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Common Cocklebur (*Xanthium strumarium*) Response to Nicosulfuron

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Abstract

The response of two populations (CC1, 43.59°N & 20.40°E; CC2, 44.46°N & 20.17°E) of common cocklebur (*Xanthium strumarium* L.) to nicosulfuron was investigated both in field experiments and in the laboratory. Population CC1 had no history of treatment with any herbicide, while population CC2 was treated with ALS inhibitor herbicides for six consecutive years. In the field, plants were treated post-emergence with nicosulfuron (0, 10, 20, 40, 60 and 80 g ai ha^{-1}) at four true leaves. Visual injury estimation and vegetative parameters (plant height, fresh weight, leaf area) were recorded about month after herbicide application. The acetolactate synthase (ALS) enzyme activity in response to herbicide concentrations of 0, 0.01, 0.1, 1, 10, 100 µM was determined *in vitro*. GR_{50} values for vegetative parameters and I_{50} values for ALS activity were slightly greater for the CC2 than for the CC1 population, but the results confirmed that neither population was susceptible to nicosulfuron. Namely, based on results for fresh weight, the population CC1 was about 3.9 and 2.6-fold more susceptible to nicosulfuron than population CC2 in two consecutive years, but differences were not so prominent for other parameters (plant height, leaf area and ALS activity), ranging from 1.18 to 1.8-fold. The differences between population CC1 and CC2 could be attributed to inter-population variability in susceptibility to nicosulfuron or could be the consequence of repeated application of ALS herbicides to the CC2 population during the six previous years. Future investigations are necessary in order to clarify this dilemma.

Keywords: ALS enzyme activity, herbicide, vegetative parameters, weed

Introduction

Nicosulfuron is a POST-applied sulfonylurea herbicide that effectively controls numerous perennial and annual grasses and some broadleaf weeds in corn (Baghestani *et al*., 2007; Nosratti *et al*., 2007). The mode of action of nicosulfuron is through the inhibition of the enzyme acetolactate synthase (ALS), which is essential for the production of branched-chain amino acids (leucine, isoleucine, and valine) needed for protein biosynthesis. This group of herbicides has become popular due to their effectiveness, relatively low use rates, and low mammalian toxicity (Schmidt *et al*., 2004). Populations of several grass and broad-leaved weed species (*Setaria viridis* (L.) Beauv., *Setaria faberi* Herrm., *Conyza albida* Willd., *Amaranthus retroflexus* L.) resistant to nicosulfuron have been found (Volenberg *et al*., 2001; Osuna and De Prado, 2003; Scarabel *et al*., 2007; Laplante *et al*., 2009).

Common cocklebur (*X. strumarium* L.) is a large-seeded annual broad-leaf weed, which has the ability to germinate under a broad range of temperatures, while requirements for maximum germination are a relatively high (25 °C) (Saric *et al*., 2012). This species is generally highly-competitive for nutrients, light and moisture and can drastically reduce yield, harvesting efficiency, and crop quality due to seed contamination (Gossett, 1971). In soybean fields, this species can reduce yields up to 80% (Bloomberg *et al*., 1982), while yield reduction in corn ranged from 30 to 40% (Karimmojeni *et al*., 2010). Nakova *et al*. (2004) confirmed the significance of this invasive species as a weed in corn across the Balkan Peninsula. This species is increasing in importance due to population expansion and the development of resistance to different types of herbicide such as MSMA (sodium hydrogen methylarsenate) (Nimbal *et al*., 1995), imazaquin (Barrentine, 1994) chlorimuron and imazethapyr (Sprague *et al*., 1997).

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In the fields beside susceptible weeds, constantly present species less susceptible or tolerant to herbicides which are used for weed control. In Serbia, *Sorghum halepense* L. (Pers.) and *X. strumarium* L. are dominant weeds in many corn fields. Previous studies have shown that nicosulfuron effectively controls *S. halepense* (Baghestani *et al*., 2007; Nosratti *et al*., 2007), even at low rates of application (Rosales Robles *et al*., 2001). Dobbels and Kapusta (1993) reported that nicosulfuron provided greater than 80% control of *Setaria faberi* Herrm. and *Abutilon theophrasti* Medik. However, Lueschen *et al*. (1992) and Dogan *et al*. (2005) showed that many broadleaf weeds, including *X. strumarium* could not be controlled satisfactory with nicosulfuron. Nevertheless, Bozic *et al*. (2013) showed that nicosulfuron could have a major impact on *X. strumarium* production, reducing growth and competitiveness, as well as, bur production and the seed bank of *X. strumarium* in the soil. The repeated use of the same herbicide or herbicides with the same mode of action during several consecutive years for the control of susceptible weed species leads to development of resistant populations. According to our knowledge, there are no reports on the effect of repeated use of the same herbicide or herbicides with the same mode of action on a weed species which is not susceptible to that herbicide. This kind of investigation could be helpful for better understanding of weed suppression measures in the field, which is necessary for establishing the best strategies for weed control. Therefore, the objectives of this research were to: (1) determine response of common cocklebur populations to nicosulfuron and (2) test whether a history of herbicide (acetolactate synthase (ALS)- inhibitor) application has an impact on the response of common cocklebur to nicosulfuron.

Materials and methods

Plant sources and herbicide history

Two populations of common cocklebur (CC1- Oplanic, 43.59°N & 20.40°E; CC2- Surcin, 44.46°N & 20.17°E) were used in this research. Population CC1 was collected from an area that had not been treated previously with any herbicide, while population CC2 was collected from a corn field with a history of treatment with ALS-inhibitors (Table 1). Burs of both populations were collected in the autumn from several randomly-selected plants within each above mentioned locality. The collected burs were stored at room temperature (approximately 25±3 °C) until the use.

Field studies

Field experiments were conducted in two consecutive years. The soil was an alluvial black marsh soil with pH 8.0 and 2.5% organic matter. Common cocklebur was sown in containers and transplanted to the field when the first pair of leaves developed. The experimental design was a completely randomized block design with four replications. Plot size was 5x4.2 m, with within-row spacing of 24 cm and 70 cm between rows. The meteorological conditions during the field trial study are given in Table 2. Growing degree days (GDD) were calculated using the formula GDD (°C) = Σ [(T_{max} + T_{min}) / 2-Tbase], where Tbase = 10 °C. The time line and additional information about the experiments are shown in Table 3.

Table 1. Field history at collection sites of common cocklebur populations (CC1, CC2)

Population	Collection site	Years of ALS-inhibitor treatment	Herbicide applied in collection year
CC1	Oplanic	0	-
CC2	Surcin	6	nicosulfuron

Plants were treated with herbicide at the four true leaf growth stage using a Neptune 15, Kwazar® knapsack sprayer and TeeJet 1004 flat-fan nozzles. The sprayer was calibrated to deliver 300 L water per hectare. Nicosulfuron (Motivell, 40 g ai L^{-1}, BASF, Ludvigmmmm, Germany) was applied at 0 (control), 10, 20, 40, 60 and 80 g ai ha^{-1}. Other weed species in plots that were not controlled with herbicide applications were removed by hand weeding. Visual damage estimation and plant height, fresh weight and leaf area measurements were made about month after herbicide application (1st year: 30 days after herbicide treatment (DAHT); 2nd year: 33 DAHT). Leaf area was measured using a Delta-T leaf area meter (Delta-T Devices, Burwell, Cambridge, UK).

Table 2. GDD and precipitation at the experimental site

Month	GDD † (°C)		Rainfall (mm)	
	1st year	2nd year	1st year	2nd year
April	11.2	19.1	1.2	9.4
May	218.6	229.8	49.0	29.2
June	320.1	270.6	39.6	98.2
July	333.7	366.3	42.0	35.8
August	356.4	358.4	35.0	76.2
Total	1240.0	1244.2	166.8	248.8

Table 3. Time line and additional information about the trials

Year	1st year	2nd year
Preceding crop	soybean	corn
Sowing date (in containers)	17 April	17 April
Date of transplantation	27 April	25 April
Date of herbicide application	13 May	17 May
Plant stage when herbicide applied	2 pairs of leaves	
Date of assessment	12 June	19 June

In vitro ALS assay

An *in vitro* ALS activity study was conducted using a modification of the procedure described by Ray (1984). ALS activity was assayed by measuring the amount of acetoin, which is the product of acetolactate decarboxylation.

Common cocklebur plants for ALS activity assay were grown in a controlled environment chamber under 300 µmol $m^{-2}s^{-1}$ photosynthetic photon-flux density (PPFD), 16 h photoperiod and 28 °C average temperature. Seeds were sown in pots (38 cm^2 surface area) containing a commercial potting mix (Flora Gard TKS1, Germany). The plants were irrigated manually when needed. Leaves from seedlings with two pairs of true leaves were harvested and used for studies. All procedures were performed at 4°C unless otherwise stated.

Two grams of fresh leaf tissue were homogenized with a mortar and pestle in 6 ml extraction buffer (0.1 M potassium phosphate (pH 7.5) containing sodium pyruvate (1 mM), thiamine pyrophosphate (0.5 mM), $MgCl_2$ (0.5 mM), flavine adenine dinucleotide (FAD, 10 µM) and glycerol (100 mL L^{-1})). The homogenates were filtered through cheesecloth and centrifuged for 25 min at 14000 x *g*. Proteins were precipitated from the supernatant with 50% saturated ammonium sulfate and the solution centrifuged at 14000 x g for 25 min. The supernatant was discarded and pellets were resuspended in potassium phosphate (0.12 M, pH 7.0) containing sodium pyruvate (20 mM), and $MgCl_2$ (0.5 mM). Protein concentrations of the crude enzyme extracts were quantified using bovine serum albumin as a standard (Bradford, 1976).

ALS activity was assayed by adding 0.1 ml of enzyme preparation to 0.5 mL reaction mixture (20 mM potassium phosphate (pH 7.0) containing sodium pyruvate (20 mM), thiamine pyrophosphate (0.5 mM), $MgCl_2$ (0.5 mM) and FAD (10 μM)) containing 0, 0.01, 0.1, 1, 10, 100 μM of nicosulfuron. After incubation of reaction mixtures at 30 °C for 1 h, 0.05 mL H_2SO_4 (3 M) was added to terminate the reaction. Then, 0.5 mL creatine (5 g L^{-1} in water) was added and the solution was incubated at 60°C for 15 min. Acetoin was detected at A_{525nm} as a coloured complex formed after adding 0.5 ml α-naphthol (50 g L^{-1} freshly prepared in 2.5 M NaOH) and incubating at 60 °C for 15 min. A standard curve was constructed using commercial acetoin. The experiment was conducted twice with three replicates per treatment.

Statistical analysis

Dose-response was used to analyze the following response parameters: plant height, fresh weight and leaf area from the field trials as well as acetoin levels from the ALS assay. More specifically, dose-response analysis was based on the three-parameter log-logistic model:

$$y = d/(1 + exp(b(log(x) - e)))$$

where, x and y denote the rate applied and the resulting observed response, respectively. The parameter d denotes the average response for rate 0 whereas the parameter e corresponds to the rate that reduces the response by 50% and the parameter b is proportional to the slope of the dose-response curve at a rate equal to e, i.e., the larger the value of b the more steep is the dose-response curve (Ritz and Streibig, 2005; Ritz, 2010). For each response parameter, simultaneous models for both populations were considered. Field trials repeated in two consecutive years were analyzed using separate models for the two years. Parameter estimates with corresponding estimated standard errors are summarized in tables and the fitted dose-response curves are shown with average response values in graphs. The delta method was used to calculate the standard errors of the ratios of GR_{50} (I_{50}) values (Ritz and Streibig, 2005). The statistical analyses and visualization of the fitted curves were carried out using the statistical environment **R** (R Development Core Team, 2013) and in particular the extension package *drc* (Ritz and Streibig, 2005).

Results

Visual observation indicated that nicosulfuron had a comparable effect on both common cocklebur populations. Assessment of visual damage showed that herbicide application to the plants of both populations caused transient damage expressed as chlorosis of tissue between leaf veins and leaf deformations several DAHT, but plants recovered and continued to grow. The log-logistic model accurately described the response of common cocklebur based on vegetative parameters 30 (1st year) and 33 (2nd year) DAHT (Fig. 1). Both populations (CC1 and CC2), showed different levels of damage

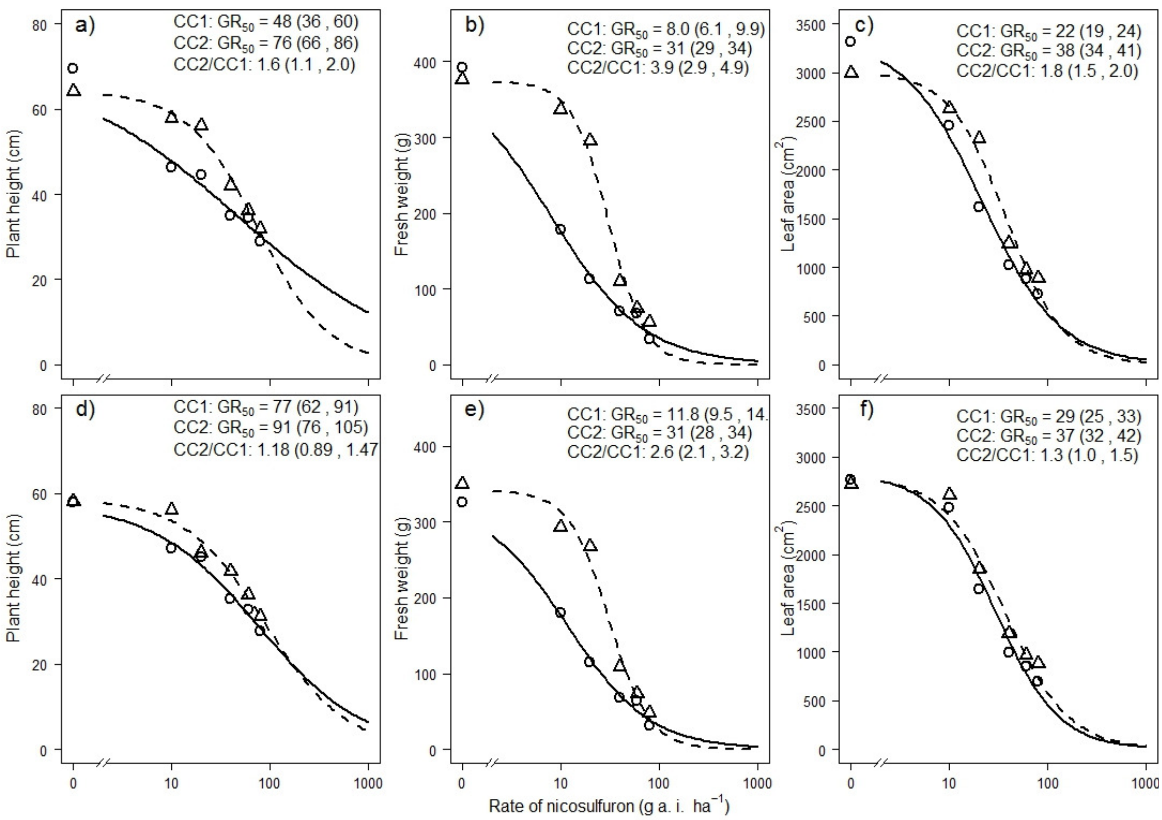

Fig. 1. Field trial - effect of increasing rates of nicosulfuron in 1st year on: a) plant height, b) fresh weight, c) leaf area and in 2nd year on: d) plant height, e) fresh weight, f) leaf area of CC1 (o) and CC2 (Δ) common cocklebur populations

as the herbicide rate increased from 10 to 80 g ai ha^{-1}. Namely, as the rate increased, the CC1 population showed reductions in fresh weight of 54-91% (1st year) and 80-96% (2nd year), plant height 33-59% (1st year) and 59-76% (2nd year) and in leaf area 26-78% (1st year) and 54-87% (2nd year) in comparison with untreated control. The decline in these parameters was slightly less in the CC2 population, where fresh weight decreased by 10-85% (1st year) and 67-94% (2nd year), height by 10-50% (1st year) and 51-73% (2nd year) and leaf area by 12-70% (1st year) and 52-84% (2nd year). Furthermore, dose-response analysis showed that slope parameters significantly differed between populations CC1 and CC2 (parameter *b* in Table 4) for plant height and leaf area in 1st year and fresh weight in both years (P<0.001), while differences between populations for plant height (P=0.09) and leaf area (P=0.98) in 2nd year were not significant.

Estimates (parameter *e* in Table 4) of the rates of nicosulfuron needed to reduce the vegetative parameters (plant height, fresh weight, leaf area) by half (GR_{50}) varied from 8 to 91 g ai ha^{-1}. Namely, the nicosulfuron GR_{50} values for fresh weight were 8 g ha^{-1} (1st year) and 11.8 g ha^{-1} (2nd year) for CC1 and 31 g ha^{-1} (both years) for CC2. Therefore, population CC1 was 3.9- and 2.6-fold more susceptible to nicosulfuron than population CC2. The GR_{50} values for plant height and leaf area differed only slightly between CC1 and CC2 populations (plant height: 1st year - 1.6-fold; 2nd year- 1.18-fold and leaf area: 1st year - 1.8-fold; 2nd year - 1.3-fold). Also, estimated parameters of log-logistic equations confirmed that GR_{50} of the CC2 population was significantly greater than the CC1 population for most vegetative parameters (height: P=0.0033 (1st year), fresh weight: P<0.001 (both years), leaf area: P<0.001 (1st year); P=0.011 (2nd year)), except plant height in 2nd year (P=0.19).

In vitro incubation of the ALS enzyme extracted from leaves of CC1 and CC2 populations with a range of nicosulfuron concentrations (from 0.01 to 100 μM) showed similar responses of the CC1 and CC2 plants, causing 25 to 97% and 19 to 97% enzyme activity inhibition, respectively (Fig. 2). The concentration of nicosulfuron needed to obtain 50% inhibition of ALS-specific activity (production of acetoin per minute per mg of protein) of the untreated control plants for population CC1 was 0.11 μM, compared with 0.16 μM for population CC2. Therefore, ALS enzyme from the CC2 population was 1.42-fold less nicosulfuron sensitive than that of the CC1 population. The slopes were also insignificantly different (as indicated by the common parameter *b* in Table 4). Therefore, the difference in sensitivity between CC1 and CC2 populations was not significant (P=0.21).

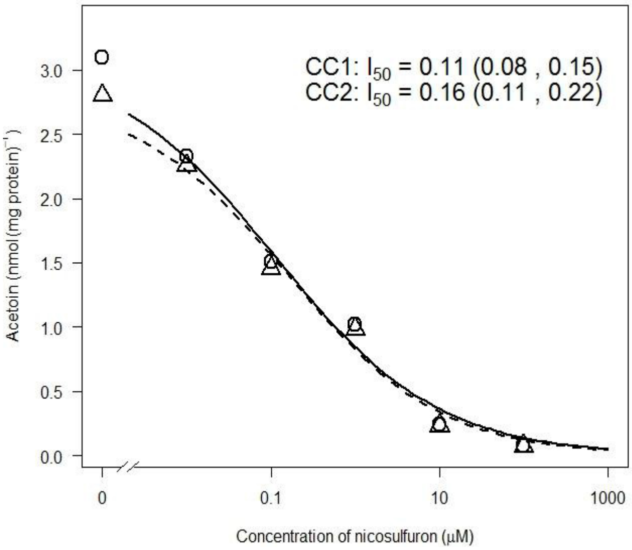

Fig. 2. Effect of nicosulfuron on *in vitro* ALS activity of CC1 (o) and CC2 (Δ) common cocklebur populations

Discussions

Our results indicate that common cocklebur is not susceptible to nicosulfuron. Namely, double the recommended rate of this herbicide (80 g ha^{-1}) was not lethal to either common cocklebur population (data not shown). Visual damage of plants of both populations although present, was only transient, after which plants recovered and continued to growth. Those findings are consistent with Lueschen *et al.* (1992) and Dogan *et al.* (2005), who confirmed that many broadleaf weeds, including common cocklebur could not be controlled satisfactorily with nicosulfuron.

Generally, nicosulfuron GR_{50} values were greater for the CC2 than for the CC1 population (parameter *e* in Table 4)

Table 4. Parameters (±SE) of the log-logistic equation† used to calculate nicosulfuron rate required for 50% reduction of plant height, fresh weight and leaf area (GR_{50}) and ALS activity (I_{50}) of common cocklebur populations

	Parameter	Population	*d*‡	*b*‡	*e*‡
1st year	Plant height (cm)	CC1	69.51±1.54	0.51±0.06	47.70±5.99
		CC2	64.08±1.44	1.21±0.14	75.77±4.92
	Fresh weight (g)	CC1	391.97±9.43	0.92±0.09	8.01±0.97
		CC2	373.61±8.19	2.33±0.18	31.17±1.28
	Leaf area (cm^2)	CC1	3341±63.92	1.10±0.06	21.56±1.20
		CC2	3007±60.25	1.50±0.10	37.79±1.76
2nd year	Plant height (cm)	CC1	57.71±1.34	0.81±0.09	76.85±7.27
		CC2	58.75±1.25	1.06±0.12	90.57±7.12
	Fresh weight (g)	CC1	326.06±8.89	1.04±0.09	11.82±1.15
		CC2	341.33±8.23	2.13±0.18	31.25±1.49
	Leaf area (cm^2)	CC1	2827.32±75.17	1.35±0.10	29.35±1.94
		CC2	2800.93±73.06	1.35±0.11	37.10±2.41
	ALS assay (*in vitro*)	CC1	3.09±0.06	0.45±0.02	0.11±0.02
		CC2	2.80±0.06	0.48±0.03	0.16±0.03

† Parameter estimates are for the log-logistic equation described in the text;
‡ *d* - average response for rate 0; *b* - the slope of the dose-response curve at a rate equal to *e*; *e* - rate that reduces the response by 50% (log(GR_{50}) for vegetative parameters and log(I_{50}) for ALS activity).

for each of the vegetative parameters. Differences between populations were most pronounced for fresh weight (1st year: 3.9-fold; 2nd year: 2.6-fold) which was the most sensitive of the three growth parameters to this herbicide based on GR_{50} values (CC1: 8 (1st year) and 11.8 (2nd year) g nicosulfuron ha^{-1}; CC2: 31 (both years) g nicosulfuron ha^{-1}). The highest GR_{50} values were estimated for plant height (CC1: 48 (1st year) and 77 (2nd year) g nicosulfuron ha^{-1}; CC2: 76 (1st year) and 91 (2nd year) g nicosulfuron ha^{-1}), indicating that this parameter was the least sensitive to nicosulfuron. This is not in accordance with findings of Abbas *et al.* (2005), who showed that plant height reduction was a more effective measure of herbicide (chlorimuron, imazaquin, sodium hydrogen methylarsenate and imazethapyr) activity on common cocklebur than dry weight or mortality. Based on plant dry weight, GR_{50} values for susceptible populations of *Setaria faberi* Herrm. were <0.5 g nicosulfuron ha^{-1}, while for resistant populations of the same species they were between 2 and 9 g nicosulfuron ha^{-1} (Volenberg *et al.*, 2001). In addition, nicosulfuron GR_{50} value based on fresh weight for susceptible *Amaranthus retroflexus* was 4.11 g ha^{-1}, while for resistant plants it was 139.2 g ha^{-1} (Scarabel *et al.*, 2007). In our study, GR_{50} values for all vegetative parameters (parameter *e* in Table 4) were higher than for resistant populations of *S. faberi* and between GR_{50} values for susceptible and resistant *A. retroflexus*. Based on that we can conclude both our common cocklebur populations were very much less susceptible to nicosulfuron than those species.

Common cocklebur response to nicosulfuron differed between years, probably because of differences in environmental conditions. Namely, in the part of the season when nicosulfuron was applied and responses estimated (between sowing and June 30), the first experimental year was characterized by low precipitation, while precipitation in the second experimental year was relatively high. Therefore, our findings are in accordance with previous investigations demonstrating that environmental conditions significantly affect the activity and effect of foliage-applied herbicides (Kudsk and Kristensen, 1992; Bozic *et al.*, 2012).

Responses of ALS enzyme extracted from both common cocklebur populations to nicosulfuron were similar (parameter *e* in Table 4) (CC1: I_{50}=0.12 μM; CC2: I_{50}=0.16 μM). Also, ALS I_{50} values were similar to those for *Ambrosia artemisiifolia* (I_{50}=0.137 μM), *Avena fatua* (I_{50}=0.104 μM), *Panicum miliaceum* (I_{50}=0.135 μM) and *A. retroflexus* (I_{50}=0.104 μM) incubated with nicosulfuron (Mekki and Leroux, 1994). This is in accordance with findings of Green and Ulrich (1993) who concluded that ALS from tolerant and susceptible plants is generally similar in sensitivity to nicosulfuron.

The possible reasons for non-susceptibility of common cocklebur to nicosulfuron could be enhanced metabolism of the herbicide in comparison with susceptible species (Brown and Neighbors, 1987), low uptake (Askew and Wilcut, 2002) or low translocation of herbicide (Carey *et al.*, 1997). Also, morphological characteristics of plants like morpho-anatomical characteristics of leaves (Lisek *et al.*, 2002), stomata characteristics (Schreiber, 2005) and the presence of hairs on the leaves (Grangeot *et al.*, 2006) could be responsible for the susceptibility of plants to herbicides.

The dose response curves (Figs. 1 and 2), GR_{50} and I_{50}, as well as, its ratios between CC2 and CC1 populations (plant height: 1.60 and 1.18, fresh weight: 3.90 and 2.60, leaf area: 1.80 and 1.30 in 1st and 2nd year, respectively; while ratio for ALS enzyme acidity was 1.42) showed slightly different responses between populations treated with nicosulfuron. Namely, based on these GR_{50} values for fresh weight, the population CC1 was about 3.9 and 2.6-fold more susceptible to nicosulfuron than population CC2 in 1st and 2nd year, respectively, but differences were not so prominent when based on other parameters (plant height, leaf area and ALS activity), ranging from 1.18 to 1.8-fold. As previous investigations have shown that weed populations can vary greatly in susceptibility to herbicides (DeGennaro and Weller, 1984; Gillespie and Vitolo, 1993; Patzoldt *et al.*, 2002), the differences between population CC1 and CC2 could be attributed to inter-population variability in susceptibility to nicosulfuron, though these differences could be the consequence of repeated application of ALS herbicides to the CC2 population during the six previous years. Future investigations are necessary in order to clarify this dilemma and give recommendation for their control in integrated weed management system.

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