THE STUDY OF METHODS FOR DETERMINATION OF METABOLISM BASED RESISTANCE OF ABUTILON THEOPHRASTI MEDIC. TO ATRAZINE

PREUČEVANJE METOD ZA UGOTAVLJANJE ODPORNOSTI ABUTILON THEOPHRASTI MEDIC. NA ATRAZIN NA PODLAGI METABOLIZMA

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ABSTRACT

Different methods for recording morphological and physiological parameters were used to define the level of susceptibility/resistance of Abutilon theophrasti populations to atrazine, in the period between 2002 and 2003. Three different populations of A. theophrasti from the fields near Belgrade were investigated. Total leaf weight and fresh and dry shoot weight among morphological parameters as well as transpiration, water vapour diffusion, chlorophyll fluorescence and total amount of chlorophyll among physiological parameters were measured. Minor differences to atrazine application between the tested populations of A. theophrasti were observed. There are some differences in all morphological parameters, but the most informative ones were the total fresh and dry shoot weight. Although the measurements of chlorophyll fluorescence caused lower differences between tested populations, this method proved itself as most sensitive among physiological methods. Calculated resistance indexes based on different parameters ranged from 2.2 to 15.9.

Keywords: Abutilon theophrasti/ herbicide resistance/ triazine resistance/ morphological parameters/ physiological parameters/ chlorophyl fluorescence

IZVLEČEK

V prispevku so prikazane različne metode za določanje morfoloških in fizioloških parametrov. Z njimi smo želeli v obdobju med 2002 in 2003 ugotoviti razmerja občutljivih in odpornih populacij vrste Abutilon theophrasti na atrazin. V okviru morfoloških parametrov smo ugotavljali skupno listno maso plevelov ter svežo in suho maso nadzemnih delov, v okviru fizioloških parametrov pa smo spremljali transpiracijo, stomatalno prepustnost vodne pare ter fluorescenco in količino klorofila. V raziskavo so bile vključene tri različne populacije vrste A. theophrasti iz okolice Beograda. Iz rezultatov je razvidno, da med populacijami preučevane vrste, tretiranimi z atrazinom, ni bilo velikih razlik. Pri vseh morfoloških dejavnikih, ki smo jih spremljali, smo ugotovili le manjše razlike. Najbolj značilne razlike smo zaznali pri sveži in suhi masi nadzemnih delov plevela. Kljub temu, da smo pri meritvah fluorescence klorofila ugotovili majhne razlike med preučevanimi populacijami plevela, se je ta metoda izkazala med fiziološkimi metodami kot najbolj občutljiva. Indeks odpornosti preučevanih populacij plevelov je bil med 2.2 in 15.9.

Ključne besede: Abutilon theophrasti/ odpornost na herbicide/ odpornost na triazine/morfološki parametri/ fiziološki parametri/ fluorescenca klorofila

POVZETEK

Odpornost plevelov na herbicide, vključno z atrazinom, je pojav, ki močno otežuje pridelovanje gojenih rastlin v številnih državah sveta. Način delovanja herbicidov, število aplikacij, ozek kolobar in pridelava z minimalno obdelavo v veliki meri pripomorejo k razvoju odpornosti. V nasprotju s toleranco, ki je dedna, se odpornost razvija v razmerah visokega selekcijskega pritiska, ki ga na populacije plevelov, ki so naravno občutljivi, izvajamo z uporabo herbicidov. Pridelovalci v Srbiji že več desetletij v glavnem uporabljajo herbicide na podlagi atrazina, predvsem zaradi njihove dobre učinkovitosti in selektivnosti, enostavnosti uporabe in nizke cene. Zaradi tega je delež teh pripravkov, ki jih v Srbiji uporabljajo pri pridelovanju koruze, večji kot 70 %. V zadnjih letih opažamo, da na njivah, kljub uporabi atrazina, ostajajo pleveli, ki so sicer občutljivi na atrazin. Med temi pleveli je eden najkonkurenčnejših baržunasti oslez (Abutilon theophrasti Medic.). Z namenom, da bi ugotovili ali so z baržunastim oslezom zapleveljene njive posledica nepravilne rabe atrazina ali pojava odpornosti, smo v obdobju med 2002 in 2003 testirali različne metode, s katerimi smo ugotavljali morebitno odpornost vrste A. theophrasti na atrazin. Pri določanju razmerja med občutljivimi in na atrazin odpornimi populacijami smo med morfološkimi parametri ugotavljali skupno listno maso plevelov, svežo in suho maso nadzemnih delov ter fiziološke parametre kot so intenzivnost izhlapevanja, stomatalna odpornost za prepustnost vodne pare in fluorescenca ter količina klorofila. V raziskavo smo vključili dve domnevno odporni populaciji plevelne vrste A. theophrasti z njiv, kjer se koruza prideluje v monokulturi že več kot 5 let, pri čemer se uporabljajo zgolj atrazinski pripravki (Rvc, Rgr). V raziskavo je bila vključena tudi ena domnevno občutljiva populacija vrste A. theophrasti z njive, kjer pridelujejo koruzo v kolobarju brez uporabe atrazina (Sps). Iz rezultatov lahko razberemo, da pri uporabi atrazina obstajajo majhne razlike pri preučevanih parametrih med različnimi populacijami plevelne vrste A. theophrasti. Vsi morfološki parametri se v manjši meri sicer razlikujejo, pri čemer statistične značilnosti nismo dokazali. Največje razlike smo ugotovili pri skupni sveži in suhi masi nadzemnih delov rastlin. Pri spremljanju fizioloških parametrov pa sta se kot najbolj zanesljivi metodi izkazali meritvi fuorescence in količine klorofila, čeprav tudi v tem primeru med preučevanimi populacijami vrste A. theophrasti ni bilo večjih razlik. Potrdili smo, da je populacija plevelov iz Padinske Skele občutljiva na atrazin, prav tako kot tudi domnevno odporna populacija plevelov iz Glogonjskega rita. Nasprotno pa smo pri populaciji plevelov iz lokacije Veliki Crljeni ugotovili povečano odpornost, pri čemer je bil indeks odpornosti za omenjeno populacijo Rvc dokaj nizek, IR=3.

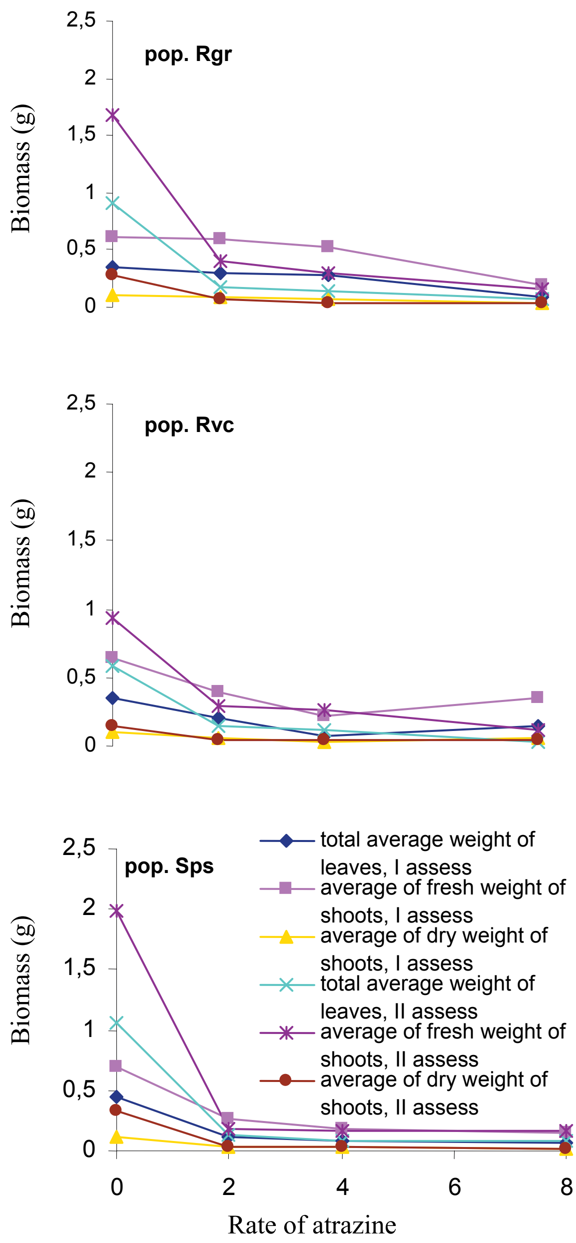
INTRODUCTION

Basic principles in weed control like crop rotation, low herbicide dose rates and the use of combinations of herbicides with different modes of action are not always a guarantee in the prevention of weed resistance. The efforts of lower use of herbicides led to the development of new herbicide survival mechanisms like cross and multiple resistance [6]. This problem led to the fact that science gave a great contribution to the control of resistant weeds. Analyzing triazine group of herbicides and resistance mechanisms plants developed, two basic mechanisms of resistance were determined: mutations at the target site of herbicide activity [7], and enhanced metabolism [9]. Glutation S transferase (GST) [16] or cytochrome P450 [17] are two basic enzymes that get activated in weeds under stress conditions, e.g. herbicide application. To confirm degree of weed susceptibility/resistance to atrazine, different morphological and physiological parameters have been studied. Many authors agree that the easiest way to confirm the resistance caused by mutation at the target place of herbicide activity is to measure the changes in fresh and dry plant weight [2, 13, 14] and by chlorophyll fluorescence [1, 5]. It is generally known that resistance based on enhanced metabolism appears at a lower level [8, 9] and also when herbicide is applied through the soil [8]. Recently we have observed in Serbia many fields infested with A. theophrasti despite the use of atrazine. It was suggested that lower herbicide efficacy was due to the atrazine resistance. The aim of this study was to define the level of susceptibility/resistance of A. theophrasti by monitoring different morphological and physiological parameters. The reliability of chlorophyll fluorescence, total amount of chlorophyll and chlorophyll extraction used for determining the resistance of A. theophrasti based on enhanced metabolism resistance was also studied.

MATERIAL AND METHODS

The responses of A. theophrasti populations to atrazine under controlled conditions were compared using the measurements of morphological and physiological parameters. The experiment was repeated two times as a completely randomized design with three replications per treatment.

Plant Collection. Seeds of A. theophrasti surviving in a 5-10-year atrazine-treated maize were collected in Glogonjski rit (Rgr) and Veliki Crljeni (Rvc) near Belgrade in 2002. A susceptible population (Sps) originated from



Rgr - presumable resistant pop. Glogonjski rit, presumable resistant Rvc - pop. Veliki Crljeni, Sps - susceptible pop. Padinska Skela. First assessment: 7 days after treatment, Second assessment: 15 days after treatment.

Figure1: Average value for the biomass of Rgr, Rvc and Sps populations of A. theophrasti treated with atrazine.
Slika1: Povprečne mase 3 populacij plevelov A. theophrasti Rgr, Rvc in Sps, treatiranih z atrazinom.

field near Belgrade (Padinska Skela) which had never been treated with atrazine. The experiments were conducted in the laboratories of the Agricultural Research Station BASF in Dinuba, California, USA, and in the Institute for Plant Protection and Environment in Zemun, Serbia in 2003.

Morphological and physiological responses. Plants were grown from seeds in a growth chamber in plastic pots (0.063 × 0.076 × 0.083 m) filled with a Sunshine 3® potting mix (Sun Gro Horticulture 15831 N.E. 8th Street, Suite 100 Bellevue, WA 98008 U.S.A.). The average daily temperatures ranged from 28 to 33 °C and the relative humidity from 75 to 80 %. Additional 350 μmol.m^{-2}.s^{-1} photon flux density (PPFD) was delivered by Sylvania cool white fluorescent light source (115 W), and Silvania 50 W standard light bulbs to complete a 15 h day. The plants were sub-irrigated as needed. At the 2nd leaf growth stage, plants were treated with atrazine (900 g a.i.kg^{-1}, trade name Gesaprim) at 2, 4, and 8 kg.ha^{-1} using a laboratory sprayer equipped with an 8001-E nozzle delivering 200 L.ha^{-1} at 276 kPa. Besides an untreated control was included. Seven and 15 days after the treatment (DAT) the total average weight of leaves, and the fresh and dry weight of shoots were measured. The fresh weights were measured immediately after harvesting. Leaf area was measured using a Delta-T leaf area-meter. Differences in transpiration intensity and stomatal resistance to water vapour diffusion were measured in the growth chamber between 9:00 and 11:00 h using a LI-1600 steady state porometer (LI-COR Lincoln, USA) at 7 and 15 DAT. Measurements were taken between the mid-vein and the margin at the widest portion of fully expanded leaves.

Chlorophyll fluorescence response. Three A. theophrasti populations were grown from seeds in 250 ml plastic pots filled with flower substrate "Floris" and placed in a growth chamber under 350 μmol.m^{-2}.s^{-1} PPFD delivered by Philips TLD 58 w/33 white fluorescent tubes and Tungsram 40 W incandescent light bulbs to complete a 15-h photoperiod. Day/night temperatures were 28/20 ± 2 °C, and relative humidity 70-75 %. Plants were sub-irrigated with 30 ml tap water every other day. Atrazine (900 g a.i. kg^{-1}) was applied at 2, 4, and 8 kg ha^{-1} onto 2 leaf growth stage plants using a sprayer for thin-layered chromatography. Fluorescence measurements were performed on the widest portion of the youngest fully expanded leaves, between the midrib and the margin, prior to atrazine application and at 1, 2, 3, 4, and 5 DAT. Intensity of chlorophyll fluorescence (Fv/Fm) was measured on each plant after 10-min dark adaptation using PAM 101 Fluorometer (Heinz Walz, Germany).

Data analysis. ANOVA, t-test and LSD test were used for

Table 1: Level of the differences between 3 *A. theophrasti* populations Rgr, Rvc and Sps, treated with atrazine for morphological parameters
Preglednica 1: Morfološke razlike med 3 populacijami *A. theophrasti* Rgr, Rvc in Sps, treatiranimi z atrazinom

Parameters	Average of fresh weight of shoot		Total average weight of leaves		Average of dry weight of shoot		Number of leaves per plant		Total average of leaf area		Average of plant height	
Treatment	7 DAT	15 DAT	7 DAT	15 DAT	7 DAT	15 DAT	7 DAT	15 DAT	7 DAT	15 DAT	7 DAT	15 DAT
[a]C - 2 $kg.ha^{-1}$	**	**	**	**	**	**	**	**	**	**	**	**
C - 4 $kg.ha^{-1}$	**	**	**	**	**	**	**	**	**	**	**	**
C - 8 $kg.ha^{-1}$	**	**	**	**	**	**	**	**	**	**	**	**
[b]Rvc : Rgr	*	**NS**		**NS**	**NS**	**NS**	**NS**	**	**NS**	**	**	**NS**
Rvc : Sps	**	**	**NS**	**	**NS**	**	**NS**	**	**	**	**	**
Rgr : Sps	**	**	**	**	**	**	**NS**	**	**	**	**	**NS**

C- untreated plants, NS- insignificant differences, DAT- days after the treatment, Rgr- presumable resistant pop. Glogonjski rit, presumable resistant Rvc- pop.Veliki Crljeni, Sps- susceptible pop. Padinska Skela, * - $p<0.05$, ** - $P<0.01$, [a]Comparison between treated and untreated plants for morphological parameters, average value, ANOVA, [b]Comparison between tested populations for morphological parameters, average value, ANOVA.

Table 2: Average of the resistance to diffusion and intensity of transpiration for 3 *A. theophrasti* populations Rgr, Rvc and Sps treated with atrazine
Preglednica 2: Povprečje odpornosti za difuzijo in intenzivnost izhlapevanja pri 3 populacijah *A. theophrasti,* Rgr, Rvc and Sps, tretiranih z atrazinom

		First assessment				Second assessment			
		Rates of atrazine ($kg.ha^{-1}$)				Rates of atrazine ($kg.ha^{-1}$)			
Parameters	Treatments	C	2	4	8	C	2	4	8
Rgr	Resistance to diffusion ($s.cm^{-1}$)	3.580	1.467	3.627	3.402	0.687	0.711	0.607	4.207
	Intensity of transp. ($micromol.cm^{-1}.s^{-1}$)	5.382	11.725	7.325	8.344	16.619	16.120	13.817	6.047
Rvc	Resistance to diffusion ($s.cm^{-1}$)	3.447	1.055	1.615	1.085	0.727	0.787	0.705	1.995
	Intensity of transp. ($micromol.cm^{-1}.s^{-1}$)	21.925	14.392	7.921	5.917	14.805	14.500	11.130	7.105
Sps	Resistance to diffusion ($s.cm^{-1}$)	2.370	14.737	8.067	2.960	0.512	0.557	0.397	11.560
	Intensity of transp. ($micromol.cm^{-1}.s^{-1}$)	5.055	1.099	5.617	6.115	20.687	19.282	20.662	1.389

C- untreated plants, Rgr- presumable resistant pop. Glogonjski rit, presumable resistant Rvc- pop. Veliki Crljeni, Sps- susceptible pop. Padinska Skela, Rate of atrazine: 2, 4 and 8 $kg.ha^{-1}$, First assessment: 7 days after treatment, Second assessment: 15 days after treatment.

data analysis and significant differences among treatments were considered at $p<0.05$ and $p <0.01$ level [20].

RESULTS

Morphological and physiological responses. Visual estimation of atrazine effect on tested populations of A. theophrasti (Rvc, Rgr, Sps) showed no differences between Rvc and Rgr. There were no statistically significant differences between analyzed populations in experiments with whole plants (data not shown). Due to resistance based on enhanced metabolism and time necessary for metabolic processes for this weed species (using GST enzyme resistant plants create conjugate) [8], the second evaluation (15 DAT) was chosen as more reliable plant response to atrazine application.

The inhibition percent of morphological parameters for all tested populations ranged in similar intervals: 42.86 to 94.50 % for Rvc, 66.67 to 92.13 % for Rgr, 62.50 to 95.67 % for Rps (Figure 1).

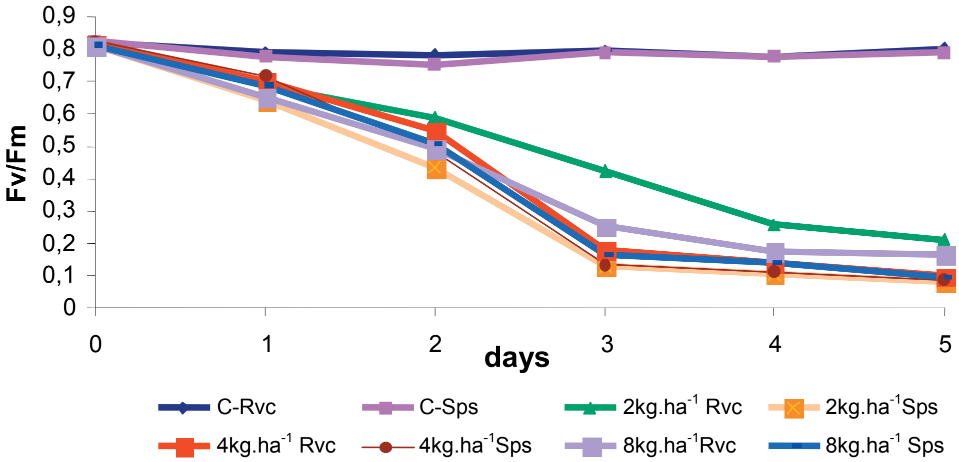
Comparing all morphological parameters, the plants height was least affected by atrazine application (ranged from 37.36 to 43.41 % for Rvc, 45.27 to 55.87 % for Rgr, 31.37 to 42.80 % for Sps related to untreated plants). Pavlović et al. (2006) reached similar conclusions when detecting higher differences in resistance to atrazine in tested populations of Chenopodium album and Amaranthus retroflexus.

There were no significant differences between Rvc and Rgr populations after atrazine application for total leaf weight, and total fresh and dry mass of plant parts.

Table 3: Index of resistance for morphological parameters of *A. theophrasti* populations Rgr, Rvc and Sps, treated with atrazine.
Preglednica 3: Index odpornosti za morfološke parametre pri 3 populacijah *A. theophrasti* Rgr, Rvc in Sps, treatiranih z atrazinom.

Parameters / Populations	Rvc:Rgr	Rvc:Sps	Rgr:Sps
Number of leaves per plant	2.5	14.8	5.8
Total average of leaf area	-	17	7.7
Total average weight of leaves	2.9	6.4	2.2
Average of fresh weight of shoot	4	15.9	3.9
Average of dry weight of shoot	4	14.7	3.6

Rgr - presumable resistant pop. Glogonjski rit, presumable resistant Rvc - pop. Veliki Crljeni, Sps - susceptible pop. Padinska Skela, Level of differences between the tested populations expressed as IR-index of resistance, ratio LD_{50} of presumable resistant pop. and susceptible pop. for morphological parameters. The highest differences between pop. Veliki Crljeni and Padinska Skela for all parameters are shown except total average weight of leaves.



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C - untreated plants, Rgr - presumable resistant pop. Glogonjski rit, presumable resistant Rvc - pop. Veliki Crljeni, Sps - susceptible pop. Padinska Skela, Rate of atrazine: 2, 4 and 8 kg.ha^{-1}, Average values of Fv/Fm for all unetaed plants were near 0.8, After 3th day the value of Fv/Fm decreased in all treated plants due to damage of FS II (ranged from 0.2 to 0.1).

Figure 2: Average value for the chlorophyll fluorescence of Rvc population of *A. theophrasti* treated with atrazine.
Slika 2: Povprečne vrednosti za fluorescenco klorofila pri populaciji *A. theophrasti* Rgr, tretirani z atrazinom.

Differences in measured morphological parameters were statistically significant ($p<0.01$) compared to untreated control, while between treatments differences were not always significant (Table 1).

Characterisation of tested A. theophrasti populations (Rvc, Rgr and Sps) by their susceptibility to atrazine was performed based on total fresh weight and according to scale suggested by Moss et al. (1999). Based on these criteria, early indication of resistance was confirmed in Rvc population.

Results showing atrazine influence to physiological parameters of A.theophrasti varied greatly in the first week of our experiments (Table 2). The second evaluation showed the tendency of increased resistance to stomatal diffusion and decrease of transpiration intensity with the increased application rates of atrazine. This is considered as a normal plant response to stress [19]. No significant differences between the untreated and treated plants were observed.

Based on correlation analysis of transpiration intensity and stomatal resistance to water vapour diffusion for Rvc, Rgr and Sps populations, statistically significant differences ($p<0.01$) of Rvc and Rgr populations compared to Sps population were observed. However differences between Rvc and Rgr populations were not statistically significant. A clear difference between A. theophrasti populations (Rvc, Rgr, Sps) based on different susceptibility to atrazine was shown by index of resistance calculated based on changes of morphological parameters (Table 3).

Chlorophyll fluorescence response. Changes in chlorophyll fluorescence were recorded based on the relationship between variable and maximum fluorescence (Fv/Fm) [3, 12]. There were no significant differences in Fv/Fm between Rvc and Rgr populations. Due to previously determined lower susceptibility of Rvc based on changes in fresh weight, further analysis was performed between Rvc and Sps populations (Table 1). The Fv/Fm values for both populations were significantly different in comparison to the untreated control values already after 24 h. The highest levels of fluorescence yield changes were recorded on a third day for both populations. Five DAT fluorescence yield for Sps population was 0.15. The results suggest the susceptibility of this population to atrazine (Figure 2). Differences in fluorescence yield in the first 24 h and five DAT were statistically significant ($p<0.01$). Small differences between Rvc and Rps populations were confirmed by the resistance index, IR=3.

DISCUSSION

Resistance can be confirmed when statistically significant differences between tested and susceptible populations exist, and when the resistant population can not be controlled using prescribed herbicide dose rates that control the susceptible population [2]. Parameters that define the total fresh and dry weight of plant shoots and total leaf weight have shown to be the most informative in determining the resistance and susceptibility of weeds to atrazine application. According to visual estimation of Rvc, Rgr and Sps A. theophrasti populations only minor differences in the susceptibility/resistance to the applied rates of atrazine were observed. Clear distinction between susceptible and resistant populations could be defined by parameters that define the mass (Table 1, Figure 1). All tested populations were susceptible to atrazine, still a 100 % plant destruction at the highest applied rate of atrazine (8 kg ha^{-1}) during the experiment (15 days) was not obtained. At 7 DAT and at highest applied atrazine rate the total fresh weight of Sps population was reduced by 78.70 % compared to untreated plants, while the reduction level for Rvc and Rgr populations was 44.60 % and 69.40 %, respectively. Similar results were observed for the total dry weight. Plants from Sps population had 80.40 % lower total dry weight compared to untreated plants, while plants from Rvc and Rgr populations had higher values (the level of inhibition compared to untreated plants was 50 % and 70 %). After Yaacoby (1986) more relevant confirmation of resistance is the one that develops by mutation of the target place of herbicide activity, which results in complete destruction or survival of plants – 100 % effect of atrazine. In cases where resistance is based on herbicide enhanced metabolism, plants need time to degrade the herbicide [4, 8, 9] or to die if they are susceptible. The resistance index of tested populations in our research revealed small differences in the susceptibility of Rvc and Rgr populations (Rvc/Rgr = 4 based on total dry weight; Rvc/Rgr = 4 based on total fresh green weight). The same conclusion applies to Rgr and Sps populations (Rgr/Sps = 3.6 based on total dry weight; Rgr/Sps = 3.9 based on total fresh shoot weight). Gray et al. (1995) reported that significant injury of S population (50 %) occurred using 1.1 kg.ha^{-1}, while resistant population needed 4.5 kg.ha^{-1} of atrazine. The differences between the resistant and susceptible populations can also be observed in plant parts, e.g. leaves, because of differences in their rate of metabolism [11]. Resistant plants under stress condition will have higher activity of the GST enzyme [15, 16]. Plaisance et al. (1995) reported that resistant plants with twice the amount of enzyme can be up to five times more active. The changes in physiological parameters in treated plants in our experiment were typical for the reactions of plants under stress conditions. The transpiration intensity decreased while the resistance to stomatal diffusion increased. Also daily changes of these parameters (not including the natural genetic plant variability) influenced significantly the variations in these responses and in that way reduced their reliability in resistance determination. No statistical differences between untreated and treated plants of A. theophrasti were observed even after the application of the highest atrazine rate (Table 2). Chlorophyll fluorescence measuring was most sensitive method for monitoring response of different A. theophrasti populations to atrazine. It is an easy, fast and reliable technique for determining the resistance as a consequence of mutation at the target site and/or increased metabolism [8]. Chlorophyll fluorescence (Fv/Fm) for untreated plants was 0.8 which is characteristic for plants that are not under stress conditions [3]. Values lower than 0.8 show that there was a damage to photosystem, which means that the electron transport has been blocked. In our study, the treated plants have shown changes in fluorescence yield already 24 h after atrazine application (Figure 2). Fv/Fm value for susceptible plants at lowest atrazine rate (2 $kg.ha^{-1}$) was 82.73 % lower, and presumably resistant population Rvc had 70.12 % lower values compared to untreated plants. As expected, the fluorescence yield at 8 $kg.h^{-1}$ of atrazine for both populations was the lowest (Sps= 84.76 %, Rvc= 74.91 %). Gronwald et al. (1989) showed similar level of initial fluorescence yield inhibition with R and S populations of A. theophrasti. Index resistance values for Sps and Rvc populations was 3 (IR=3), assuming this to be the initial resistance. This means that on the same fields, with the same weed management year after year, we can expect very soon to observe the development of resistance of A. theophrasti to atrazine.

CONCLUSIONS

- The most reliable parameters for distinguishing susceptibility/resistance of tested populations to atrazine in our study were the total fresh and dry shoot weight.
- Transpiration intensity and stomatal resistance to water vapour diffusion were not reliable enough for distinguishing susceptibility/resistance A. theophrasti to atrazine. Contrary chlorophyll fluorescence was found more reliable and sensitive method.
- Susceptibility of weed population from Padinska Skela was confirmed, as well as susceptibility of presumably resistant weed population from Glogonjski rit.
- An early resistance of weed population from Veliki Crljeni to atrazine was indicated.

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