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Full Length Research Paper

Activity of acetolactate synthase (ALS) of redroot pigweed in relation to imazethapyr application

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Seed of weed species redroot pigweed for which there exist possibility of resistance occurrence were collected from different localities in Autonomous Province of Vojvodina, the northern part of the Republic of Serbia (Krivaja, Kikinda, Vrbas and Kačarevo). Studies on herbicide resistance were performed in the period of 2003 to 2009. Biological studies included whole plant studies on plants grown in net-house and biochemical researches confirmed the activity of acetolactate synthase enzyme *in vivo*. In order to study the occurrence of resistance during the tests, herbicide products based upon active ingredient imazethapyr were used. Values of resistance index were calculated in relation to the referent population collected from the ruderal sites. Based upon the results of biological studies in the redroot pigweed plants from the locality of Krivaja, the presence of resistance was established. Immunological tests confirmed the results of biological assays given that the activity of ALS enzyme was found in all the studied populations. Established resistance is a consequence of physiological changes and accelerated metabolism.

Key words: Redroot pigweed, imazetaphyr, acetolactate synthase activity, dose response curve, resistance.

INTRODUCTION

Acetolactate synthase (ALS) inhibiting herbicides, due to their practical use, are among the most selling ones in the world in quantities of several g/ha. In contrast to previously used herbicides whose rates per ha were measured in kg, in the eighties of the last century, implementation of ALS enzyme inhibitors has led to reduced amounts of the applied herbicides in the field (Bellinder et al., 1994). Defect of these herbicides is their ability to select resistant weed population (R), which was not only a threat to their future use, but also for sustainable agricultural production (Tranel and Wight, 2002). This feature is associated with their very specific reaction and inhibition of ALS enzyme. ALS, also described as acetohydroxy acid synthase or AHAS, is the first enzyme that catalyzes biosynthesis of branched amino acids isoleucine, valine and leucine (Umbarger, 1978). In addition, in cases of the herbicide soil application, in susceptible plants they cause damages in the form of necrosis of apical meristems that cease the growth of plants while their foliar use cause purple colour of the leaf along the central nerve (Lovell et al., 1996a).

Today, there are over 50 herbicides from the group of ALS inhibitors that are used for selective control of weeds in many crops (Saari et al., 1994; Heap, 2010) in almost all parts of the world. They are grouped into five structurally different chemical classes: imidazolinones, sulfonylureas, triazolopyrimidines, pyrimidinyl thiobenzoates and sulfonylamino-triazolinones (Tranel and Wright, 2002) and according to the association of herbicide resistance active committee (HRAC), they are classified in group B of herbicide compounds (Heap, 2010). The most intensively are applied herbicides from the group of sulfonylureas and imidazolinones, and the widest use of the given active ingredients has nicosulphuron and imazethapyr (Duggleby et al., 2003). There are 107 weed species resistant to the herbicides belonging to this mode of action group (Heap, 2010).

Resistance to herbicides from the group ALS inhibitors is common throughout the world, which threatens the use of these herbicides. Primarily, resistance has emerged as a result of reduced susceptibility of ALS enzyme target site which prevents the action of herbicides. Secondary mechanism of resistance is triggered by enhanced

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herbicide metabolism resulting in faster detoxification of herbicides. The enhanced herbicide metabolism is the most common cause of the occurrence of low intensity cross-resistance (resistance index (RI) is 10-fold lower) of herbicides with different mechanisms of action (Hall et al., 1994). Within the genus, *Amaranthus* 47 ALS resistant populations were determined (Heap, 2010). Manlez et al. (1998) proved resistance of *Amaranthus hybridus* after six years of imazaquine use in combination with pendimethalin or trifularin. In *Amaranthus rudis,* (Sprague et al., 1997) population occurrence of resistance was recorded after only two years of suflonylureas use and after three years from using combination sulfometuron-methyl and simazine in redroot pigweed (*Amaranthus retroflexus* L.) population (Sibony et al., 2001). Due to the high number of cases in species from the genus *Amaranthus,* it is considered that they have predisposition for resistance development, such as tendency toward mutations, which is also confirmed by resistant types *Amaranthus palmeri* (Gaeddert et al., 1997; Sprague et al., 1997), *A. retroflexus* (Sibony et al., 2001; Ferguson et al., 2001), *Amaranthus blitoides* (Sibony and Rubin, 2003), *A. rudis* (Lovell et al., 1996a; Sprague et al., 1997), *A. hybridus* (Maertens et al., 2004) and *Amaranthus powelii* (Ferguson et al., 2001).

In the territory of Serbia, the problem of weed resistance was studied for the first time by the method of leaf fluorescence and by measuring quant yield of different *A. retroflexus* populations' resistance to atrazine (Janjić et al., 1988). In the region of Vojvodina, resistance to atrazine, the herbicide belonging to the group of inhibitors of photosynthesis was established in weed populations of *A. retroflexus*, *Chenopodium hybridum, Setaria viridis* and *Abutilon theophrasti* (Konstantinović and Meseldžija, 2001), as well as to ALS inhibitors in weed species *A. retroflexus* and *Panicum crus-galli* (Meseldžija, 2009). With the aim of testing resistance of weed species to herbicide imazethapyr from the group of acetolactate synthase inhibitors, studies of morphometric differences were performed in biological tests between referent, that is, susceptible populations of *A. retroflexus* and populations for there exist an assumption that, they may have obtained capability to survive treatments by a range of imazetaphyr rates; values of the middle efficient rate (ED_{50}) and RI (index of resistance) have been determined upon dose-response curve. For the first time, resistance was studied also by use of biochemical methods, by testing ALS activity *in vivo.*

MATERIALS AND METHODS

In the period of 2003 to 2009, studies were performed in field and laboratory conditions. They included field observation; collecting more seed of *A. retroflexus* L. populations and biological assays for ALS enzyme inhibiting herbicides. After measurements and systematization, data were statistically processed. Statistical processing was performed by analysis of variance (ANOVA); significant differences were evaluated by t-test and individual comparisons by LSD values in the program Sigma Plot 10.0.

Resistance studies in field

In resistance studies, the method of field studies proved insufficient, but also unavoidable as basic for several factors may indicate development of resistance in the field. This method (Moss, 1995) was applied for terrain survey; data on use of ALS enzyme inhibiting herbicides in the studied region were collected for several previous years and seed of more populations of *A. retroflexus* L. for which there exist the assumption of evolved resistance to the studied herbicides were collected in order to perform bioassays for its confirmation. Seed was collected in the period of August to October from plots with the long history of ALS inhibiting herbicides use in control of weedy vegetation and on which low efficiency to the monitored weed species was observed (Table 1). Seed were collected from different localities in the region of Vojvodina (North Serbia); Kikinda, Bečej, Krivaja, Vrbas and Kačarevo. For susceptibility, referent population seeds were taken from ruderal sites that had never been treated by herbicides. All seeds were collected manually from over 100 individuals of each population of the studied weed species. They were collected from 15 plots within each locality, in which great presence of *A. retroflexus* L. weedy populations were observed after herbicide treatment. Cleared seeds were dried on 25°C during 5 days period and then exposed to the temperature of 17°C and 54% of relative air humidity until the trial according to Adkins et al. (1997). After maturation was confirmed by germination test, whole plant bioassays were set up in net-house.

Table 1 shows the use of herbicides belonging to the group of ALS enzyme inhibitors (*) at localities from which seed were collected for analysis. During studies on the territory of Serbia, commercially used herbicides based upon active ingredient imazetaphyr from ALS enzyme inhibiting group of herbicides were applied.

Whole plant bioassay

Whole plant bioassay on weed species *A. retroflexus* L. was set up in uncontrolled conditions of the net-house, in which weeds were treated by a range of imazethapyr rates; the herbicide from the group of imidazolinones. Herbicide rates were chosen with the aim of obtaining plant reactions from ''unharmful'' to ''complete death''. The herbicide was applied in rates of: 0, 8, 40, 100, 200, 400, 800 and 2000 g of active ingredient imazethapyr/ha. The effect of the treatment on plants was studied 2 to 3 weeks after herbicide application by measuring sprouts fresh foliage weight and the number of survived plants (Moss, 1995). In data processing, results were presented on the dose-response curve that enables better designation of the susceptibility level by calculation of the quantity coefficient that is needed to produce identical effect in resistant and susceptible population (ED_{50}); that is, quantity needed to achieve 50% of reduction in the measured parameters (leaf mass or number of survived plants) in relation to the untreated control, and it was obtained by interpolation of the curve. Based upon coefficients of these evaluations (ED_{50}) concerning those susceptible populations, resistance index (RI) that enables relatively simple description of resistance level was calculated (Moss, 1995). Biochemical tests of ALS enzyme activity *in vivo* were performed according to the method of Lovell et al. (1996b) in four replications on weed species *A. retroflexus* L. 24 h after application of the studied imazethapyr rates, solution of 1.1-cyclopropanedicarboxylic acid (CPCA) (0.26 g/ml) in 0.25% non-ionic surfactant Trend 90 was applied foliary. 3 h after application of CPCA to each plant, 0.2 g of the previously (-20°C) frozen youngest leaf tissue was taken in order to enhance destruction of cells. In each frozen sample, 3 ml distilled water was added, after which they were incubated for 5 min at 60°C and 45 min at 25°C with the alternate shaking up of the samples. In 3 ml of each sample 75 µl H_2SO_4 (3 mol/dm^3) was added, after which they

Table 1. Review of use of herbicides belonging to the group of ALS enzyme inhibitors (*) at localities from where seed for the analysis were collected.

Locality	Year	Crop	Herbicide	Rate
	1998	Soybean	Imazethapyr*+Bentazone	40 g a.i./ha+720 g a.i./ha
	1999	Maize	Rimsulfuron*	15 g a.i. /ha
	2000	Wheat	2.4-d	838.05 g a.i./ha
Krivaja	2001	Wheat	Amidosulfuron*+Tribenuron methyl*	15 g a.i./ha+15g a.i./ha
	2002	Sugar beet	Triflusulfuron methyl*+ metamitron	2x15g a.i./ha+1.05 kg a.i./ha
	2003	Soybean	Imazethapyr*	100 g a.i./ha
	2004	Maize	Rimsulfuron*	15 g a.i. /ha
	1999	Maize	Prosulfuron*+ primisulfuron methyl*	12.5 g a.i./ha + 7.5 g a.i./ha
Kikinda	2000	-	-	-
	2001	Soybean	Imazethapyr*+ Bentazone	40 g a.i./ha+720 g a.i./ha
	1998	Soybean	Imazethapyr*	100 g a.i./ha
	1999	Maize	Rimsulfuron*	10 g a.i. /ha
	2000	Wheat	Tribenuron methyl*	12 g a.i. /ha
Bečej	2001	Soybean	Imazethapyr*+ oxasulfuron*	60 g a.i./ha+ 60 g a.i./ha
	2002	Maize	Nicosulfuron* +Prosulfuron*+ Primisulfuron methyl *	20 g a.i./ha + 12.5 g a.i. +7.5 g a.i./ha
	2003	Wheat	Tribenuron methyl*	12 g a.i./ha
	2001	Maize	Rimsulfuron*+Dicamba	15 g a.i./ha+384 g a.i./ha
	2002	Soybean	Imazethapyr*+Bentazone	40 g a.i./ha+ 960 g a.i./ha
Vrbas	2003	Maize	Rimsulfuron* + 2.4-d	12.5 g a.i./ha + 558.7 g a.i./ha
	2004	Soybean	Oxasulfuron*+ imazethapyr*	37.5 g a.i./ha + 40 g a.i./ha
	2005	Maize	Rimsulfuron*	12.5 g a.i./ha
	1995	Soybean	Imazethapyr*	100 g a.i./ha
	1996	Winter wheat	-	-
	1997	Maize	Nicosulfuron*	40 g a.i./ha
	1998	Sunflower	Prometrine	1000 g a.i./ha
Kačarevo	1999	Winter wheat	-	-
	2000	Maize	Nicosulfuron*	40 g a.i./ha
	2001	Soybean	Oxasulfuron* + Imazethapyr *	37.5 g a.i./ha +100 g a.i./ha
	2002	Maize	Nicosulfuron*	20 g a.i./ha
	2003	Maize	Nicosulfuron*	40 g a.i./ha

were incubated for 30 min at 60°C in order to avoid decarboxylization of acetolactate into acetoin. This was followed by adding 1.5 ml solution of NaOH (2.5 mol/dm^3) that contained 90 g/l of naftol and 9 g/l of creatine. The samples were then incubated for 30 min at 60°C, after which they changed colour. After cooling at room temperature, the samples were centrifuged for 5 min at 24 *g*. Their light absorbance was read on spectrophotometer at wavelength of 525 nm. The measured absorption values were converted into µg acetoin by standard curve.

RESULTS

Based upon field observations, populations of *A. retroflexus* L. were separated from localities Krivaja, Kačarevo, Kikinda and Vrbas, in order to perform biological assays and biochemical ALS enzyme resistance studies. Whole plant bioassays were performed in net-house by a range of imazethapyr rates. Imazethapyr was the chosen representative of the imidazolinone group. The measured parameters were fresh foliage weight and number of survived plants treated by a range of imazethapyr rates (Figure 1).

Values of ED_{50} and RI presented in Table 2 were calculated upon dose-response curve that represents plant reaction to the applied range of herbicide rates.

DISCUSSION

During the three years studies of weed plant *A. retroflexus*

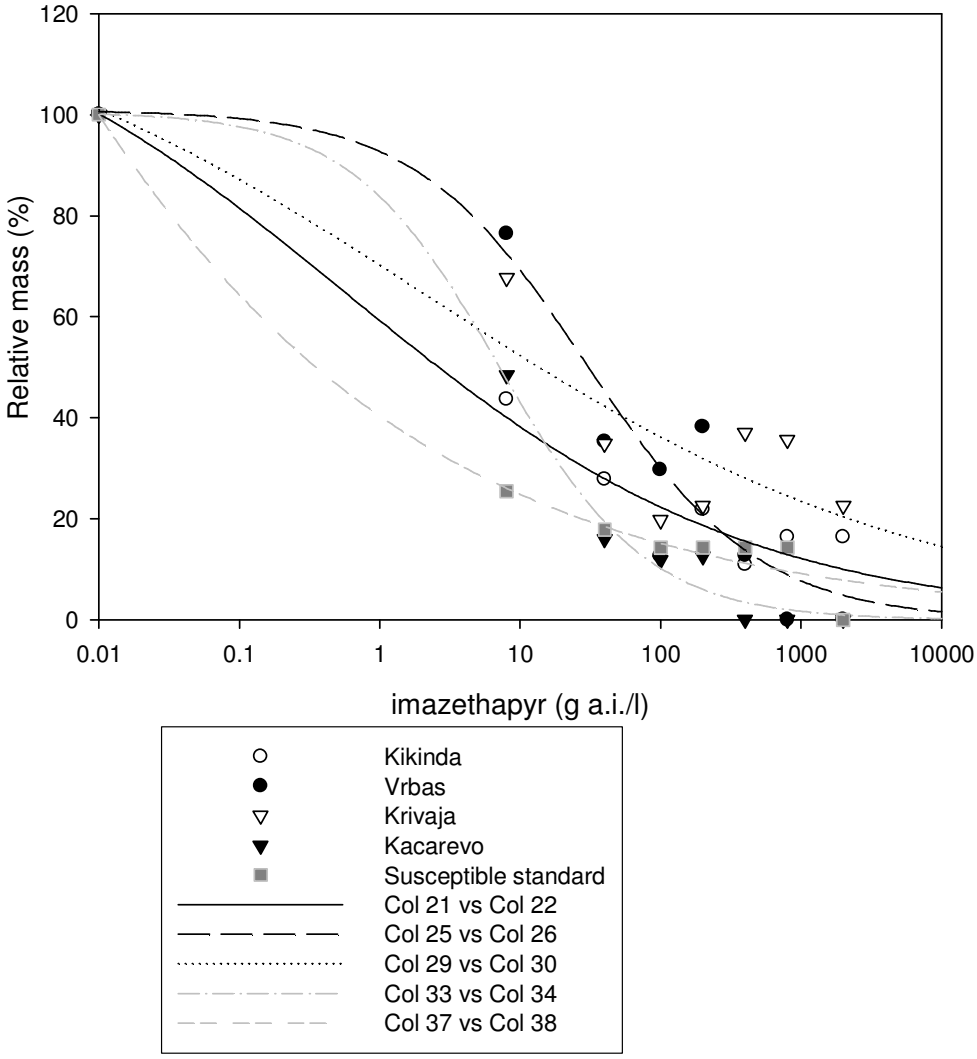

Figure 1. Relative plant mass in a range of imazethapyr rates.

Table 2. Values of ED_{50} and resistance index for relative plant mass in a range of imazethapyr rates.

Population	ED_{50} value	RI value
Kikinda	2.62	7.46
Vrbas	30.79	87.68
Krivaja	13.57	38.66
Kačarevo	7.15	20.35
Susceptible standard	0.35	-

L. resistance occurrence and development to ALS enzyme inhibiting herbicides, preliminary field studies were performed after which plant material was tested by various laboratory methods. Plant material was collected from different locations in which ALS inhibiting herbicides were used during several years period. Seeds of redroot pigweed individuals that survived herbicide treatments at the chosen plots were collected during autumn. Since there are many factors that may be responsible for inadequate herbicide efficiency, it is often difficult to determine the exact cause of the herbicide inefficiency in the field. At localities with long-standing application of ALS enzyme inhibiting herbicides (Bečej, Krivaja, Kikinda, Kačarevo and Vrbas), observed changes in field

populations were also confirmed by laboratory assays. Populations of *A. retroflexus* L. survived treatments by herbicides from HRAC group B in the field.

From Table 1, it is obvious that in the period of 1998 to 2004 at locality Krivaja with the aim of weed control, six herbicides from HRAC group B were applied (marked by*). The used herbicides were based upon the following active ingredients: imazethapyr (Pivot 100E), rimsulfuron (Tarot 25 WG), amidosulfuron (Grodyl), tribenuron-methyl (Granstar 75WG) and triflusulfuron-methyl (Safari 50WG). During 2000, herbicides from SU or IMI groups were not used in only one crop in crop rotation followed by high selection pressure to the present weed species. For Kikinda locality, the presented data are for three-years period during which herbicides Ring 80 WG (prosulfuron+primsulfuron methyl) and Pivot 100E (imazethapyr) were applied. In the six year lasting period at Bečej locality, there were three crops in crop rotation. In each of the crop, ALS enzyme inhibitors were used; in soybean imazethapyr and oxasulfuron, in wheat tribenuron-methyl and in maize rimsulfuron, nicosulfuron and combination prosulfuron+primsulfuron-methyl were used. According to the anti-resistance strategy which is implemented with the aim of prevention of resistance occurrence, beside crop rotation, it is also unnecessary to apply rotation of herbicides of different modes of action (Jutsum and Graham, 1995; Konstantinović et al., 2000). Data record on herbicide use at Kačarevo locality, also indicated frequent use of ALS inhibitors. For nine years, in this locality, imazethapyr (IMI) was applied twice and five herbicides from SU group, of which nicousulfuron was applied for four years and oxasulfuron once. In the period of 2001 to 2005 at Vrbas locality, the following herbicides were used: Tarot 75WG (rimsulfuron) during three years, Pivot 100E (imazethapyr) for two years and Dynam 75 WG (oxasulfuron), once. Development of resistance to more than one group of herbicide modes of action is shifted by monoculture and minimum tillage (Rubin, 1997; Gasquez, 1997). Heap and Knight (1986) and LeBaron (1987) observed the occurrence of cross-resistance in which weed population is resistant to two or more herbicides because of the presence of one resistance mechanism and multiple resistance that refers to the cases in which resistant plants possess one or more resistance mechanisms. Some *Lolium rigidum* Gaud. populations (Powles, 1997) develop resistance to several different herbicide groups that act at different target sites and/or metabolize by different enzymes. Possibility of polymorph population development indicates to the capacity of the species to resist against different measures of weed control (Powles, 1997). Existence of one such mechanism may complicate choice of the alternative herbicide for control in the case of resistance and control strategy must include much more than one opposite product. Therefore, in order to achieve rational program of weed protection, herbicide of different herbicides or even rotation of herbicides with different modes of action is needed (Konstantinović et al., 2000).

Any manner of selection pressure reduction that leads toward resistance occurrence and development will also reduce the level of resistance evolution. Integral approach in weed control that includes physical, chemical and biological measures, without excessive reliance to any of these methods is the most efficient manner in resistance prevention (Mallory-Smith et al., 1999). Chances of induction of resistance by this would be reduced, for there is small probability that at the same time plants would develop resistance to several herbicides, although, there are exceptions in the case of multiple resistance (Schmidt, 1997). Summer post-emergence herbicides enable susceptible weeds that produce seed before or after herbicide use to dilute quantity of seeds of resistant plants that significantly lowers selection pressure. It means that length of residual activity and time of herbicide application may have impact to the level of resistance evolution (Konstantinović et al., 2000).

Biological assays were performed by the method of net-house trials in which number of survived plants and fresh foliage weight were recorded. In all the assays, imazethapyr was applied in a range of rates, including rates under and above recommended field ones. Curves herbicide-plant reaction enabled calculation of ED_{50} values and resistance index in the studied populations. In very resistant populations, it might be impossible to obtain ED_{50} values; therefore, precise resistance index cannot be calculated (Letouze et al., 1997). The higher resistance index is the higher level of resistance (Moss, 1995). Generally, susceptible populations have smaller biomass and seed production. However, the result is often also uniform. Many assays described in literature have not included isogenic lines or field populations or they were carried out in glasshouses under growth-limiting conditions (Holt, 1997). According to Kremer and Kropff (1998), correct comparison of growth should be made under conditions for optimal growth, with susceptible and resistant populations of different geographic origin, in order to establish variation in parameters of growth within and between populations.

Based upon results of the biological assays on studied populations of *A. retroflexus* L., differences in susceptibility to imazethapyr were established in relation to the referent population. Morphometric parameters were analyzed in many methods that are applied for weed resistance to herbicides from different chemical groups (Beckie et al., 2000; Hanson et al., 2004; Corbett and Tardif, 2006).

Whole plant assays were performed in the uncontrolled conditions of net-house by a range of imazethapyr rates that was chosen as the representative of the group. The obtained results of the fresh foliage weight and the number of survived plants were presented by relative number (% in relation to susceptible standard) on

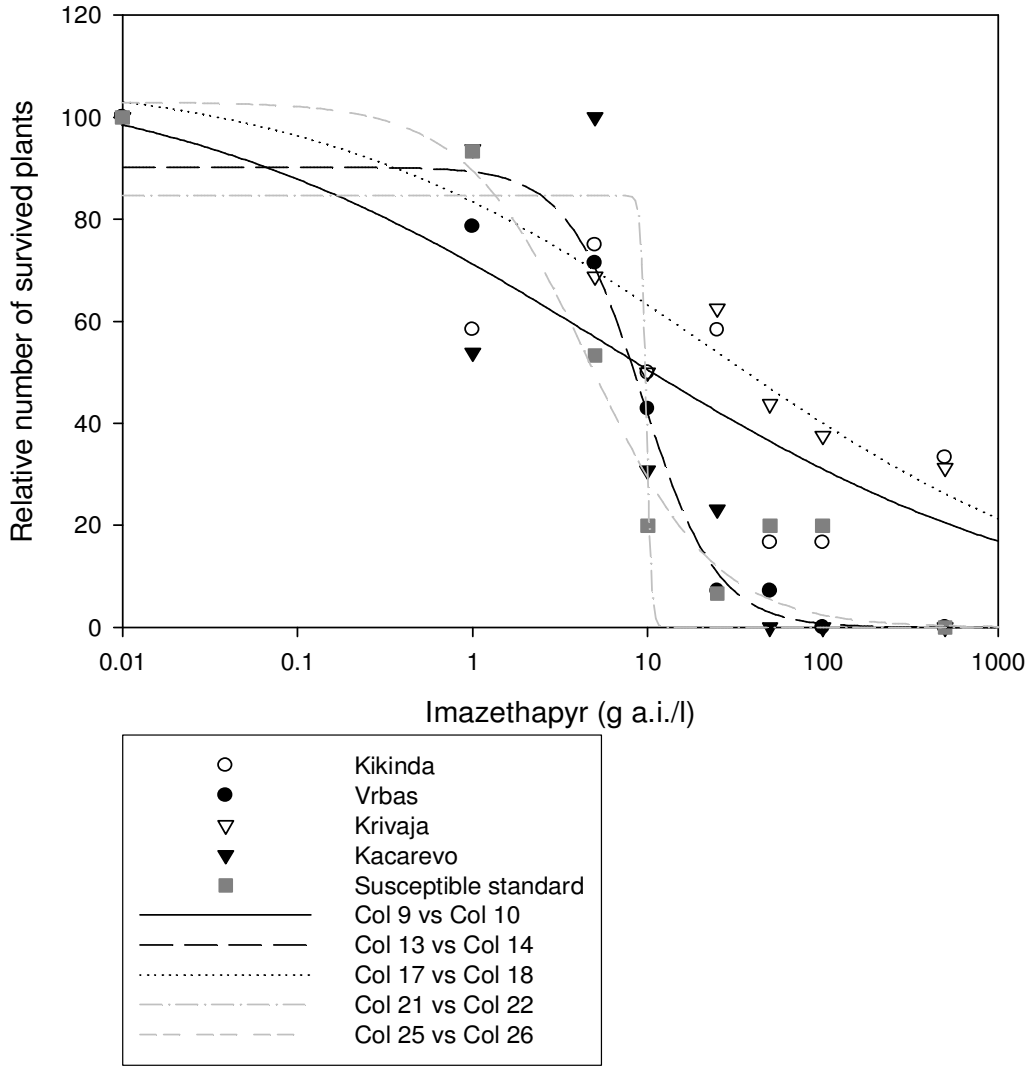

Figure 2. Relative numbers of survived plants in a range of imazethapyr rates.

Table 3. Values of ED_{50} and resistance index for relative number of survived plants in a range of imazethapyr rates.

Population	ED_{50} value	RI value
Kikinda	10.59	2.14
Vrbas	8.40	1.69
Krivaja	36.45	7.36
Kačarevo	9.70	1.96
Susceptible standard	4.95	-

logarithmic doze imazethapyr-response curve (Figures 1 and 2). The calculated IR based upon ED_{50} values were between 1.69-87.68. The highest IR was determined for *A. retroflexus* L. from Vrbas (87.68) and Kačarevo localities (7.36) (Tables 2 and 3). According to the results of the whole plant assay in the uncontrolled conditions with a range of imazethapyr rates (Figure 1) and for fresh foliage weight, the highest IR was established in populations from Vrbas (87.68), Krivaja (38.66) and Kačarevo (20.35) localities. Resistance index for population from Kikinda locality was 7.46 (Table 2). Based upon the relative number of the survived plants in a range of imazethapyr rates, the highest IR value had population from Krivaja locality (7.35). Population from Kikinda locality had IR of 2.13, population from Kačarevo had 1.95 and Carnex had 1.69 (Table 3). Values of resistance index for fresh foliage weight is considered the most reliable parameter for separation of susceptible and resistant populations of *A. retroflexus* L. As ALS enzyme is a site of action of the studied herbicide, activity of this plant enzyme was also studied by *in vivo* method. The method was based upon quantity of acetoin in fresh foliage weight of the plant material, which enables establishment of the results of herbicide performance 24 h after its application. According to Gerwick et al. (1993), R and S populations can be differed even by the colour of

Table 4. Acetoin accumulation (µg g^{-1} of fresh foliage weight h^{-1}) of different *A. retroflexus* L. populations after imazethapyr application.

Population	Imazethapyr rate (kg a.i./ha)						
	0	**0.04**	**0.08**	**0.10**	**0.15**	**0.20**	**0.40**
1 Kikinda	12.05	9.32	8.66	7.94	7.38	6.51	5.30
2 Vrbas	13.22	9.03	7.88	6.50	4.98	3.71	3.20
3 Krivaja	11.34	10.96	9.73	9.50	8.76	8.41	7.63
4 Kačarevo	14.01	10.23	9.80	9.22	8.53	8.02	7.78
Susceptible standard	12.56	3.71	3.02	2.54	1.94	0.82	0.34

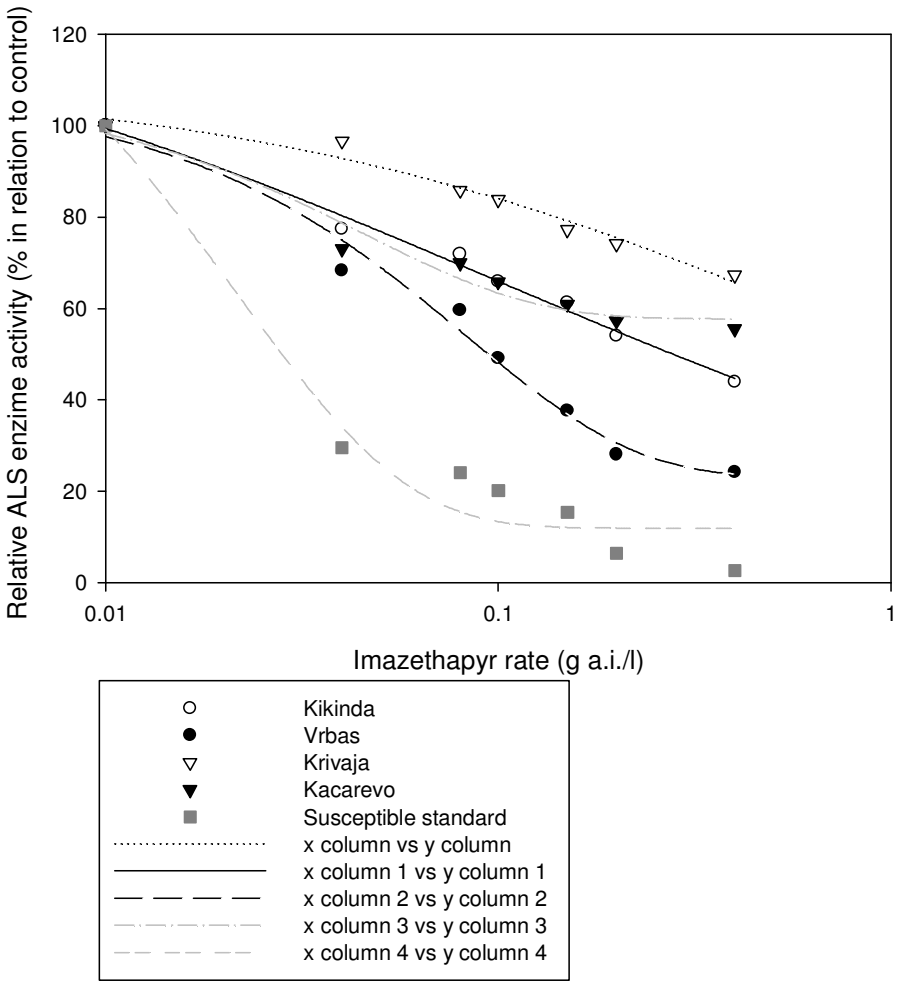

Figure 3. Activity of ALS enzyme after application of a range of imazethapyr rates.

the samples. Samples with higher quantity of acetoin are red or pink and in the absence of acetoin yellow colour occurs (S population). Based upon activity of ALS enzyme, this method enables detection of resistance occurrence (Lovell et al., 1996b). Other authors also applied modification of this method in order to establish resistance induced by insensitivity of ALS enzyme (Gerwick et al., 1993; Simpson et al., 1995; Sprague et al., 1997).

In all the studied control populations, where no herbicides were applied, acetoin rates in fresh foliage weight were high (11.34 to 14.01) which confirmed validity of the assay according to the described method (Table 4). By increase in rates of both of the applied herbicides, acetoin rates reduced, which is obvious on the logarithmic dose-response curve on which relative activity of ALS enzyme were presented in relation to the control (Figure 3). In populations from Kikinda, Krivaja

Table 5. Significant differences between different populations and susceptible standard treated by imazethapyr.

Population	Activity of ALS enzyme
Kikinda-S	*
Vrbas-S	ID
Krivaja-S	*
Kačarevo-S	*

p < 0.05*; ID, statistically insignificant differences; S, susceptible standard.

and Kačarevo localities, statistically significant differences were established during changes of a range of imazethapyr rates in relation to the susceptible standard (Table 5). Similar results were obtained by Sprague et al. (1997), who studied two populations of *A. rudus* in a range of different hlorimuron, primisulfuron and halosulfuron rates, the herbicides from the group of sulfonylureas. Resistant population of *A. rudis* was highly resistant to hlorimuron at enzyme level and was based upon expressed activity of ALS enzymes at all applied rates of this sulfonylurea. At the highest dose of imazethapyr (0.40 kg a.i./ha), acetoin rate in the populations from Kačarevo (7.78 µg g^{-1} fresh foliage weight h^{-1}), Krivaja (7.63 µg g^{-1} fresh foliage weight h^{-1}) and Kikinda localities (5,30 µg g^{-1} fresh foliage weight h^{-1}) was respectively 22.88, 22.44 and 15.58 times higher in correlation to the susceptible standard (0.34 µg g^{-1} fresh foliage weight h^{-1}) (Table 4).

Based upon ALS enzyme activity studies *in vitro*, Sibony et al. (2001) established that, ALS R population of *A. retroflexus* was 11 to 14 fold more active in relation to S population. Higher IR values for the activity of ALS enzyme in R population and in relation to IR for morphometric parameters were obtained by many authors (Sibony et al*.,* 2001; Osuna and Prado, 2003; Hanson et al., 2004). This method of study of enzyme activity is more sensitive and a precise method in relation to those that follow morphometric changes in plants and results that suggest that in *A. retroflexus* populations from Krivaja, Kačarevo and Kikinda localities exist resistance to herbicide imazethapyr action. The results of ALS enzyme activity suggest that, resistance can be the consequence of reduced activity of ALS enzyme. This conclusion is derived in many studies in which high activity of ALS enzymes was confirmed in R populations (Saari et al*.,* 1994; Lovell et al., 1996 a; Sprague et al*.,* 1997).

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