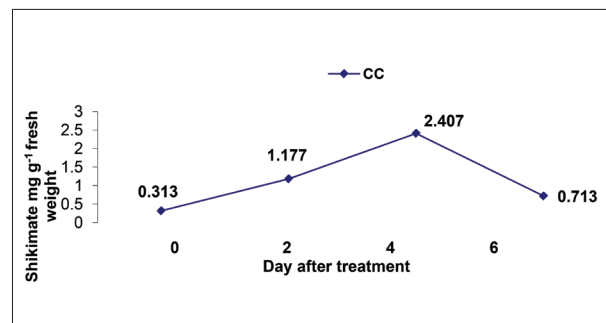

Figure 4. Shikimate content of CC pop. Of C. canadensis vs day of sampling after treatment with 1 kg a.e. ha-1 glyphosate
Slika 4. Sadržaj šikiminske kiseline kod CC pop. C. canadensis u odnosu na dan uzorkovanja posle primene 1 kg a.m. ha-1 glifosata

The chlorophyll content extracted by methanol from the treated plants was statistically significantly lower than in the non-treated plants, with all plants tested, except for maize (where this difference was not statistically significant) (Table 1). Differences between treated and control plants were somewhat more pronounced with the SS soybean line ($p<0,001$) that with the GRS line, in all measurement periods and with all chlorophyll parameters measured (except for chlorophyll *a* content, where these differences were at the $p<0,05$ and $p<0,01$ levels of significance, Table 1, Figure 5a,b).
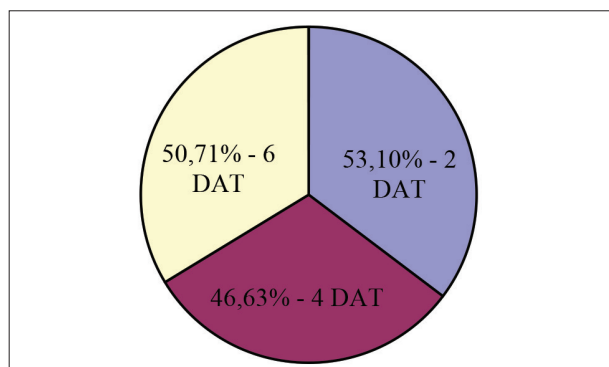
Figure 5a. Totall amount of chlorophyll vs. days after treatment, S soybean line
Slika 5a. Ukupan hlorofil vs. dani uzorkovanja posle primene, S linija soje
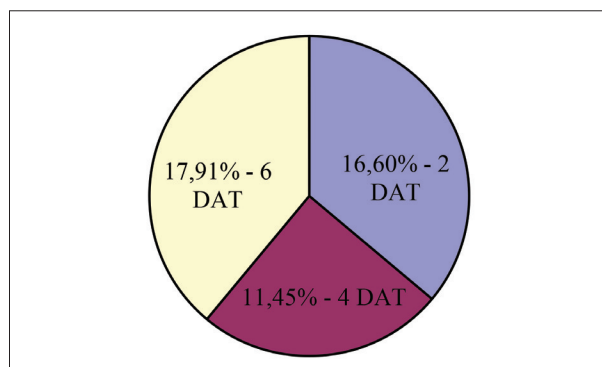

Figure 5b. Totall amount of chlorophyll vs. days after treatment, R soybean line
Slika 5b. Ukupan hlorofila vs. dani uzorkovanja posle primene, R linija soje

Table 1. Amount of chlorophyll (mg g-1 fresh weight) in control and treated plants with 1 kg a.e. ha-1 glyphosate population of weeds and crops (LSD test).
Tabela 1. Sadržaj hlorofila (mg g-1 sveže mase) u kontroli i tretiranim biljkama sa 1 kg a.m. ha-1 glifosata populacija korova i useva(LSD test).

		Average			Sataistical differences		
		Soybean	Mayze	*C.canadensis*	Soybean	Mayze	*C.canadensis*
			6 day after treatment				
			Resistant populations				
Totall chl	C	62,82±2,86	39,83±8,21	62,08±0,71	**	NS	***
	T	51,57±9,83	45,02±10,44	59,70±0,63			
Chl.a	C	22,82±0,77	24,25±1,41	23,66±0,47	**	NS	NS
	T	24,47±1,05	24,44±2,43	24,11±0,38			
Chl.b	C	40,00±3,61	15,57±7,48	38,43±0,91	**	NS	**
	T	27,18±10,64	20,58±9,27	35,70±0,89			
a:b	C	0,57±0,08	1,56±0,75	0,61±0,06	*	NS	NS
	T	0,90±0,52	1,19±0,57	0,67±0,06			
			Susceptible populations				
Totall chl	C	55,94±8,23	41,75±6,53	-	***	NS	-
	T	27,56±11,05	33,37±15,24				
Chl.a	C	24,33±1,72	24,90±2,17	-	*	**	-
	T	18,11±4,92	19,03±4,83				
Chl.b	C	31,60±9,89	16,85±4,67	-	***	NS	-
	T	9,46±6,29	14,34±11,32				
a:b	C	0,77±0,39	1,48±0,31	-	***	NS	-
	T	1,91±0,58	1,33±0,57				

p<0,001 ***; p<0,01**; p<0,05*; NS - not statistically significant

By analysing the results obtained for total chlorophyll content (Figure 6a,b), chlorophyll *a* and chlorophyll *b* content and chlorophyll *a:b* ratio, in both maize hybrids, no statistically significant differences were confirmed for the values registered before and after herbicide treatment, with the exception of GRM hybid 2 DAT for chlorophyll *a* content (p<0,01) and SM hybrid 2 and 6 DAT, also for chlorophyll *a* content (p<0,05, p<0,01) (Table 1).
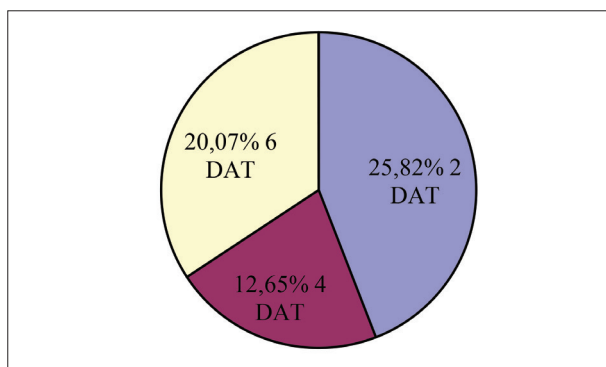
Figure 6a. Totall amount of chlorophyll vs. days after treatment, S maize hybrid
Figure 6a. Ukupan hlorofil vs. dani uzrkovanja posle tretmana, S hibrid kukuruza
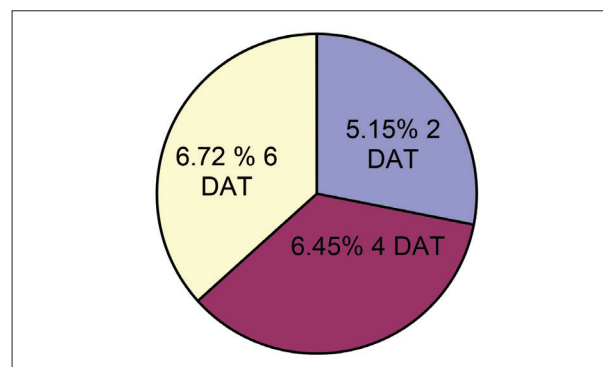

Figure 6b. Totall amount of chlorophyll vs. days after treatment, R maize hybrid
Figura 6b. Ukupan hlorofil vs. Dani uzorkovanja posle tretmana, R hibrid kukuruza

Based on the results obtained and data analysed for *C. canadensis* population, it was determined that the results are statistically significantly different or highly different, for nearly all the chlorophyll parameters analysed in treated plants, when compared to the values measured before 1 kg a.e. ha^{-1} glyphosate application (p<0,05, p<0,01, p<0,001), except for 6 DAT for chlorophyll *a* content and chlorophyll *a:b* ratio (Table 1, Figure 7).

DISCUSION

Thanks to modern biotechnologies (i.e. GMO crops) examples of mixed and multi resistance of weeds have been recorded. As a result of this, nowadays in many developed and developing countries of the world the development of herbicide resistance in weeds has become a major problem, on a local, but also on a global scale.

In all tested populations, low shikimate content in nontreated plants confirmed that the amount of shikimate is not connected with the resistance and susceptibility of plants. This method is used as an early and highly sensitive indicator of the effect of glyphosate on sensitive plants (Komossa 1992). Changes in the shikimate content in treated plants vs control plants were significant for S plants and nonsignificant for R plants. Differential shikimate accumulation can be used to differentiate between glyphosate S and R plants, although it is not an indication of the mechanism responsible for such resistance (Mueller et al., 2003). HPLC determination of shikimate levels can be conducted at different growth stages of plants, and even on dead plant material (Singh and Shaner, 1998). In our experiment by shikimate method we confirmed the resistance of GR populations and resistance of LR population
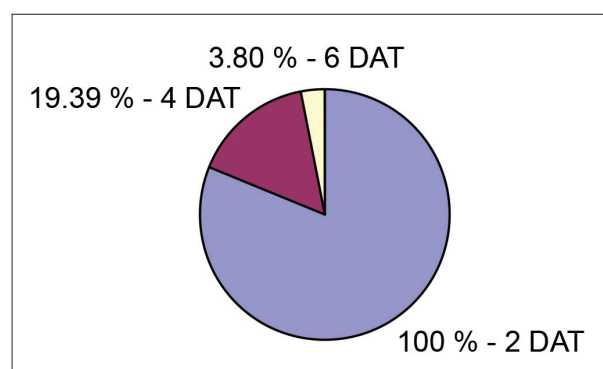

Figure 7. Totall amount of chlorophyll vs. days after treatment, C. canadensis
Figura 7. Ukupan hlorofil vs. dani uzorkovanja posle tretmana, C. canadensis

of *L. rigidum*. Shaner et al. (2005) confirmed that leaf area is posititvely correlaed with shikimate accumulation. With regards to this, they confirmed a greater amount of shikimate after glyphosate application in sunflower plant tissue, as opposed to the amount measured in wheat and *Proso millet* tissue. This fact was also confirmed in our research, where a greater amount of shikimate (2,3-3,6 mg g^{-1} fresh weight) was recorded in the sensitive line of soybean compared to the susceptible maize hybrid (0,4-0,6 mg g^{-1} fresh weight), after the same dose of glyphosate. Perez-Jones et al. (2007) have come to similar results when testing *Lolium multiflorum* for glypfosat resistence. Shikimate amount was 2-3x greater in the leaves of sensitive *L. multiflorum* population, when compared to the amount measured in the resistent population. Also, Michitte et al. (2007) have noticed the same regularity as did we in our research, by registering the increase in shikimate content over time in the susceptible populations of *L.*

multiflorum, and more or less unchanged amount of shikimate in the R populations of *L. multiflorum* after glyphosate treatment. The shikimate amount was 8x greater in the LS than in the LR population on the last day of analysis (6 DAT). Certain changes in the shikimate content in the LR population can be explained as the effect of stress in the presence of herbicides, but the plants have very quickly overcome this state of stress. Baerson et al. (2002) have explained the differences between the S and R populations of *L. rigidum* as a result of increased EPSPS enzyme activity, which was 2-3x greater in treated than in control plants. High initial levels of shikimate measured in R population (10x greater than in the control) can be explained by stronger plant activity, which enables it to overcome the state of stress caused by glyphosate treatment. Our results for populations of *C. canadensis* are consistent with the results of Mueller et al. (2003). They compared R and S populations of *C. canadensis* and observed an increasing amount of shikimate time consuming after glyphosate treatment (2-4 DAT) when compared with control. However, in the analysis of shikimate trend in 2-6 DAT period (Fig. 4), a slight tendency for a decrease in its amount can be observed (2 DAT: 3,8x greater, 4 DAT: 7,7x greater and 6 DAT: 2,5x greater), compared to the values measured before herbicide application. Based on this, it can be presumed that the plant activates certain enzymes and tries to overcome the state of stress, even though the amounts are still high, compared to the control.

Chlorophyll content extracted by methanol from the treated plants was statistically significantly lower than in the non-treated plants, with all the plants tested, except for maize (where these differences were not statistically significant) (Table 1). In their research Reddy et al. (2004) came to the conclusion that a greater amount of glyphosate (1,12 – 13,4 kg ha^{-1}) causes a milder inhibition of chlorophyll content in soybean plants (12% after 7 DAT), and a mild reduction in fresh matter (8% after 14 DAT). Based on these research, it can be concluded that the environmental conditions can also influence the chlorophyll content after glyphosat application. With regard to this matter, Pline et al. (1999) have confirmed that high temperatres cause significant damage to plants of the glyphosat resistant soybean line, and a significant reduction of chlorophyll content in high temperatures (35°C) when compared to the changes measured on 15^{0}C.

Research conducted and the results obtained have shown that, based on the analysis of shikimate content, differences in the sensitivity of plants to glyphosate can be easily registered and resistance to it can be proved. Measurments of chlorophyll content have also proved to be a good method for registering the presence of glyphosate, but not for differentiating the sensitivity of R and S plants and proving the plant's resistence.

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ODGOVOR KOROVSKIH POPULACIJA I GAJENIH USEVA NA PRISUSTVO GLIFOSATA

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REZIME

IMerenje sadržaja šikiminske kiseline i hlorofila kod hibrida kukuruza, linija soje, populacija *C. canadensis* and *L. rigidum* je obavljeno nakon primene 2 kg a.m. ha^{-1} herbicida TOU-CHDOWN® Šaktivna materija: glifosat trimezijum so (sin. sulfosat), 500 g L^{-1}Ć. Uzorkovanje je urađeno 2, 4 i 6 dana posle primene. Promene sadržaja šikiminske kiseline kod tretiranih biljaka u odnosu na ne tretirane su bile značajne kod svih S biljaka i nisu imale značaja kod R biljaka. Sadržaj hlorofila kod tretiranih biljaka je statistički bio niži u odnosu na sadržaj kod ne teretiranih biljaka kod svih testiranih populacija/linija osim kod hibrida kukuruza (razlike nisu bile statistički značajne).

Ključne reči: korovi, usevi, sikiminska kiselina, hlorofil