INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
12–1206/2013/15–6–1091–1097
http://www.fspublishers.org

Full Length Article

Determination of *Conyza canadensis* Levels of Sensitivity to Glyphosate Trimesium Sulphosate

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Abstract

Changes in leaf anatomy were examined in two S. African populations of *Conyza canadensis* of which one was presumed to be resistant (CCPR) and the other susceptible (CCS) to glyphosate. Glyphosate was applied to plants, which were grown from seed collected from these populations, at rates of 1, 2 and 4 kg a.e. ha^{-1} of TOUCHDOWN® [active substance: glyphosate trimesium salt, 500 g L^{-1}] that are equivalent to 2, 4 and 8 L ha^{-1} of the herbicide Touchdown. Leaf samples for the light microscope (LM) analysis were collected 3, 7 and 24 h after treatment (HAT). Changes in chlorophyll and shikimate content of leaf material were also examined. Changes in the palisade and pith tissue of leaves were not detected in the investigated populations at 3 and 7 HAT. However, at 24 HAT the different herbicide doses caused changes in leaf anatomy. These changes (injuries) were detected in the CCS at all tested doses, but in the CCPR population of *C. canadensis* the injuries were observed at only the two highest rates, 2 and 4 kg a.e. ha^{-1}. Chlorophyll and shikimate contents indicated significant differences between the treated and untreated plants of susceptible population only. Difference in glyphosate resistance between the CCS and CCPR populations was confirmed with an index of resistance of 1.58. This value of the index of resistance indicates that CCS population is 1.58 times more susceptible to glyphosate compared to CCPR population.

Keywords: Chlorophyll; Horseweed; Leaf anatomy; Shikimate; Glyphosate tolerance

Introduction

Conyza canadensis (L.) Cronq. (ERICA) (syn. *Erigeron canadensis* L.; family: Asteraceae; common name: horseweed, commonly occurs as a weed in grain crops, orchards, vineyards, pastures, abandoned fields, roadsides, railroads, stream banks and urban areas (Koger *et al*., 2004). *Conyza* spp. typically inhabits dry places and is mostly an urban and agricultural weed of highly disturbed and often compacted soils throughout the North Temperate Zone, with a more moderate distribution in the South Temperate Zone. *Conyza* spp. Occur as noxious weeds in more than 40 crops in 70 countries (Holm *et al*., 1996). Some quantities of allergenic pollen are produced by the plant causing severe hay fever symptoms in human population. *Conyza* spp. reproduce by seed, and one plant under non-competitive conditions can produce around 65 000 seeds (Vrbnicanin *et al*., 2004). Achene dispersal occurs with wind, soil movement, water and human activities. This weed species is not easily controlled by many herbicides and is therefore among the top 10 weed species to develop resistance to herbicides (Heap, 2012). The question often is how it can be most effectively controlled. Glyphosate is the preferred herbicide for vegetation control prior to planting, and after emergence of glyphosate-resistant crops, where it has provided effective broad-spectrum post-emergence control of annual and perennial broadleaf weed species and grasses for over 20 years (Koger *et al*., 2004).

Glyphosate, as an inhibitor of metabolims, inhibits the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSP), which is necessary for the formation of the aromatic amino acids tyrosine, tryptophan, and phenylalanine. These amino acids are important in the synthesis of proteins that link primary and secondary metabolism (Carlisle and Trevors, 1988).

This study was conducted to confirm glyphosate trimesium sulphosate sensitivity/ resistance in two separate populations of *C. canadensis* in South Africa, where for about three decades other glyphosate salt formulations had been used at high rates and high frequency in orchards in particular. The objective of the present study was to determine the extent of induced reduction in biomass, the contents of chlorophyll and shikimate, and leaf anatomical changes caused by a glyphosate trimesium sulphosate formulation in two *C. canadensis* populations of which one was presumed to be resistant to glyphosate and the other susceptible.

To cite this paper: Pavlovic, D., C.F. Reinhardt, D. Bozic and S. Vrbnicanin, 2013. Determination of *Conyza canadensis* levels of sensitivity to glyphosate trimesium sulphosate. *Int. J. Agric. Biol*., 15: 1091–1097

Materials and Methods

Experiments were conducted in a greenhouse and a laboratory at the University of Pretoria, Pretoria, South Africa. Horseweed seeds were accessed from orchards in Pretoria. The two populations are henceforth referred to as *C. canadensis* presumably susceptible population (CCS) and *C. canadensis* presumably resistant population (CCPR). For the dose-response experiment six seeds were planted 0.5 cm deep in 1 L plastic pots in a natural soil from the university's experimental farm. Key properties of soil were: hutton form, sandy clay loam (23% clay), 0.6% carbon, pH_{water} 6.5. At the cotyledon stage, plants were thinned to three per pot. Plants were grown in a greenhouse and maintained at a mean maximum/minimum temperature regime of 22.8/10.5°C (day/night), 54.6% RH, and 12:12 h light/dark period. Plants were irrigated on a daily basis in such a way that water stress was avoided by maintaining soil moisture at near field capacity level (15% v/m basis), and fertilized with a complete nutrient solution by applying 200 mL nutrient solution to each pot every 15 days.

Morpho-anatomical Study

For assessment of morpho-anatomical changes Touchdown Forte HighTech® (500 g a.e. L^{-1} glyphosate as the trimesium sulphosate salt) from Syngenta was applied at rates of 0, 0.5, 1 and 2 kg a.e. ha^{-1} (equivalent product rates: 0, 1, 2 and 4 L ha^{-1}) when CCS and CCPR plants had reached a height of about 15 cm. This factorial experiment comprised two *C. canadensis* biotypes and three glyphosate rates that were arranged in a completely randomized design in which each treatment combination was replicated five times. The experiment was repeated in 2007. Samples were collected 3, 7 and 24 h after treatment (HAT). Segments (4 mm^2) of treated and non-treated leaves were kept in fixsative (2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.4) until preparation for transmision electron microscopy (TEM) and light microscopy (LM) was done according to Glauret (1975) and Coetzee and Van der Merwe (2007). Samples were rinsed three times for 10 min each time in 0.075 M phosphate buffer and fixed in 0.5% water solution of osmium tetraoxide for 2 h, followed by rinsing three times for 10 min each with distilled water. Samples were then dehidrated in increasing concentrations of ethanol: 30%, 50%, 70%, 90%, 3 x 100% for 30 min at each grade. Subsequently, samples were first infiltrated in 50% quetol (1 h) and then in 100% quetol (1 h), and polimerized at 60°C for 39 h in a special mould (Fig. 1). For LM sections of 0.5-1 µm thickness were stained with toluidine blue in 1% tap water. Samples were examined under a light microscope (Nikon Optiphod-Nikon Instech Co., Kanagawa, Japan).

Shikimate Analysis

The method described by Mueller *et al.* (2003) was used to determine the shikimate accumulation in CCS and CCPR plants at 2, 4 and 6 days after treatment (DAT).

Fig. 1: Mould with samples: pieces of leaf

Fig. 2: Dose response test

A single treatment of 1 kg a.e. L^{-1} glyphosate in the form of the same formulation described above was applied to obtained the following number of samples per biotype: 3 plants x 5 replications x 3 times repeated. Plants were harvested by cutting stems at ground level, followed by weighing, freezing and storage at -20°C until samples were prepared for analysis. An amount of 1.5 g of finely grounded material per sample were powdered with liquid nitrogen, mixed with 10 mL 1 M HCl, and shaken 24 h for extraction of shikimate. The pH was adjusted with 1 M NaOH and 0.1 M NaOH to 3.0-3.5. The homogenates were filtered through a layer of cheese cloth (0.45 µm pore), and the supernatant kept in a refrigirator at 4°C until analysis. Shikimate content was determined using High Performance Liquid Chromatography (Hewlett Packard Agilent 1100 series, DAD (Diode Array Detector), Luna-NH_2 column diameter 5 µm, flow 1 mL min^{-1}). The retention time of shikimate was approximately 7 min.

Chlorophyll Analyses

Chorophyll content of intact leaves of CCS and CCPR plants treated as described above were measured 2, 4 and 6 days DAT using a Minolta SPAD 502 chlorophyll meter (Konica Minolta Sensing, Inc., Osaka, Japan). Two measurements were taken between the midrib and margin of a single leaf were taken and averaged. After each SPAD reading leaves were collected for chlorophyll extraction with methanol as described by Wellburn (1994).

Leaf tissue (0.5 g) per sample was powdered in liquid nitrogen under low light condition and extraction was done with 5 mL 80% methanol per sample. Samples were stored

in a refrigirator at 4°C until analysis. Before taking a reading samples were centrifuged at 2 000 rpm for 5 min. Total chlorophyll content, chlorophyll *a* and *b* were determined with a spectrophotometer (Beckman DU 530, Coluter Life Science UV/VIS Spectrophotometer), as described by Wellburn (1994).

Dose-response Test

Plants of both *C. canadensis* biotypes were treated with eight doses of glyphosate: 0.5, 1, 2, 3, 4, 5, 6 and 7 kg a.e. ha^{-1} through application of: 1, 2, 4, 6, 8, 10, 12 and 14 L ha^{-1} Touchdown®. The control was not treated with the herbicide. Treatments were made 42 days after seeds were sown in soil contained in pots. Herbicide application was done with a hand-held Oxford small-plot sprayer equipped with an RS-MM 110°/04 nozzle, which applied 300 L water per ha at 276 kPa. Plants were harvested 17 DAT, and oven-dried (at 65°C) for 48 h to determine dry weight. Three replicates of each treatment were arranged according to a completely randomized design, and the experiment was repeated once.

Statistical Analyses

Statistical analysis was performed with Sigma Plot V. 4.0 software. Results of the microscopy study were assessed visually. Dose-response curves were fitted according to the non-linear regression model that accounts for hormetic effects as proposed by Brain and Cousens (1989) and Schabenberger *et al*. (1999). Index of resistance (IR) was calculated as LD_{50} resistant population relative to LD_{50} of susceptible populations.

Results

Morpho-anatomical Study

Effects of glyphosate on leaf anatomy of CCPR and CCS populations were distinct. Cross section of untreated leaves clearly showed differentiation in palisade and pith tissue, and normal vascular bundles and plate cells of the adaxial and abaxial epidermis (Fig. 3A and 3E). Differentiation were not seen in mesophyll at 3 and 7 HAT. Changes in leaf tissue became apparent 24 h after glyphosate application in both biotypes. In the CCS population the lowest dose of 0.5 kg a.e. ha^{-1} caused significant changes on morpho-anatomical level (Fig. 3B) and no effects on the leaf tissue of the CCPR population (Fig. 3F). Cells of palisade and pith tissue appeared disoriented – cells lost their shape, walls of cells were twisted and rolled, the number of chlorophyll grains was reduced, and there was citolysis of some pith cells. However, changes in the adaxial and abaxial epidermis were not definitive.

Dose of 1 kg a.e. ha^{-1} dose caused more or less the same changes as the lowest dose in both populations, but effects were generally stronger (Fig. 3C and 3G).

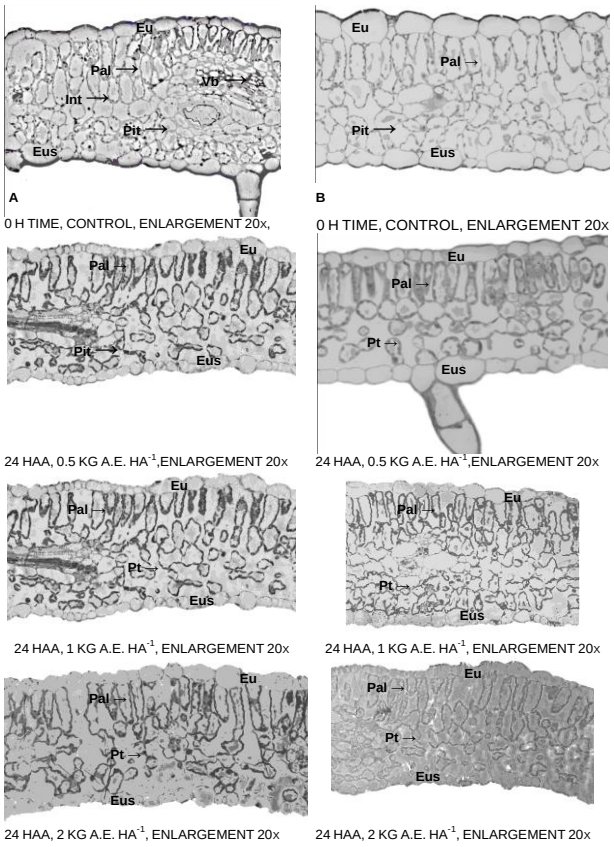

Fig. 3: Light micrographs of cross sections of leaves of the ccs (a, b, c and d) and ccpr (e, f, g and h) populations at various times after treatment with different rates of a commercial glyphosate formulation at half leaf stage of growth showing effects on upper (ue) and lower (use) epidermal cells, palisade (pal), and pith (pt) tissue; vb-vascular bound, int-intercelular spaces

The highest dose of glyphosate (2 kg a.e. ha^{-1}) almost completely destroyed leaf tissue in the both populations (Fig. 3D and 3H). Most notable effects were cytolysis and total disorganization of the arrangement of mesophyll cells. Some cell walls disintegrated and cells appeared fused, with total loss of cell integrity and function. In these instances, big intercellular spaces appeared. The same or significant levels of injury, at the highest dose of the herbicide, in leaf tissue of CCPR and CCS populations were confirmed by the images taken by TEM (Fig. 4A and 4C). The critical cellular components of untreated leaves of *C. canadensis* appeared normal (Fig. 4B). Most damage to palisade cells occured in leaf tissue of the CCS population, where the greatest damage is revealed in dead palisade cells (Fig. 4C).

Shikimate Content

Accumulation of shikimate due to glyphosate treatment was confirmed with the HPLC analysis of the leaf extracts

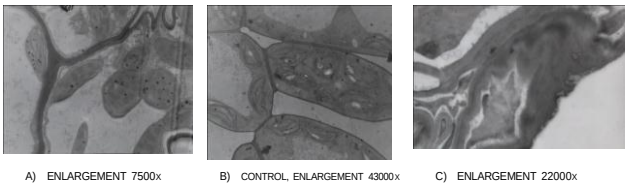

Fig. 4: Transmision electron micrographs of cross sections of leaves of the ccpr (a), control (b) and ccs (c) populations at after treatment with 1 kg a.e. Ha^{-1} of a commercial glyphosate formulation: chl-chloroplast, cw-cell wall, chlg-chlorophyll grains, pl-plasmalema and ins-intercelular spaces

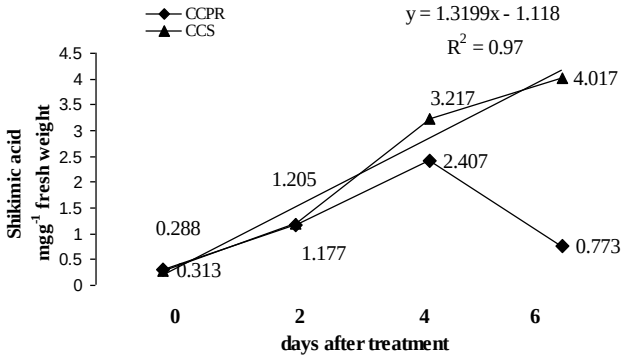

Fig. 5: Shikimate content of ccs and ccpr populations of *c. Canadensis* vs dose of glyphosate

(Graph 1). In response to 1 kg a.e. ha^{-1} glyphosate the amount of sikimate in both populations of *C.* canadensis tended to increase in treated plants and not in untreated plants (Fig. 5). At 6 DAT the concentration of shikimate reached 4.017 mg g^{-1} fresh weight in CCS plants and 0.773 mg g^{-1} fresh weight in CCPR plants. The amount of shikimate in the CCPR population had a decreasing trend in the period 4-6 DAT compared to the CCS population. Despite a decreasing trend in shikimate concentration in CCPR plants, the estimated level of shikimate is still higher than in untreated plants. In CCS plants the increase of shikimate was 3.8 times (2 DAT), 7.7 times (4 DAT) and 2.5 times (6 DAT) that of the pre-treatment levels in this population.

Chlorophyll Content

In both the CCPR and CCS populations, at 1 kg a.e. ha^{-1} glyphosate, significant differences ($p < 0.05$, $p < 0.01$, $p<0.001$) were observed in the amount of chlorophyll in treated plants compared to those levels that were measured prior to treatment with glyphosate levels. In the CCPR population, at 6 DAT, this tedency was not seen only in chlorophyll *a* and for the chlorophyll *a:b* in CCPR population (Fig. 6). The total amount of chlorophyll in CCS plants were significantly lower than in CCPR plants. The total amount of chlorophyll in the CCPR population showed a slight decrease after application of 1 kg a.e. ha^{-1} glyphosate (b = -2.155) (Fig. 6A). Across the measurement intervals (2, 4 and 6 DAT) the amount of chlorophyll *a* increased slightly in CCS population, which was confirmed by the coefficient 'b' from regression equation (b = 0.524) and low coefficient of correlation ($R^2 = 0.18$) (Fig. 6B).

In contrast to chlorophyll *a*, the amount of chlorophyll *b* changed after the application of 1 kg a.e. ha^{-1} of glyphosate (Fig. 6C) in both tested populations. But, amount of chlorophyll *b* decreased strongly (b=-8,509) in CCS pop. vs CCPR pop. (b = -2,679). Similarly to the changes of amount of chlorophyll *a* and *b*, we also determined a slight increase of chlorophyll *a:b* ratio in both tested populations (b = 0.073 of CCPR, b = 0.106 of CCS pop., Fig. 6D).

Amounts of chlorophyll that were measured with non-destructive method (SPAD meter) showed no significant differences in relative amount of chlorophyll between values estimated before and after the application of glyphosate, except at 2 DAT in the CCPR population ($p < 0.01$). SPAD readings of the CCPR population at 2 DAT were significantly lower than readings taken before the application of glyphosate. Over time those values increased, and at 6 DAT they were the highest compared to measurements taken prior to herbicide application. This finding indicated recovery of the CCPR population after glyphosate application. Based on the values obtained for relative amount of chlorophyll (SPAD meter) there appeared no correlation between these values and quantification of chlorophyll with methanol extraction.

Dose-response Test

Differences in response of the CCS and CCPR populations to glyphosate trimesium sulphosate were observed in dry weight data. Dose response test were showed 1.58 times susceptibility of CCS population (Table 2; Fig. 7, Fig. 2) vs CCPR population on applied dose. Non-linear regression curves determined the lethal dose of glyphosate of the CCS population to be 0.280 kg a.e. ha^{-1}, and that of the CCPR population as 0.442 kg a.e. ha^{-1}. Visual estimation revealed injury at 17 DAA on both populations of *C. canadensis*. A 20% of CCS plants were killed by 1 kg a.e. ha^{-1}glyphosate whereas 20% plants of the CCPR population were killed by 4 kg a.e. ha^{-1} (Fig. 2). Based on the visual injury evaluation system of Frans (1972) 0% corresponds to absence of visible foliar and stem symptoms and 100% to death of plants.

Discussion

Certain species of *Conyza* genus are relatively tolerant to herbicides and can easily develop resistance to some herbicides (Buhler *et al.*, 1997; Owen and Zelaya, 2005). The *C. canadensis* population, which we used to confirm, susceptibility to glyphosate of a population, did not demonstrate tolerance to this herbicide.

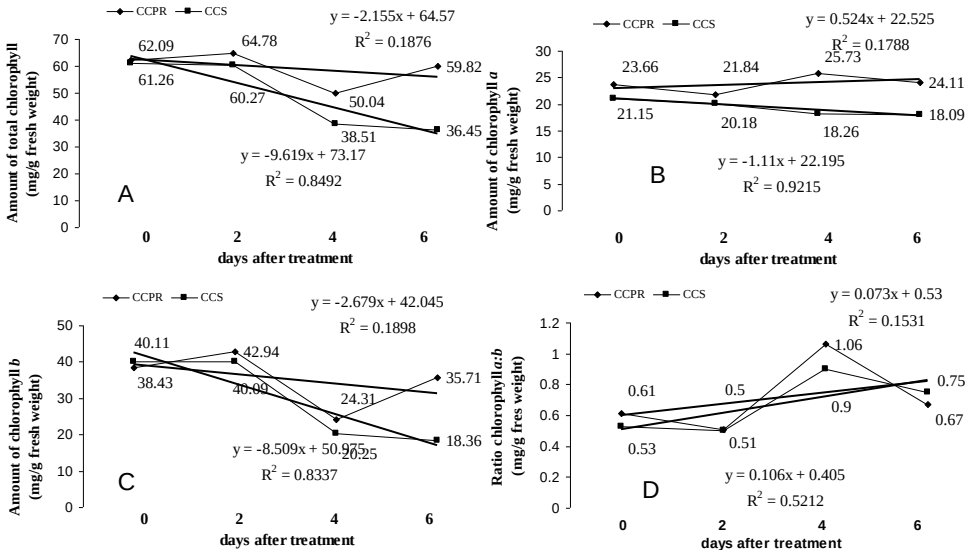

Fig. 6: Changes amount of chlorophyll in treated plants ccpr and ccs pop. 0, 2, 4 and 6 dat 1 kg a.e. Ha^{-1} of glyphosate: (a) amount of total chlorophyll, (b) amount of chlorophyll *a*, (c) amount of chlorophyll *b* and (d) ratio of chlorophyll *a:b*

Changes in anatomy of leaves after herbicide treatment were not confirmed at 3 and 7 HAT, which indicated that was sufficient time for herbicide uptake and translocation to the target site (Feng *et al*., 1998). In relation to this, we assumed that the hairiness of *C. canadensis* leaves could impair uptake of the herbicide (Gutschick, 1999). Preliminary tests with *Conyza* spp confirmed that the first symptom of injury, after application of low glyphosate dose occurred at 8 HAT, but at the high dose injury could be seen at 6 HAT. Based on this result it can be concluded that a sensitive *C. canadensis* population could be affected by glyphosate within 7 HAT. In their work, Feng *et al*. (1998) concluded that glyphosate uptake occurred at different rates depending on the formulation and was rapid during the first 6 to 8 HAT. Lorentz *et al*. (2011) found that changes in phloem of different weed species became visible after 3 days.

Analysis of leaves in cross section (LM), after treatment with lowest dose of the herbicide (0.5 kg a.e. ha^{-1}), confirmed the susceptibility of the CCS population. The lowest dose caused deformations of the cell walls, changes in chloroplasts and a reduction in number of chlorophyll grains (Fig. 3B and 3D), while the highest dose caused few, but pronounced symptoms. However, there were no clear anatomical changes in the CCPR population after the treatment of 1 L ha^{-1} of the herbicide and this was confirmed with a low index of resistance $IR_{CCPR:CCS}$ = 1.58. Visual estimations did not show clear differences between CCS and CCPR populations after treatment with low doses of glyphosate. Clear pictures were gotten after a treatment with the highest doses of herbicide. Plants of the CCS population were 90% destroyed after the application of 1 kg a.e. ha^{-1} glyphosate, and in plants of the CCPR population after the application of 4 kg a.e. ha^{-1} of the herbicide. Nonlinear regression confirmed a lethal dose of glyphosate for the CCS population to be 0.280 kg a.e. ha^{-1}, and 0.442 kg a.e. ha^{-1} for the CCPR population. Because there were minor differences in susceptibility between the tested populations (IR = 1.58) it can be concluded that the CCPR population was intrinsically resistant to glyphosate. Other research has shown variations in levels of lethal doses depending on the plant growth stage (Dinelli *et al*., 2006). Values for both tested populations at 2-leaf growth stage ranged from 0.110 to 0.140 kg a.e. ha^{-1}, while in the rosette growth stage the values ranged from 1.36 to 1.61 kg a.e. ha^{-1} for the resistant biotype, and 0.340 - 0.530 kg a.e. ha^{-1} for the susceptible biotype. The index of resistance 4-4.7 indicated 5 times greater susceptibility of S vs R populations to glyphosate.

Changes in the amount of shikimate in plants of *C. canadensis* after treatment with 1 kg a.e. ha^{-1} of glyphosate were correlated to determined changes on morpho-anatomical characters and the amount of chlorophyll. It should be pointed out that when compared to other research with species of *Conyza*, we came to interesting conclusions. In *C. bonariensis* the level of shikimic acid was below the detection limit (also in control plants) and for that reason we did not accept this method as a tool for determination of R vs S plants of *C. bonariensis* (results not shown). We considered sufficiently definitive the morpho-anatomical effects (SM, TEM images) after treatment with the lowest dose of glyphosate (0.5 kg a.e. ha^{-1}). Contrary to the CCPR populations, the susceptible *C. canadensis* population the amount of shikimate increased at 2, 4 and 6 DAT and the values for CCPR populations were variable (Fig. 5). Amount of sikimate in CCPR population showed a slight decrease at 2 DAT (3.8 times), 4 DAT (7.7 times) and 6 DAT (2.5 times) vs values estimated before the treatment.

Table 1: amount of the total chlorophyll based on extraction by methanol and by spad meter readings

	Spad: total chlorophyll	
Days after treatment	Ccpr	Ccs
0	**	**
2	**	**
4	Ns	**
6	*	Ns

*p < 0.05, **p < 0.01, ns – non significant, ccpr - presumable resistant pop., ccs – susceptible pop., 0 – before treatment, 2, 4 and 6 dat

Table 2: ld_{50} and ir for glyphosate based on dry weight of *c. Canadensis* populations

Populations	Ld_{50}	Ci	Ir
	Kg a.e. Ha^{-1}		
Ccpr	0.442	0.195	
Ccs	0.280	0.127	1.58

Ir – index of resistance, ci – interval of confidence, ccpr – presumable resistant; pop., ccs – susceptible pop

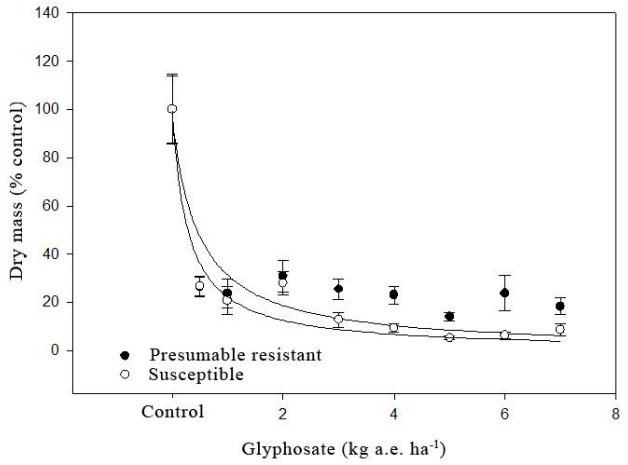

Fig. 7: Dray mass of ccs and ccpr populations of *c. Canadensis* vs dose of glyphosate

Based on this we assume that the plant processes were activated by enzymes and tried to recover from the stressful situation despite the highest amount levels of shikimate vs control plants. This response in the CCS population may be related to the disrupted metabolism of plants, which are trying to overcome the stressed state induced by glyphosate (Mannlerof *et al*., 1997). Mueller *et al*. (2003) compared R and S populations of *C. canadensis* and found an increasing amount of shikimate in treated plants vs untreated plants during the estimation period 2-4 DAT in susceptible population. However, we should take in consideration the fact that shikimate in plant tissue is available only for a certain time period after glyphosate treatment (Shaner *et al*., 2005; Henry *et al*., 2007). Also, Lorentz *et al.* (2011) indicated that after 4 days shikimate accumulation was highest in stem tips and decreased towards the base of the plant. The necrosis of pith cells started at the same time. There are numerous theories about the mechanism of resistance of *C. canadensis* (Mannlerof *et al*., 1997; Bourque *et al*., 2002; Mueller *et al*., 2003; Dinelli *et al*., 2006) to glyphosate. Some of them state that the R plants can contain an insensitive type of EPSPS enzyme, and have significant accumulation of shikimate in plant tissue. This theory is based on the fact that accumulated shikimic acid binds with the alternative type of EPSPS, which discharges. Also there is on the possibility that other enzymes e.g. glyphosate oxidase/reductase (GOX enzymes), can decompose herbicides like glyphosate (Mannlerof *et al.*, 1997). Warwick and Black (1994) noted that the investigation of resistance has to be considered on a case to case basis, according to the plant species, and according to the circumstances when resistance is developing, and that it can be confirmed only when there are significant differences between tested populations (Beckie *et al*., 1990).

Changes in cell construction after the treatment with glyphosate affected the amount of chlorophyll. Amount of total chlorophyll and chlorophyll *b* in the CCPR population decreased over time after treatment with 1 kg a.e. ha^{-1} of Touchdown (Fig. 6A and 6C). This tendency caused the increase in chlorophyll *a:b* ratio of the CCPR population. Muñoz-Rueda *et al*. (1986) showed similar results in alfalfa and shamrock. During the first 24 HAT the amount of total chlorophyll and chlorophyll a were reduced and 7 DAT the amount of chlorophyll *b* significantly reduced. The amount of chlorophyll varied over time showing that changes in relative amount of chlorophyll depended on plant responses to stress (Morales *et al*., 2002) and also on different factors, e.g. temperature, sun light, relative humidity, genotype, herbicide application etc., (Anderson *et al*., 1993). Measuring the relative amount of chlorophyll with the SPAD meter is an acceptable indirect parameter of plants response to stress (Pavlovic, 2005; Pavlovic *et al*., 2006; Bozic *et al*., 2007).

In conclusion, glyphosate at 0.5, 1 and 2 kg a.e. ha^{-1} (half, recommended and double rate) caused damages to *C. canadensis* susceptible population and confirmed initial level of resistance of *C. canadensis* presumable resistant population to glyphosate. Observations in our experiments are consistent with the accepted mechanism of action (EPSPS inhibition) of glyphosate. We conclude that these assays can be used for rapid detection of injury due to glyphosate and resistance to glyphosate.

Acknowledgements

We thank the Ministry of Education and Science of the Republic of Serbia Project III 46008 and Syngenta company, S. Africa for providing funding for this study.

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(Received 10 Novemner 2012; Accepted 08 April 2013)