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Article

The Transfer of ALS Gene Responsible for the Tolerance to ALS Inhibiting Herbicides between Different Sunflower (*Helianthus annuus* L.) Forms

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Abstract: Weedy sunflower is an invasive plant on the territory of the Republic of Serbia, which causes high yield losses in many crops. During the harvesting of the sunflower crops the dispersal of the seeds occurs, and as a result- the volunteer plants appear next year. Weedy sunflowers originate from volunteer plants that live through a longer period in one place. Spontaneous hybridization of weedy sunflower with other sunflower forms makes them more aggressive. If the volunteer plants originate from the hybrids tolerant to ALS inhibiting herbicides, they can be the carriers of herbicide tolerance genes and thus will not be sensitive to these herbicides. The exchange of the genetic material also enables the transfer of the ALS (AHAS) gene (responsible for the tolerance to the ALS inhibiting herbicides) to the progeny. In this study we have examined the spontaneous hybridization between different sunflower forms (volunteer sunflowers, weedy sunflowers, susceptible and tolerant sunflower hybrids to ALS inhibiting herbicides) in field conditions during three years. The progeny (F1 generation), which was assumed to possess the ALS gene, was tested with the application of the recommended doses of the Express (a.i. tribenuron-methyl) and Pulsar 40 herbicides (a.i. imazamox). The significant percent of the progeny of different forms of sunflowers, survived the herbicide treatment (6-31%). Molecular analysis of the ALS gene sequence in weedy sunflower progeny confirmed gene transfer in two cases at a distance of 30 and 120 m from the gene donor, i.e. tolerant hybrid Sumo 1 PR.

Keywords: hybridization; gene flow; different sunflower forms; imazamox; tribenuron-methyl; ALS gene

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1. Introduction

The creation of herbicide-tolerant crops has resulted from a good knowledge of the mechanisms of the herbicide activity at the molecular level, as well as the development of modern biotechnologies [1]. The herbicide-tolerant crops can be obtained through the application of genetic engineering methods, creating transgenes, i.e. genetically modified (GM) crops, or through the application of conventional selection techniques (tissue cultures, mutagenesis, and the use of resistant weed populations as gene donors for tolerance) [2], thereby creating non-transgenic herbicide-tolerant crops. In Serbia, the production of non-transgenic sunflower hybrids tolerant to ALS (acetolactate synthase) inhibiting herbicides (imidazolines) started in 2003 at the Institute of Field and Vegetable Crops, Novi Sad. The cultivation of tolerant hybrids obtained this way is widely accepted in Europe. Conventional selection has so far created a number of sunflower hybrids tolerant to imazethapyr, imazamox, and tribenuron-methyl, thereby solving the problem of a significant number of economically important weeds (*Ambrosia artemisiifolia*, *Xanthium strumarium*, *Orobanhe cumana*, etc), which cannot be effectively suppressed in non-tolerant sunflower crops to these herbicides. This is one of the key reasons why these hybrids have been accepted quickly and have occupied wide areas of the crop production in Serbia, as well as in many other parts of the world where the sunflower is traditionally grown, squeeze out the non-tolerant sunflower hybrids from the production.

During the sunflower crop harvesting, a dissipation of seeds occurs, which results in the growth of volunteer sunflower plants the following year. If the volunteer sunflower plants originate from the herbicide-tolerant hybrids they could become troublesome weeds in the subsequent crops, because they can be the carriers of tolerance genes and are therefore insensitive to those herbicides [3]. Also, due to genetic compatibility and overlapping of the flowering time, spontaneous hybridization between the volunteer, as well as weedy sunflower, with the tolerant cultivated sunflower, can occur. The transfer of genes responsible for herbicide tolerance is possible by pollen, because sunflower is an allogamous plant [4], as well as by seeds, due to their easy dispersal [5]. The exchange of genetic material also enables the transfer of ALS (also known as AHAS- acetohydroxy acid synthase) gene to the progeny in which this gene can persist for at least 5 years in volunteer plants [6], and considerably longer if the tolerant sunflower hybrids are grown in the vicinity. The possibility of the transfer of genes responsible for the sunflower resistance to herbicides into the weedy sunflower has been confirmed by many authors [4, 7-11].

The aim of this study was: a) to determine if spontaneous hybridization leads to the transfer of ALS gene from the tolerant to the susceptible sunflower hybrids, weedy and volunteer sunflowers; b) to estimate what percentage of the progeny from mentioned crossing shall survive the treatment by the recommended doses of the herbicides Express (a.i. tribenuron-methyl, 22.5 g/ha) and Pulsar (a.i. imazamox, 48 g/ha); c) to monitor the effects of applied herbicide on the treated plants, during a certain period of time after application; and finally d) to detect a mutation that causes tolerance to a.m. tribenuron-methyl using molecular analysis of the ALS gene sequence from progeny obtained in field experiments.

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2. Materials and Methods

Field experiments were conducted on the territory of Požarevac (on two sites) in 2013 and 2014, in order to record the spontaneous hybridization (gene transfer) between the tolerant (Sumo 1 PR (THS- tribenuron-methyl) and Rimi (THR- imazamox)) sunflower hybrids and susceptible sunflower forms (susceptible hybrid Duško (SHD), volunteer sunflowers originating from the susceptible hybrid Sremac (VS) and weedy sunflower (WS). All sunflower forms included in the study belong to the *Helianthus annuus* species. The seeds of all three crop hybrids were obtained from the Institute of Field and Vegetable Crops, Novi Sad. The seeds of the volunteer progeny of the hybrid Sremac were obtained from the school farm Radmilovac (wherein the previous year (2012), hybrid Sremac was cultivated), while the seeds of the weedy sunflower were also collected in 2012, on the territory of the agricultural areas of Surčin. The basic data of the experiment is shown in Table 1. The experiments were organized in 350 m long rows (3 rows), where the sowing was done by the pneumatic seeding machine with 27 cm spacing between plants, and 70 cm between the rows. In 2013, on-site No.1 (Požarevac, Metkor area) the tolerant hybrid Sumo 1 PR was sown in the first 50 m, and in the following 300 m SHD was sown in the first row, VS in the second and WS was sown in the third row. Following the same method, the second experiment was conducted on site No.2 (Toponica, Gaj area), only instead of the hybrid Sumo 1 PR, a tolerant hybrid Rimi was sown. In 2014, following the same method, the field experiments were conducted, with the rotation of the tolerant hybrids. On-site No.1 (Požarevac, Tulba area - about 4 km distance from Metkor) hybrid Rimi was sown, while on-site No.2 (Toponica, Selište area - at a 4 km distance from Gaj) Sumo 1 PR hybrid was sown.

Table 1. General data of the experiment for monitoring the transfer of genes resposible for the tolerance to ALS inhibitor herbicides, from tolerant sunflower hybrids to their congeners.

Sunflower form	2013				2014			
	Site	Sowing time	Flowering time	Harvesting time	Site	Sowing time	Flowering time	Harvesting time
THS	I	April 26	July 15-25	August 28	II	April 30	July 21-31	Sept. 7
THR	II	April 26	July 15-25	August 28	I	April 30	July 21-31	Sept. 7
SHD	I, II	April 26	July 15-25	August 28	I, II	April 30	July 21-31	Sept. 7
VS	I, II	April 26	July 18-30	August 28	I, II	April 30	July 23-August 7	Sept. 7
WS	I, II	April 18	July 15-Sept. 15	August 28	I, II	April 23	July 19–Sept. 6	Sept. 7

THS - tribenuron-methyl tolerant sunflower hybrid, THR - imazamox tolerant sunflower hybrid, SHD - susceptible sunflower hybrid, VS - volunteer sunflowers originating from the susceptible sunflower hybrid, WS - weedy sunflower.

In order to coordinate the flowering times of all four sunflower forms, the sowing of the weedy sunflower was done 7-10 days prior to the others (previous research has shown that the flowering of the weedy sunflower often starts later). The spatial isolation of both experiments was provided by the fact that within the radius of 3 km, there were no other sunflower fields, thereby excluding the possibility of existence of another donor of herbicide-tolerant genes in the vicinity. After the flowering, at different distances from the tolerant hybrids (30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 m) 10 plants in each of the three rows were marked on both sites, and in both years, following the same method. Each marked head, i.e. plant in hybrids, or more heads on a single plant, in the case of VS and WS, represented a single specimen, which was collected in the stage of full maturity, its seeds dried to 14% of moisture and stored at room temperature, for the next season, for further examination.

In the third year, at the site Toponica, the sensitivity of the progeny of SHD, VS, and WS to ALS inhibitor herbicides was tested. The results of the test were evaluated based on the plant's reaction to the application of the recommended doses of Express and Pulsar 40 herbicides. Tolerant hybrids THS and THR were used as a sensibility control, as well as the seeds of WS. The experimental plot was divided into two subplots. In one of the subplots the progeny from 2013 and in the other the progeny from 2014, were sown, respectively. Each subplot was further divided in two parts (sub-sub plots) for both hybrids THS and THR. For each combination, 100 seeds were sown, except for the progeny of WS, where 200 seeds were sown, as they are smaller and have a more pronounced dormancy. The sowing was done by hand in 5 m long rows, with a distance of 20 cm between the rows. After that, germination of the seeds of progeny was assessed. When the plants have reached the growth stage of 2 leaves pairs (BBCH 12) they were treated by the recommended doses of the appropriate herbicide- Express (a.i. tribenuron-methyl, 22.5 g/ha) or Pulsar 40 (a.i. imazamox, 48 g/ha). The herbicides were applied by a knapsack sprayer Solo 425 and XR TeeJet flat-fan nozzles to deliver a spray volume of 300 L water per hectare. The treatment effects were assessed 7, 14, 21, and 28 days after the application of herbicides. Weather conditions during the vegetation period for the area of Požarevac are provided in Table 2.

Table 2. Weather data for the area of Požarevac at the time of the experiment.

Year	GDD					Monthly rainfall (mm)				
	April	May	June	July	August	April	May	June	July	August
2013	145.7	289.2	336.5	436.6	476.6	21.8	104.0	49.3	2.7	43.3
2014	124.9	230.2	347.4	410.9	399.5	85.4	268.3	59.5	246.4	63.3
2015	100.8	278.8	351.0	508.1	479.9	30.2	82.1	38.0	11.0	49.5

GDD - Growing Degree Days.

The temperatures have been converted into GDD (Growing Degree Days), i.e. the sum of effective temperatures, using the following equation [12]:

$$GDD = \sum [(T_{max} + T_{min})/2 - T_{base}] \quad (1)$$

where T_{max} and T_{min} are daily maximum and minimum air temperatures (°C), and T_{base} is the basic temperature for the crop germination (10°C).

Progeny from spontaneous hybridization of weedy, volunteer sunflower, and susceptible sunflower hybrids with THS as the potential donor of the tolerance gene (field experiments in 2014) were studied using molecular analyzes of the sequence of the *Ahas1-2* [13], which controls tolerance to tribenuron-methyl.

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DNA isolation was performed according to the CTAB-KK v2 protocol [14]. The quality and quantity of isolated DNA was determined spectrophotometrically using NanoPhotometer® P-Class 300 devices (IMPLEN), by measuring absorbance at three wavelengths: A230 nm, A260 nm and A280 nm and by calculating the ratio A260 / 280 and A260 / 230. In order to amplify the sequence of the ALS gene (Table 6) within which the mutation that forms the basis of sunflower tolerance to herbicides ALS inhibiting herbicides occurs, the following pair of primers was used: the sequence of the forward primer 5- TCCGCAATGTGCTCCCACGT and the reverse 4- CACTCGAAGCCGGGTA CGCT [15-16]. Mentioned primer pair amplifies the AHAS 1, haplotype 3 gene sequence (https://www.ncbi.nlm.nih.gov/nuccore/AY541453.1).

PCR amplification was performed on a Mastercycler device (Eppendorf) according to the "TOUCH DOWN 3" program described in Dimitrijević et al. [17]. After PCR amplification, parts of the ALS gene sequence were digested with the restriction enzyme Kpn2I (Thermo Scientific). For restriction was used 10 units of restriction enzyme in the duration of 4 hours. Restriction enzymes Kpn2I performed successful digestion at 55°C while inactivation was performed at 80°C. Visualization of PCR amplification products, as well as restriction enzyme digestion results, was performed by agarose gel electrophoresis. Electrophoresis was performed on 1 and 1.5% agarose gels, with the addition of ethidium bromide (0.5 μg/ml) in 1 x TBE buffer, at a constant voltage of 100 V for 2.5 h. Electrophoresis was performed in a horizontal electrophoresis tub (Compact L/XL, Whatman, Biometra GmbH, Goettingen, Germany), while the gels were visualized in a UV transilluminator (ST4 3026-WL/26M, Wilber Lourmat, Torcy, France). A 100 bp DNA marker (DNA Ladder Plus, Fermentas, Vilnius, Lithuania) was used to determine the size of the fragments.

3. Results and Discussion

3.1. Field experiments

Spontaneous hybridization is a process that happens continually between both the closely related cultivated and weed/wild species, then between the populations of the weed or wild species, as well as, between different species [4, 18]. Crossing between cultivated plants and their wild relatives, weed species can be formed that have some properties, such as e.g. resistance to herbicides [19-20], and thus become resistant and very aggressive weed biotypes [21]. The theoretical transfer of genes between different forms, i.e. congeners of sunflower *H. annuus* (hybrid, weedy, volunteer, and wild sunflower), which grew at a relatively small distance of each other (min. distance 27 cm and max. 300 m), was possible between all forms, considering the genetic compatibility and overlap of the flowering and pollinating times. Therefore, there was a real possibility that, in a certain percentage, the gene responsible for the tolerance to ALS inhibitor herbicides was transmitted, in the process of spontaneous hybridization, from the tolerant hybrids Sumo 1 PR and Rimi to the individuals of other sunflower forms, such as the susceptible hybrid Duško, the volunteer plants of the susceptible hybrid Sremac and weedy sunflower. In favor of this claim, there is also the fact that the transfer of genes responsible for the herbicide tolerance occurs through pollen, because sunflower is a foreign pollinated plant [4]. The presence of alleles in the progeny resulting from the crossing of the hybrids and the weedy sunflower has been proven. Witton et al. [6] have found the alleles from the hybrid sunflower in 42% of plants of the weedy sunflower, found at a 3 m distance from the hybrid. With increasing the distance of the weedy sunflower plants from the hybrids, the frequency of the hybrid alleles in the progeny reduces. Witton et al. [6] have established the presence of the allele in the hybrid sunflower in 10% of the progeny at a distance of 200 m from the hybrids, and at a distance of 400 m in only 4% of progeny, which confirms the possible gene transfer. The transfer of pollen from the tolerant crops to the closely related species does not depend completely on the distance, but also on: the size of the populations from which the pollen originates and which it reaches, depends on the population density, the number of flowers per individual, the environmental conditions etc. [22]. Therefore, the working hypothesis of this research was a transfer of the gene responsible for the tolerance to ALS inhibitor herbicides occurred between the tolerant hybrids Sumo 1 PR and Rimi and the congeners of the same species, in this case the susceptible hybrid Duško (SHD), the volunteer plants of the hybrid Sremac (VS), and weedy sunflower (WS).

In general, the germination of the progeny from the spontaneous hybridization of the tolerant hybrid Sumo 1 PR with the susceptible hybrid (SHD), volunteer plants (VS) and weedy sunflower (WS) from 2014 was better, when compared to those from 2013, with the exception of the progeny of the WS where the opposite situation was recorded. The number and the percentage (in 100 sown seeds) of the germinated seeds were 70-82% in SHD, i.e. 68-75% in VS, and the lowest germination was recorded in the progeny of the WS (16-48%). Lower germination of the VS progeny, and especially WS is a trait that is characteristic for the forms which are genetically more distant from the sunflower hybrids, i.e. which are at the lower degree of domestication [23-25]. Also, there was no correlation between the number/percentage of the germinated seeds and the physical distance from the tolerant hybrid where the sample had been taken. However, cases there have been recorded where the germination of certain samples from the WS progeny was high (e.g. 158 germinated seeds in 200 sown seeds in a sample, at a 180 m distance from the THS), which can be related to two times greater number of seeds sown, as well as a clear population variability of the weedy sunflower (WS). However, in general, the progeny of the WS had higher germination in 2013, on average 48%, and in 2014- 16%, of the 200 seeds sown in total (Table 3).

Table 3. Germination and the percentage of the plants which have survived the herbicide application in the progeny of the spontaneous hybridization between the tolerant hybrid Sumo 1 PR (THS) and the other sunflower forms.

Sunflower forms	Year		Distance diferent sunflower forms from hybrid Sumo 1 PR at which the heads (seeds) sampled after spontaneous crossing (m)										G (%)	P (%)
			30	60	90	120	150	180	210	240	270	300		
SHD	2013	Ng	55	87	82	57	72	63	52	71	76	79	70	
		Ps	0	0	0	0	0	1.6	0	1.4	3.9	19.0		2.6
	2014	Ng	87	84	79	81	92	83	77	83	83	72	82	
		Ps	1.1	11.9	0	1.2	0	0	0	0	0	15.3		2.9
VS	2013	Ng	56	69	77	71	56	77	68	70	61	74	68	

		Ps	0	0	19.5	0	0	0	7.3	0	14.7	0		4.1
	2014	Ng	84	62	60	66	79	77	65	92	79	90	75	
		Ps	5.9	0	0	7.6	0	1.3	0	7.6	12.6	18.9		5.4
WS	2013	Ng	89	47	158	95	68	68	146	*	98	*	48	
		Ps	22.5	12.8	7.6	12.6	8.8	14.7	6.8	*	9.2	*		9.5
	2014	Ng	28	79	19	6	54	31	31	6	53	12	16	
		Ps	17.8	2.5	5.3	16.7	3.7	32.2	19.3	0	20.7	0		11.8

SHD - susceptible sunflower hybrid, VS - volunteer sunflowers originating from the susceptible sunflower hybrid, WS - weedy sunflower, Ng - the number of germinated seeds per sample, Ps - the percentage of plants which have survived the herbicide application per sample, G - average germination, P – the percent of plants which have survived the herbicide treatment, * - the plants that did not survive for other reasons.

The progeny of the SHD, VS, and WS responded differently to the application of the recommended doses of the herbicide Express (tribenuron-methyl). However, there were no differences in the percentage of the progeny from 2014 and 2013 which have survived in SHD (2.6-2.9%) and VS (4.1-5.4%), while in the progeny of the WS there was a difference in the survival after the application of the recommended doses of the Express (9,5% of the plants survived in 2013 and 11.8% in 2014) (Table 3). The percentage of the survival after the application of the herbicide was different on the level of one genotype (form), when compared to the samples analyzed, that is depending at what distance the sample had been taken, compared to the THS. In this case, there was medium negative correlation (-0.58) in the progeny from crossing between THS and WS in 2013 (Table 4). This data suggested that with the increasing of the distance from the tolerant hybrid the percent of the survived progeny plants reduced significantly. In the treatment by the herbicide Express, 6% of the total number of plants (4036/242), that represented the progeny from spontaneous hybridization, survived. On the other hand, the control plants (tolerant hybrid Sumo 1 PR) have survived the herbicide treatment in a high percentage (98-100%). Al-Khatib et al. [26] also suggest a high level of survival (82-86%) of the weedy sunflower populations after the herbicide application in the soybean plantation.

Table 4. Pearson coefficient of correlation (potential gene donor hybrid Sumo 1PR-THS).

Year		Sunflower forms		
		WS	VS	SHD
2013	D/S	-0.58	0.07	0.64
	D/G	0.18	0.20	0.09
2014	D/S	-0.04	0.63	0.13
	D/G	-0.26	0.52	-0.48

WS - weedy sunflower, VS - volunteer sunflowers originating from the susceptible sunflower hybrid, SHD - susceptible sunflower hybrid, D-distance from tolerant hybrid, S-percent of survived plants, G-percent of germinated plant.

Mercer et al. [27] as well as Bozić et al. [28] showed that F1 progeny (resulting from the crossing of sunflower tolerant to ALS inhibitors and wild/weed sunflower) have significantly better biological potential and significantly less sensitivity to herbicides compared to wild or weed parents. Contrary to the above, Snow et al. [29] indicate that the first generations were obtained from a cross between sensitive hybrids of cultivated sunflower and wild sunflower, mostly of poorer fitness than wild plants, even when grown in favorable conditions. However, this does not represent a barrier for further hybridization with other genotypes and strengthening of fitness and invasiveness in the following generations, especially if there is backcrossing with the wild parent [30]. In the

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progeny, where the potential gene donors were the tolerant hybrid Rimi and its congeners (SHD, VS and WS), the percent of plants that survived the imazamox treatment was significantly higher than the percent of the survivals of the progeny from the spontaneous crossing of the THS and its congeners, on which tribenuron-methyl was applied. On average, 30.5% (4233/1287) of the total number of plants (all three forms of sunflower, in both years) survived the imazamox treatment (Table 5). Presotto et al. [31] have also confirmed a certain degree of survival of the imazapyr application in the populations of weedy sunflower (16.4% at an application of 0.5x of the recommended herbicide dose). Besides, they have confirmed a high percentage of survival when applying imazapyr in the F1 generation of the weedy and tolerant sunflower hybrid crossbreeding (88.1% (0.5x), 86.7% (2x), 26.6% (8x)).

Table 5. Germination and the percent of plants which survived after the application of the herbicide in the progeny of the spontaneous hybridization between the tolerant Rimi hybrid (THR) and the other sunflower forms.

Sunflower forms	Year		Distance diferent sunflower forms from hybrid Rimi at which the heads (seeds) sampled after spontaneous crossing (m)										G (%)	P (%)
			30	60	90	120	150	180	210	240	270	300		
SHD	2013	Ng	80	81	78	75	83	84	90	77	81	69	80	
		Ps	47.5	50.6	33	26.6	39.7	44.1	51.1	42.2	25.9	26.1		38.6
	2014	Ng	69	*	74	*	77	66	74	81	82	77	75	
		Ps	21.7	*	12	*	0	0	14.8	11.1	0	0		7.3
VS	2013	Ng	88	71	69	76	60	81	75	66	64	70	72	
		Ps	75	69	48	57.8	55	72.8	65.4	74.2	81.2	71.4		67.5
	2014	Ng	71	*	66	*	71	69	61	77	74	65	69	
		Ps	45.1	*	54	*	29.6	18.9	40.9	18.2	12.3	0		27.1
WS	2013	Ng	*	88	76	72	84	102	76	81	92	98	42	
		Ps	*	14.8	6.6	18.1	14.3	31.4	9.2	0	15.2	7.1		12.9
	2014	Ng	92	*	87	86	81	90	77	84	93	102	44	
		Ps	60.8	*	54	41.8	50.6	17.8	14.3	8.3	6.4	8.8		29.2

SHD - susceptible sunflower hybrid, VS - volunteer sunflowers originating from the susceptible sunflower hybrid, WS - weedy sunflower, Ng - the number of germinated seeds per sample, Ps - the percentage of plants which have survived the herbicide application per sample, G - average germination, P – the percent of plants which have survived the herbicide treatment, * - the plants that did not survive for other reasons.

The germination of plants of the progeny (THR and SHD, as well as THR and VS) had higher values in 2013, in comparison to 2014- on the other hand, while through the hybridization of the THR and WS the progeny had approximately the same germination (about 43%) in both years. At the same time, the germination of the hybrid progeny of the THR and WS was significantly lower in comparison to the hybrid progeny of the THR and SHD (75-80%); THR and VS (69-72%).

The germination of the progeny of the SHD and VS from 2013 was higher (SHD= 80%, VS= 72%), compared to the progeny from 2014 (SHD= 75%, VS= 69%), while the germination rates of WS were lower in general, and more or less similar for both years (42% and 44%). There was no regularity or correlation between the number/percentage of the germinated seeds and the physical distance from the tolerant sunflower hybrids. Similar results regarding the germination of the weedy sunflower, as well as the germination of the progeny of the cross between the weedy and sunflower hybrid tolerant to imazapyr, have been confirmed by Presotto et al. [7]. They have also confirmed that the germination of the weedy sunflower increases with age (fresh seeds had a germination rate of 15%, 6-month-old seeds had a rate of 40-60%, and 12 months old seeds 75-90%). This confirms the hypothesis that the germination percent of the weedy sunflower depends on the seeds' dormancy. Furthermore, Presotto et al. [7] have confirmed that the progeny originating from a cross between the weedy sunflower and a hybrid tolerant to imazapyr shows a significantly higher germination rate, when compared to the weedy sunflower, regardless of its age, which also confirms the hypothesis that as the species/genotype is at a lower degree of domestication, the seeds have a more pronounced dormancy [23-24].

The progeny of the SHD, VS, and WS have reacted differently to the application of the recommended doses of imazamox. Unlike the progeny of the spontaneous cross between the THS and a congener which has been treated by the tribenuron-methyl, the progeny of the cross between the THR and a congener treated by imazamox survived the herbicide application in a greater percentage (Table 3, 4). Therefore, based on this, it can be assumed that a higher percent of genes (AHAS) responsible for the tolerance to ALS inhibiting herbicides was transferred from the THR to its congeners, than from the THS to the same congeners. Also, the percent of the progeny of the spontaneous cross between the THR, SHD, and VS from 2013, which have survived the application of herbicides was higher than the progeny from 2014 (in 2013 SHD= 38.6%, VS= 67.5%; in 2014 SHD= 7.3%, VS= 27.4%). However, with the progeny of the WS, the situation was reversed, that is the progeny of the WS from 2014 survived the application of imazamox in a much higher percentage (29,2%)- compared to the progeny from 2013 (12.4%) (Table 5). According to this, analyzed data indicate that during the crossing of THR with its congeners weak to moderate negative correlation existed (Table 4, 6) in 2013. On the other hand, in 2014 the progeny from a cross between THR and WS or THR and VS showed a strong negative correlation (-0.91 and -0.99). This data shows that the increasing the distance from the tolerant hybrid significantly reduces the survival of the progeny plant, which further increases the possibility that there has been a transfer of the ALS gene.

Table 6. Pearson coefficient of correlation (potential gene donor hybrid Rimi-THR).

Year		Sunflower forms		
		WS	VS	SHD
2013	D/S	-0.24	-0.74	-0.66
	D/G	0.43	-0.46	-0.19
2014	D/S	-0.91*	-0.99*	0.60
	D/G	0.27	0.03	-0.63

WS - weedy sunflower, VS - volunteer sunflowers originating from the susceptible sunflower hybrid, SHD - susceptible sunflower hybrid, D-distance from tolerant hybrid, S-percent of survived plants, G-percent of germinated plant, * - a statistically significant difference.

Massinga et al. [4] have, in a circle designed experiment, established a clear correlation between the percent of plants in the progeny (from the cross of the tolerant hybrid, a susceptible hybrid, and weedy sunflower) which have survived the application of imidazolinone and the distance of the congener to the tolerant hybrid, from which the seeds had been collected (at a 2.5 m distance 11-22% have survived, and at a distance of 30 m 0.3-5% percent of progeny survived). According to Hvarleva et al. [32], the transfer of genes is the strongest at a 3 m distance from the source of the pollen and is about 27%. In general, the gene transfer depends on the humidity, temperature, genotype, wind, pollinators, etc. The same authors claim that the gene transfer in sunflower is also possible at a 1.5 km distance.

Al-Khatib et al. [26] have confirmed, after 7 years of continuous application of imazethapyr, that a high percentage of the weedy sunflower plants survive the application of a 32x higher amount of herbicide than the recommended dose. Weedy sunflower populations which were not exposed to the herbicide treatment did not survive, even with the application of a 0.25x amount of the recommended dose of imazethapyr [26].

The percent of the progeny which has survived the herbicide treatment differed at the level of the same genotype between the congeners, but also between the years. This can be related to the differences in the weather data under which the plants have developed (2014 is extremely humid, 2013 is more-less dry). Furthermore, in spontaneous hybridization all the flowers in one head have not been pollinated by the pollen of the same origin (from the same plant), nor by the same pollinator (e.g. which carries the ALS gene). Therefore, all of the achenes (seeds) in the same head don't need to be the carriers of the gene responsible for the herbicide tolerance, even in the case when there is a donor of this gene (a tolerant hybrid) in the vicinity of the congener. Therefore, in theory, and in practice, the seeds from a single head can produce the progeny of different sensitivity to herbicides. In general, in our studies, a significant percentage of progeny survived herbicide application, especially when the potential donor of the tolerant ALS gene was a tolerant Rimi hybrid and this may be related to the fact that sunflower hybrids are tolerant to imidazolinone carriers of both types of tolerance: target tolerance actions (target site) and tolerance outside the target site of herbicide action (non-target site tolerance) [33-34]. The very complexity of this tolerance mechanism in Rimi hybrids, as well as its progeny after hybridization, enables a significantly reduced sensitivity, ie tolerance (resistance) to herbicides from the imidazolinone group. Cytochrome P450 is responsible for the expression of tolerance outside the target site of herbicide action, whereby inhibition of P450 monooxygenase results in a high degree of plant tolerance to herbicides due to herbicide metabolism [35].

3.2. Molecular analysis

Molecular analysis of the ALS gene in the progeny of susceptible hybrid (SHD), volunteer sunflower (VS), and weedy sunflower (2014) obtained from spontaneous hybridization, showing a very low level of gene transfer. The following profiles were obtained by combining the primer pair For 5- Rev 4 [17] and the restriction enzyme Kpn2I [15-16]:

- tolerant parent - fragments of length 437 and 183 bp were obtained,
- sensitive parent - a fragment of length 620 bp was obtained
- tolerant heterozygous progeny - contained all three mentioned fragments.

The combined primer pair is codominant and made it possible to distinguish homozygous tolerant from heterozygous tolerant genotype. Of the ten samples of weedy sunflower progeny (Figure 1), heterozygosity was observed only in sample W1, which proves gene transfer and hybridization with the tolerant Sumo 1PR sunflower hybrid. Sample W1 represented progeny from a weedy sunflower plant at a distance of 30 m from the pollen source (tolerant hybrid).

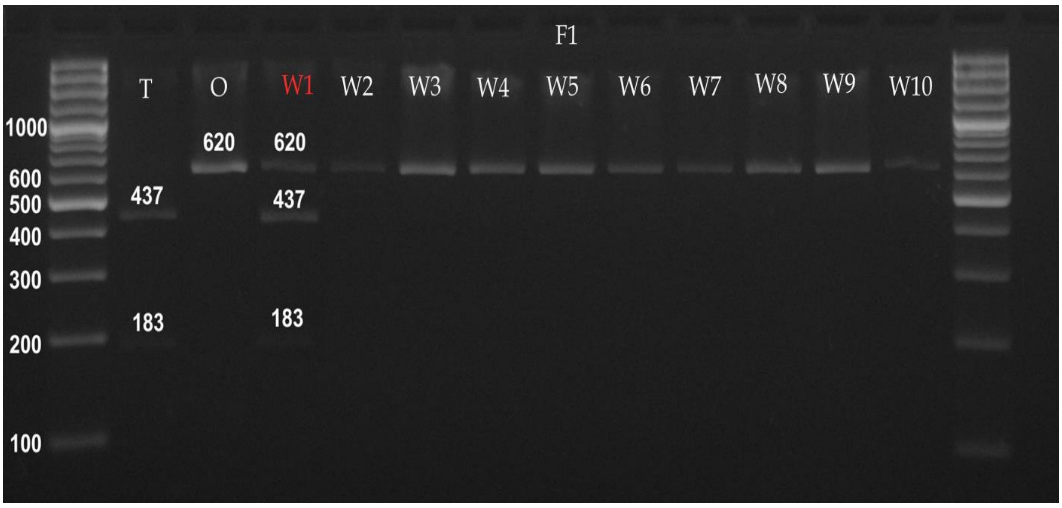

Figure 1. Profiles obtained by digestion with Kpn2I enzyme amplified by For 5- Rev 4 primer pair. A scale of 100 bp was used. T- tolerant parent, O- sensitive parent, W1-10 (progeny of weedy sunflower, 2014, from different distances).

Since gene transfer was molecularly proven only in the progeny of weedy sunflower, in the group sample from a distance of 30 m from the hybrid pollen source, the analysis of individual plants was continued. In a sample of 10 individual plants, molecular analysis revealed gene transfer in one plant, which is shown in Figure 2.

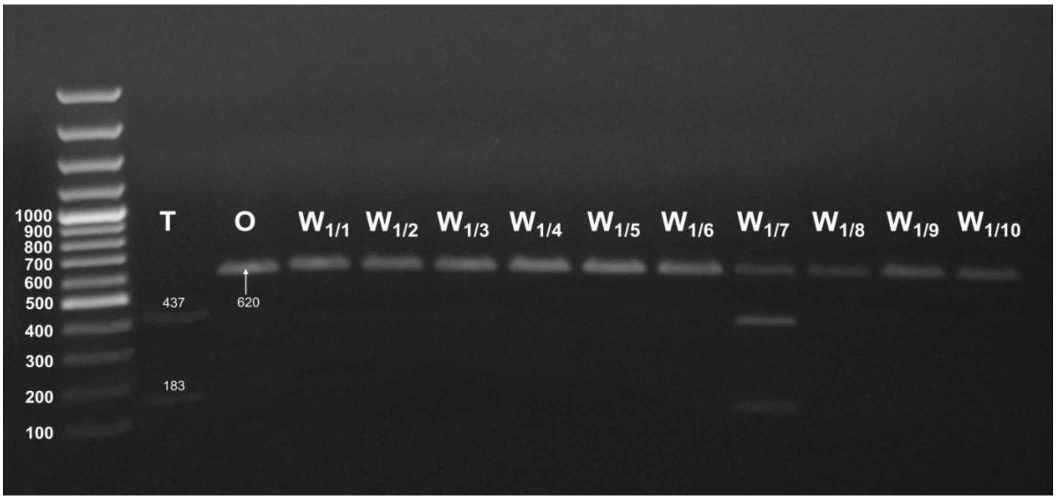

Figure 2. Profiles obtained by digestion with Kpn2I enzyme amplified by For 5- Rev 4 primer pair. A scale of 100 bp was used. T- tolerant parent, O- sensitive parent, W1- individual samples from crossing THS x weedy sunflower 30m (W1/7 hetrozygote).

Leaf samples were collected from the plants of the progeny of weedy sunflower from spontaneous hybridization, where the potential gene donor was THS (2013), which survived the application of herbicides, and their DNA was processed in the same way as in the previous case. For the four examined samples, the profiles shown in Figure 3 were obtained. Of the four tested progeny plants that survived herbicide treatment, and in which it was assumed that there was gene transfer, only in one plant this was molecularly confirmed. F1 progeny (THS x weedy sunflower) from a distance of 120 m from the pollen source, which survived herbicide treatment, showed heterozygosity based on molecular analysis.

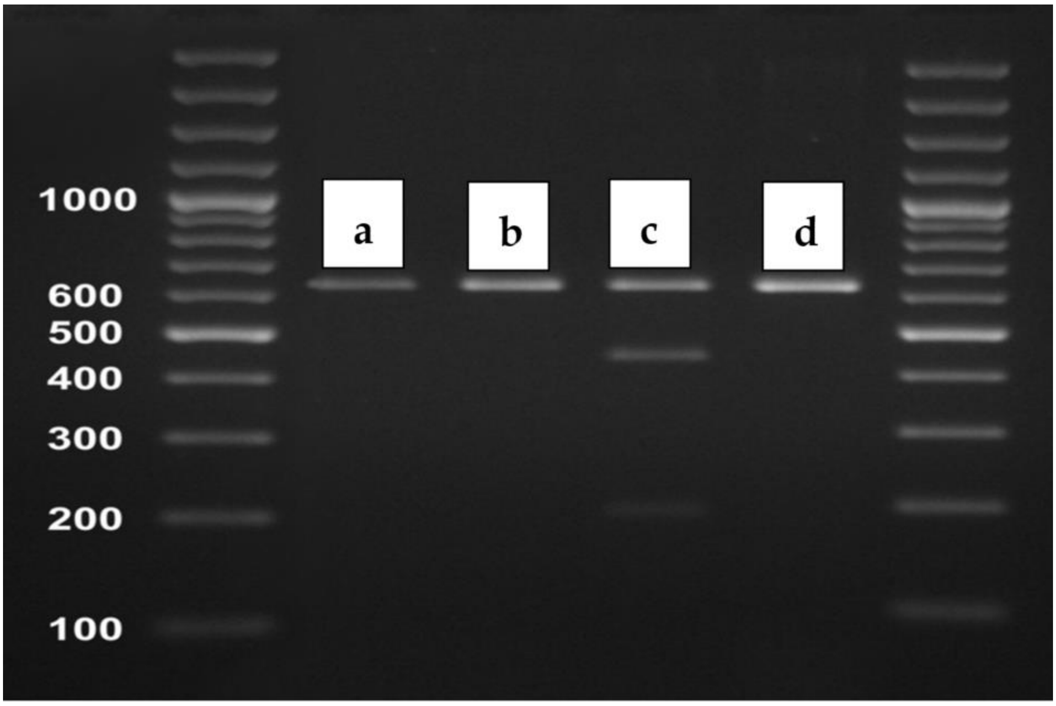

Figure 3. Profiles obtained by digestion with Kpn2I enzyme amplified by For 5- Rev 4 primer pair. A scale of 100 bp was used. T- tolerant parent, O- sensitive parent, progeny that survived herbicide treatment: a - weedy sunflower 30 m; b - weedy sunflower 90 m; c - THS x weedy sunflower 120 (progeny from a distance of 120 m from hybrids from crosses during 2013); d - weedy sunflower 180 m.

The results from field trials related to the percentage of plants that survived the use of imazamox and tribenuron-methyl do not completely coincide with molecular analyzes. Namely, gene transfer was confirmed only in two cases in the progeny of weedy sunflower, from a distance of 30 and 120 m from the pollen source (THS), while the percentage of plants that survived the application of herbicides in the field was significantly higher. On the other hand, the progeny of SHD, VS, and weedy sunflower from spontaneous hybridization, where the potential donor of the gene was a hybrid Rimi, survived the use of imazamox in a significantly higher percentage. Also, Božić et al. [11] obtained a low percentage of F1 progeny (from a cross between a tolerant hybrid of sunflower and weedy sunflower) that survived the application of herbicides (7.75%), with 48.9% of the surviving plants being heterozygous, ie. they had a tolerance gene transfer.

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Plants that survive herbicide application without molecular evidence of gene transfer may have other mechanisms to survive herbicide action such as herbicide detoxification (metabolism), altered absorption and translocation patterns, and mutations at the primary site of herbicide action. In addition to confirmed mutations at codons 197 and 205 [36-37], there are also mutations at codon 122 that cause high tolerance to imidazolinones [13], then at 574 codon that provides tolerance to 4 groups of herbicides [33]. Also, moderate tolerance to imidazolinones was confirmed as a result of a mutation at codon 203 [38].

Thus, regardless of the low level of gene transfer, there is a real risk that even such a small percentage of plants of the tolerant progeny of weedy sunflower in subsequent vegetations creates significant problems in controlling this weed in arable land. Since the genes of cultivated sunflower can persist for many years among wild populations [6, 39], weedy sunflower populations can be modified depending on their fitness [40-41], and also due to the selection pressure of predators, weedy sunflower can become significantly more invasive [31].

4. Conclusions

During field experiments in order to examine spontaneous hybridization, it was concluded that the progeny of the cross of the THS and its congener (SHD, VS, WS) has survived the application of the tribenuron-methyl in a lower percent (6% on average), while the progeny of the THR cross with its congeners has survived the application of imazamox in a higher percent (30.5% on average). Also, the progeny (field experiments from 2014) from a cross between THR and WS or THR and VS showed a strong negative correlation (-0.91 and -0.99, respectively). This data shows that increasing distance from the tolerant hybrid significantly reduces plant survival of the progeny, which further increases the likelihood that there has been a transfer of the ALS gene. Using primer pair For 5- Rev 4 and restriction enzyme Kpn2I, heterozygosity (characteristic of tolerant sunflower genotypes) was confirmed in the analyzed progeny of weedy sunflower obtained from spontaneous hybridization where the potential gene donor was a tolerant Sumo 1PR hybrid. The tolerance gene was confirmed in one group sample, ie. a weedy sunflower plant that was 30 m away from the Sumo 1PR hybrid (2014, field experiment). Heterozygosity was found in individual plants of F1 progeny (THS x weedy sunflower) in one of the ten analyzed plants. Of the four selected plants of the progeny of weedy sunflower from 2013, which survived the use of tribenuron-methyl, one plant at a distance of 120 m from the pollen source (THS) was confirmed to be heterozygous. Despite the relatively low level of ALS gene transfer from tolerant sunflower hybrids to weedy sunflower, there is a risk of further spread of this invasive weed species in Serbia, reduced sensitivity to ALS inhibiting herbicides, and the development of cross-resistance, which requires intensive work to improve and the application of integral protection measures in the control of weedy sunflower.

Author Contributions: Conceptualization, S.V.; methodology, D.B. and T.B.; software, D.S.; validation, D.M.; formal analysis, D.S.; investigation, S.V. and D.P.; resources, D.M. and D.S.; data curation, D.B.; writing—original draft preparation, D.S. and T.B.; writing—review and editing, S.V, D.B., D.M. and A.R.; visualization, D.S.; supervision, S.V.; funding acquisition, S.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant numbers: 451-03-9/2021-14/200116, 451-03-9/2021-14/200032, 451-03-9/2021-14/ 200007 and 451-03-9/2021-14/200010.

Conflicts of Interest: The authors declare no conflict of interest.

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