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Gene flow from herbicide-resistant sunflower hybrids to weedy sunflower

Dragana Bozic[1], Danijela Pavlovic[2], Valeria Bregola[3], Alessandro Di Loreto[3], Sara Bosi[3] & Sava Vrbnicanin[1]*

[1] University of Belgrade, Faculty of Agriculture, Nemanjina 6, Zemun-Belgrade, Serbia

[2] Institute for Plant Protection and Environment, Belgrade, Serbia

[3] Alma Mater Studiorum University of Bologna, Department of Agriculture Sciences, Viale Fanin 44, Bologna, Italy

* Corresponding author: sava@agrif.bg.ac.rs

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Abstract

Weedy forms of cultivated sunflower (*Helianthus annuus*) are invasive species widely distributed in several regions of the world and are commonly controlled by applying acetohydroxyacid synthase (AHAS)-inhibiting herbicides, such as imidazolinones (IMIs) or sulfonylurea (SUs). The widespread adoption of herbicide-resistant crops has exposed the weedy population to the high risk of crop-to-weedy gene flow. The aim of this study was to check and quantify the gene flow from IMI- and SU-resistant sunflower hybrids to weedy sunflower populations. Field experiments were conducted in 2008 at two sites in Serbia to evaluate the relationship of distance between the crop and the weedy sunflower and its impact on the percentage of gene flow. The weedy sunflower progenies were evaluated through herbicide resistance and SSR marker study. Hybridization with IMI-resistant hybrids was not confirmed. Conversly, SU-resistance trials and SSR marker studies partially confirmed the transfer of resistance within the weedy population.

Key words: *Helianthus annuus*, hybridization, imazamox, pollen movement, tribenuron-methyl

Introduction

Helianthus annuus (Asteraceae/Compositae) is a species which occurs in many different forms. In addition to cultivated sunflower ("normal" crop plants) sunflower fields often contain a few "off-type" crop plants. Off-types are taller, multibranched crop plants that are probably derived from wild pollen contamination during the production of hybrid crop seed. Harvesting crops inevitably results in some unintentional loss of seeds. When these seeds germinate in subsequent years, they are called "volunteers". Volunteer plants exist in regions with sunflower production. True wild sunflowers (wild type) exist in origin area of cultivated sunflower in America. As a result of hybridization between volunteer, weedy or wild sunflower populations, weedy populations of this species (weedy type of sunflower) are developed (Reagon & Snow 2006). Weedy *H. annuus* are morphologically clearly different from cultivated and wild forms (Reagon & Snow 2006). In addition, weedy populations are characterized by highly pronounced morphological variability, wherein the ratio of properties of cultivated and wild forms in them varies (Reagon & Snow 2006).

Hybridization and introgression have continuously occurred between cultivated and wild or weedy relatives (Loureiro et al. 2006, Zahareva & Monneveux 2006, Andersson & de Vicente, 2010), as well as, between relative weedy and/or wild species (Tranel et al. 2002, Trucco et al. 2005). Generally, natural hybridization between crops and weeds had significant role in evolution of many weed species (Campbell et al. 2006). In studies regarding to hybridization between cultivated and wild radish populations Snow et al. (2001), Hedge et al. (2006) and Campbell et al. (2006) found that first-generation crop-wild hybrids are relatively fecund, hybrid populations rapidly evolve increased pollen fertility and produce large quantities of seeds. Also hybrid populations can persist for many generations and their growth dynamics are similar to those of wild populations, and relative hybrid fitness may differ dramatically among environments. In the case of sunflower, molecular analysis of French and Spanish weedy populations has shown that these weeds originated from the unintentional introduction of crop-wild hybrids into farmers' fields through contaminated seed lots (Muller et al. 2011). Although wild populations of sunflower are self-incompatible (Gandhi et al. 2005), modern crop sunflower varieties are about 65% autogamous (Astiz et al. 2011); weedy population as a result of their hybridization are self-incompatible. On the other hand, gene flow from crops to weedy relatives is associated with many problems. Namely, crop to weed gene flow can create new or more harmful weeds for some of the most important crops (Ellstrand et al. 1999). Another problem is gene flow from herbicide-resistant hybrids (obtained with or without biotechnology) to weedy or wild relatives, because their progeny will be resistant weeds. Generally, the interspecies and intraspecies crossing in *H. annuus* and its close relatives were identified (Arias & Rieseberg 1994, Burke et al. 2002).

Three technologies of weed control which include crop resistance to herbicides based on mutations at the *Ahasl1* locus were developed for sunflower (Sala & Bulos 2012). The Clearfield_system (Tan et al. 2005) and the Clearfield-Plus_system (Sala et al. 2008a) have been developed for growing of sunflower hybrids resistant to IMI herbicides. Those hybrids have been developed either by subsequent crossing of resistant wild sunflower (Al-Khatib et al. 1998) with cultivated sunflower lines or as the result of seed

mutagenesis (Sala et al. 2008a). ExpresSun system has been developed for growing of sunflower hybrids resistant to tribenuron-methyl (Canadian Food Inspection Agency 2008) which were developed as the result of mutagenesis breeding (Sala et al. 2008b).

Our recent survey showed that weedy sunflower had the biggest population in north part of Serbia, particularly in southern Srem (~ 1000 ha) and southern Banat (~ 7–8000 ha) (Saulic at al. 2013). First population of weedy sunflower forms were detected by Radjenovic (1978). Due to high competitive ability, invasiveness and increasing area with tolerant sunflower hybrids problem with the weedy sunflower form had increased during the last decade. Weedy sunflower is also considered of major concern in the sunflower growing areas of the Balkan Peninsula like Hungary (Benécsné Bárdi et al. 2005), but also Croatia and Romania. Weedy sunflower populations had been detected in Spain (Poverene & Cantamutto 2010), central Italy (Vischi et al. 2006) and France (Muller et al. 2009). Risk of gene flow from sunflower hybrids to wild relatives was confirmed by some researchers (Marshall et al. 2001, Massinga et al. 2003), who found that gene flow depends on distance.

The objectives of this study were to check and quantify the gene flow from RIMI and RSU sunflower hybrids to weedy sunflower. Therefore the main hypotheses were: (*i*) gene flow between tolerant sunflower hybrids and weedy sunflower can occur in field conditions; (*ii*) distance between weedy and crop sunflower will affect gene flow; (*iii*) wind direction at the time of the pollination will affect gene flow; (*iv*) the progeny of weedy sunflowers will have a weedy-crop hybrid profile.

Materials and methods

Seeds of the sunflower hybrids RIMI (resistant to imazamox) and RSU (resistant to tribenuron-methyl) were obtained from the Institute of Field and Vegetable Crops in Novi Sad (Serbia). Sunflower hybrids resistant to sulfonylurea (SU) and imidazolinone (IMI) were developed using non-transgenic methods. The donor of genes for resistance of RIMI hybrid to the imazamox was a wild *H. annuus* population collected at Rossville, Kansas, USA (Al-Khatib et al. 1998). In this case resistance is controlled by a single gene and the mode of inheritance of resistance to the imidazolinones is partial dominance (Jocic et al. 2001). The sources of genes for resistance of RSU hybrid to tribenuron-methyl were the genetic stocks SURES-1 and SURES-2 (Miller & Al-Khatib 2004). SURES-1 and SURES-2 are homozygous for resistance to tribenuron-methyl and F1 generations produced from the crossings are completely resistant to tribenuron-methyl. The mode of inheritance of this trait is dominant and exact number of genes controlling resistance is still unknown (Jocic et al. 2011). Bozic et al. (2012) confirmed high level of resistance to imazamox for RIMI and to tribenuron-methyl for RSU hybrids. In autumn 2005, weedy sunflower seeds were collected from a rural area in Padinska Skela, where no herbicides had been applied in the previous 5 years. Collected seeds were stored at room temperature (approximately 20 to 25°C) in the laboratory until use.

Gene flow studies from RIMI and RSU sunflower hybrids to weedy sunflowers were assessed in 2008 at two sites (S1 and S2) located in Oplanic, Serbia (43°59'13 N, 20°40'44 E). Distance between the two experimental sites was 3 km. In sites S1 and S2, IMI- and SU-resistant sunflowers, respectively were sown in a 5 m diameter circle. Interrow spacing of seeds was 24 cm and the distance between rows 70 cm. Surrounding the IMI- and SU-resistant hybrids, weedy sunflower were sown in concentric circles at distances of 1, 2, 3, 4 and 5 m from the outside border of the central circle, with interplant spacing of 24 cm (Fig. 1). An overview of the time line in the trials is shown in Table 1. Precipitation and growing degree days (GDD), calculated using a baseline temperature of 10°C, are summarized in Table 2. During the sunflower flowering period, daily wind speed and prevalent direction were recorded (Table 3). The collected information on the main meteorological parameters was employed to evaluate possible pathways of gene flow between donor and acceptor sunflower. To estimate the gene flow from IMI- and SU-resistant hybrids to weedy sunflowers, fields were

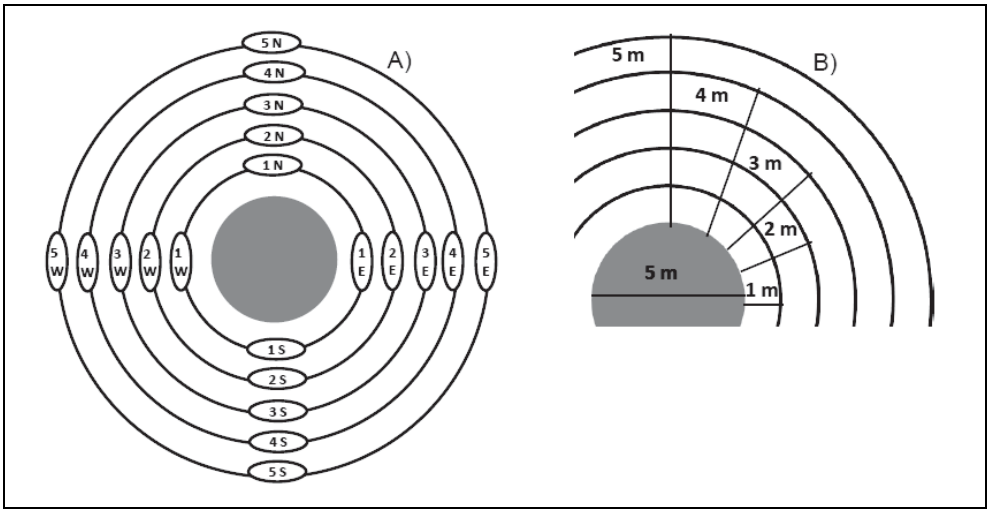

Fig. 1: Harvest diagram of field experiment. Each experimental site was divided into 4 equal slices corresponding to compass direction points (A) and each slice into 5 arc slices corresponding to distance from RSU pollen source (B).

Table 1: Time line and additional information about the gene flow experiment

	Site 1	Site 2
Preceding crop	Pea	Maize
Sowing date	April 12	
Date of the begining of sunflower hybrid flowering	July 01	July 06
Date of the end of sunflower hybrid flowering	July 09	July 17
Date of the begining of weedy sunflower flowering	July 06	July 08
Date of the end of weedy sunflower flowering	end of August	
Date of harvest	September 13	

Table 2: Monthly precipitation (mm) and growing degree days during the field experiment

Month	Precipitation (mm)	GDD
April	32.70	12.80
May	13.10	232.00
June	59.00	334.45
July	51.60	328.60
August	41.40	361.45
September	50.20	164.65
Total	248.00	1433.95

divided into 4 equal slices corresponding to each of the compass direction points (N, E, S, W) (Fig. 1). Each slice included 5 arc slices corresponding to the evaluated distance (1, 2, 3, 4, 5 m) (Fig. 1). Thirty heads of weedy sunflower (approx. 150–180 seeds per head) from each arc slice (4 slices × 5 arc slices) were collected at the maturity. After cleaning, achenes were stored at room temperature (~ 20–25°C) in the laboratory until use.

To determine gene flow from sunflower hybrids to weedy populations, achenes collected from the 20 arc slices of each experimental field (S1 and S2) were sown in pots (38 cm^2 surface area) containing a commercial potting mix (Flora Gard TKS1, Germany). Pots were placed in a controlled environment chamber set at 300 μmol $m^{-2}s^{-1}$ photosynthetic photon-flux density (PPFD), 16 h photoperiod and 24°C as average temperature. Plants were manually irrigated as required.

Imazamox (applied to the plants of weedy sunflower collected in the site S1) and tribenuron-methyl (applied to the plants of weedy sunflower collected in the site S2) were sprayed post-emergence onto plants with four true leaves. Imazamox (Pulsar-40, 40 g a.i. l^{-1}, SL, BASF, Germany) was applied at 48 g a.i. ha^{-1} and tribenuron-methyl (Express 50-SX, 500 g a.i. kg^{-1}, WG, Du Pont, Switzerland) at 22.5 g a.i. ha^{-1} with 0.1% surfactant Trend 90. Herbicides were applied using a laboratory sprayer equipped with an 8001E even-spray flat-fan nozzle delivering 200 l ha^{-1} at 276 kPa. The experiments were conducted three times in a completely randomized design with 30 plants per arc slice (15 pots × 2 plants). About 10 seeds per pot were sown and after emergence they were manually thinned to 2 plants. Visual assessment of plants was done 28 days after herbicide treatment (DAHT) and number of surviving plants was determined.

Achenes collected from the north slice (bulk sample) of the site S2 (see Fig. 1), that corresponded to the slice with the higher percentage of crop-weed hybrids, were used in the experiment. Seeds were sown in pots (30 pots × 2 plants

Table 3: Mean wind speed (m/s) and prevalent direction (%) observed during the flowering (from 06/07/2008 to 31/07/2008) for Oplanic (Kragujevac region). The frequency (%) of Beaufort wind force classes (from class 2 to class 5) observed during the flowering are reported for each wind direction.

	Wind direction	N 315°–45°	E 45°–135°	S 135°–225°°	W 225°–315°
Beaufort scale	Mean wind speed (m/s)	6.8	3.7	6.1	4.8
	Frequency (%)	53.8	15.4	15.4	15.4
	Light breeze (1.6 < m/s < 3.4)	–	25.0%	–	–
	Gentle breeze(3.5 < m/s < 5.4)	21.4%	75.0%	25.0%	50.0%
	Moderate breeze(5.5 < m/s < 7.9)	50.0%	–	75.0%	50.0%
	Fresh breeze (8.0 < m/s < 10.7)	28.6%	–	–	–

per herbicide dose) and grown in a controlled environment chamber under 760 μmol $m^{-2}s^{-1}$ photosynthetic photon-flux density (PPFD), 16/8 h photoperiod and 24°C average temperature. Tribenuron-methyl (Express 50-SX, 500 g a.i. kg^{-1}, WG, Du Pont, Switzerland) was applied at doses 11.25, 22.5 and 45 g a.i. ha^{-1} with 0.1% surfactant Trend 90, corresponding to ½, 1 and 2 times the recommended use rate of tribenuron-methyl, respectively. Plants were assessed 17 DAHT and were scored as dead or alive. Only tissues sampled from plants surviving tribenuron-methyl treatments were subjected to molecular analyses. Simple sequence repeat (SSR) markers based on pAHAS18/19 primers that identify allelic variants in the AHAS1 gene (Kolkman et al. 2004), were used to verify the percentage of gene flow from resistant hybrids to weedy sunflower. DNA extraction was carried out from leaf tissue (~ 100 mg) according to the CTAB procedure of Saghai-Maroof et al. (1984). PCR products were amplified in a 20 μl reaction containing 10× buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primers, 2 ng genomic DNA, and 0.5 U TrueStart Hot Start Taq DNA Polymerase (5 U μl^{-1}, Fermentas) (Kolkman et al. 2004). Amplification was as follows: 95°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 45 s, 72°C for 30 s and a final extension at 72°C for 10 min. PCR products were analyzed on 1.5% agarose gels.

Results and discussion

Despite the likelihood of spontaneous hybridization between the cultivated sunflower and wild relatives favoured by overlapping of flowering time, shared pollinators, self-incompatibility of wild sunflower, self compatibility of domesticated sunflower and high rate of outcrossing (Hvarleva et al. 2009), in our studies gene flow from RIMI to weedy sunflower was not confirmed. Namely, four weeks after recommended rate of imazamox treatment, all plants of weedy sunflower progeny from S1 site died (data not shown). These findings are not in agreement with previous studies (Burke et al. 2002, Massinga et al. 2003) that have detected gene transfer from domesticated sunflower to close relatives. Possible reason for the lack of gene flow can be ascribed to the short period of overlapping flowering time between the resistant hybrid and the weedy sunflower (Table 1).

Conversely, some plants of progeny of weedy sunflower, which were grown near RSU hybrid survived the recommended rate of tribenuron-methyl (Table 4). In particular, the number of hybrids between RSU hybrid and weedy sunflower ranged from 11.25% at 1 m to 5.00% at 5 m from the outside border of the pollen source. That is in agreement with other studies about gene flow between sunflower and its close relatives which reported that the number of hybrid individuals decreased as the distance from the source of the resistant pollen increased (Table 4) (Arias & Rieseberg 1994, Burke et al. 2002). Moreover, the highest percentage of surviving weedy sunflower progeny was observed for north direction (13.0%), while the smallest was on the east direction (5.0%) (Table 4). Many researchers (Marshall et al. 2001, Massinga et al. 2003) observed that wind can affected gene flow, probably through an effect on insect pollinator movement. Likewise, in the area of field experiment, the prevailing wind direction recorded during the flowering period came from south to north (53.8%), while frequencies of 15.4% were recorded for the remaining wind directions (E, S and W) (Table 3). In addition, the intensities of wind observed in the north and south direction were stronger than the other ones (east and west directions). In particular, in the north and south quadrants, the frequency of the classes 4 and 5 of the Beaufort scale was higher than 75% (78.6% and 75.0%, respectively). Data confirmed the highest levels of hybridization were noted in north and south directions. The results of the current study confirm the findings of several other field studies (Arias & Rieseberg 1994, Burke et al. 2002) and demonstrate the impact of wind direction on pollen-mediated gene flow in sunflower. However, as observed in the S1 site, because of the highly complex process of gene flow, the percentage of hybridization depends also on a number of other factors, such as distance between the crop and the weed, flowering status of source (cultivated crop) and receptors (weed) and meteorological conditions during pollen shed (Halsey et al. 2005).

For molecular analysis 44 plants which survived ½ x (14 plants), 1 x (25 plants) and 2 x (5 plants) tribenuron-methyl

Table 4: Percentage of surviving plants in progeny of weedy sunflower after application recommended rate of tribenuron-methyl

Distance	Survived plants (%)				Mean (%)
	Direction				
	North	East	South	West	
1 m	25	15	5	0	11.25
2 m	25	0	0	15	10.00
3 m	0	5	10	10	6.25
4 m	5	5	10	5	6.25
5 m	10	0	10	0	5.00
Mean (%)	13.00	5.00	7.00	6.00	7.75

30 plants were treated with tribenuron-methyl per each arc slice (30 plants × 5 arc slices = 150 plants per direction)

were used. Weedy sunflowers revealed a 328 bp single band, while the resistant RSU hybrid showed 313 bp bands. Progenies of the cross between weedy sunflowers and RSU hybrid had both 313 and 328 bp bands. Molecular analyses carried out on plants surviving tribenuron-methyl treatments demonstrated that only 48.9% of surviving plants present the hybrid profile. Of these surviving plants, about half (54.5%) were treated with the ½ x recommended use rate of tribenuron-methyl; whereas the remaining plants (45.5%) were treated with the 1 x (31.8%) and 2 x (13.7%) recommended use rate of tribenuron-methyl, respectively. As the inheritance of sunflower resistance to tribenuron-methyl is dominant we can expect that plants with hybrid profile are resistant (Jocic et al. 2011). On the other hand some plants which survived recommended and double field herbicide dose were not present hybrid profile. These results indicate that other factors may affect herbicide resistance of phenotypes. As reported by Kolkman et al. (2004), differential absorption (such as efficiency of herbicide uptake and/or rate of transport) and mode of metabolism can contribute to herbicide resistance of these populations.

In conclusion, our results suggest that the gene flow between resistant sunflower crops and Serbian weedy sunflower populations is possible and depends on flowering overlap, wind speed and prevalent direction during the flowering period and distance. Therefore development of strategies to avoid gene flow should focus on: isolation distances, pollen traps, male sterility and temporal reproductive barriers. An isolation zone – a region (distance) between pollen source and pollen acceptors depend on species. Arias & Rieseberg (1994) considered isolation distance for sunflower to be more than 1000 m. In addition, Staniland et al. (2000) showed that pollen traps (trap crop sown to surround pollen source that could "absorb" escaping genes and in destroyed after flowering) have an important role in confining the spread of transgenic *Brassica napus* pollen. In sunflower, effective barier could be male sterility (Hvarleva et al. 2009). Also, Roumet et al. (2013) concluded that reproductive time variation within weedy populations creates a partial reproductive isolation from the sunflower crop. Progress could be made through selection of genes involved in flowering time during sunflower domestication and breeding (Blackman et al. 2011).

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