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# Gene Flow from Herbicide-Resistant Crops to Wild Relatives

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Additional information is available at the end of the chapter

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## Abstract

Development of herbicide-resistant (HR) crops is way to overcome problems in weed control due to weed resistance to herbicides and absence of new herbicides with a new mode of action for their control. Three types of HR crops were developed: nontransgenic, transgenic, and multiple HR crops. Cultivation of HR crops is associated not only with many benefits (simplification of weed control, more effective and efficient weed control, higher yields, etc.) but also with various risks (development of HR weeds, development of HR volunteer crops, gene flow from HR crops to susceptible relatives, etc.). The greatest risk is gene flow from HR crops to related weed species, wild relatives or conventional crops of the same species. Unwanted gene flow could be prevented or reduced using different barriers such as isolation in space or time, protective vegetation barriers, male sterility, etc. Sunflower hybrids resistant to herbicides (imidazolinones and sulfonilureas) was developed by conventional breeding methods, and their introduction in Serbian fields has enabled a more efficient control of harmful weed species, but the presence of huge populations of weedy sunflower is the main concern associated with their cultivation, because numerous studies have confirmed gene flow from sunflower to its relatives.

**Keywords:** gene flow, herbicide resistant crops, wild relatives

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## 1. Introduction

The main aim of plant breeding is creating new varieties and hybrids, which would enable us to overcome different problems of contemporary agriculture and achieve high yields and productivity. Research in the fields of molecular genetics, biochemistry, and physiology is leading to development of plants with additional agronomic properties, such as herbicide

resistance, pathogen and pest resistance, salt and dryness tolerance, certain food quality parameters, etc. [1–4]. The predominant resistances used in crops are herbicide resistance, both in nontransgenic and in transgenic crops. Owing to the novel insights into the mechanisms and site of action of herbicides on a molecular level, and the development of new biotechnology methods, breeding of herbicide-resistant (HR) crops has been enabled. Thanks to that it is possible to use herbicides, which are preferable from agronomic, environmental, or genetic viewpoint. This new biotechnology gives many benefits in food production such as higher yield through high efficiency of weed control, less unit cost of food production, better quality through removal of existing volunteers of the some species, the possibility of using low-tillage systems, etc. But, this new biotechnology also has some disadvantages such as development herbicide-resistant weed species due to high selection pressure, potential for development of herbicide-resistant volunteer crops, risks of cross-pollination and gene flow from resistant to susceptible relatives, etc.

The focus of this chapter is review of risks associated with HR crops growing with special attention on gene flow from crops to their wild relatives. We first discuss development of HR crops and technologies of weed control based on resistant crops. Also, we briefly discuss gene flow from HR crops to their wild relatives and barriers, which can prevent it. Finally, we discuss transfer of genes responsible for resistance from sunflower hybrids (present resistant crop in Serbia and in Europe) to wild sunflower forms.

## 2. Herbicide-resistant crops

Discovery of new herbicides, especially with a new mode of action is difficult and expensive. During the last few decades, no one herbicide with novel site of action was found and there are no expectations for its appearance in the near future [5, 6]. One way to overcome this problem was development HR crops, which provide expanding the utility of existing herbicides and improve weed control with them. The study on developing HR crops started soon after the discovery of first herbicide-resistant weeds [7, 8]. These type of crops are designed to tolerate specific broad-spectrum herbicides, which kill the surrounding weeds, but leave the cultivated crop intact. There were two directions in HR crops development, which resulted with two groups of crops: transgenic (genetically modified, GM) and nontransgenic HR crops. The first nontransgenic program for HR crops breeding transferred resistance to herbicide triazines from a *Brassica rapa* to canola [9]. Although several triazine-resistant canola varieties were developed, farmer interest for these varieties was poor due to pleiotropic effects of mutation responsible for resistance, which caused lower yielding and poorer seedling vigor [10]. Also, nontransgenic methods like whole-cell selection, mutagenesis, and plant selection from natural populations have been used for breeding of crops resistant to sulfonylurea, sethoxydim, and imidazolinone herbicides. At the same period (1980s), tools for producing transgenic crops were becoming available and many companies start to work on their development. Bromoxynil-resistant cotton was one of the first transgenic HR crops available to farmers in 1995 [11], followed by glyphosate-resistant maize, canola, cotton, soybean, and other crops known as “Roundup Ready” crops. After period of effective, simple, and inexpensive weed

management with cultivation glyphosate-resistant crops, glyphosate-resistant weeds becoming a problem in weed control, which increase the use of crops resistant to glufosinate [12], followed by initiation of new approach in HR crops development, which was based on building of multiple resistance in crop plants.

Significant number of crop plants resistant to different ALS (acetolactate synthase; also known as AHAS—acetoxyacid synthase) inhibiting herbicides were developed using conventional breeding methods (**Table 1**). These groups of herbicides have very good characteristics for utilization in weed control in HR crops, which include low use rates, broad spectrum weed control, low mammalian toxicity and environmental compatibility. Immediately after discovery of this group of herbicides, ALS resistant tobacco and maize lines were developed using tissue culture selection [13, 14], while ALS-resistant soybean developed using mutagenesis [15].

	Herbicide	Crop	First market
Non-transgenic	Photosystem II inhibitors	Soybean	~1991
		Canola	1984
	Imidazolinones	Maize	1992
		Canola	1995
		Wheat	2001
		Rice	2001
		Sunflower	2003
	Sulfonylureas	Soybean	1994
		Sunflower	2006
		Sorghum	~2013
	ACCase inhibitor sethoxydim	Maize	1996
Transgenic	Glyphosate	Soybean	1996
		Canola	1996
		Cotton	1997
		Maize	1998
		Alfalfa	2006
	Glufosinate	Sugarbeet	2007
		Canola	1995
		Maize	1996
		Cotton	2005
		Rice	2006
		Soybean	2009

**Table 1.** Some commercialized HR crops, modified from reference [20].

After that, three technologies of weed control, which include crop resistance to this group of herbicides, were developed. The Clearfield® and the Clearfield Plus® system have been developed with the aim to grow crops resistant to IMI herbicides [16], while ExpresSun® system has been developed with the aim to grow sunflower hybrids resistant to tribenuron-methyl [17]. As there is no “alien” genes introduced into these crops, this group of HR crops is not considered as transgenic and has been accepted in countries where the cultivation of GM crops is prohibited [16], like many European countries, as well as in Serbia.

Transgenic (GM) crops developed based on the use of different transgenes, mainly responsible for resistance to glyphosate, which introduced into many crop species (Table 1). These crops became popular thanks to simplification of weed control and reduction of production costs, making the crop more profitable. Between more than a hundred GM products, which have been authorized for commercialization only 13 are crops [18]. The main GM crops are maize, soybean, cotton, and rapeseed, which grow on more than 90 million ha distributed in 14 countries in which these crops have been authorized [19]. These crops are grown in America, Australia, China, South Africa, but distribution is the highest in the USA, where it covers more than 49.8 million ha [19]. In Europe, GM crops (maize, rapeseed, endive, soybean, and flowers) adopted for the production and/or consumption only in few countries, between which Spain is major producer, growing GM maize on more than 100,000 ha [19].

New approach in development of HR crops is technology, which combines glyphosate resistance with resistance to other herbicides resulting in multiple HR crops (Table 2). This technology developed with the aim to overcome increasing development of multiple HR weeds and based on engineering crops that are able to express multiple HR traits and tolerate multiple herbicides. This new concept using stacked (contains more than one transgene) genes as a tool for postoccurrence and future resistance management is the equivalent to using a single herbicide in case when weed is already resistant to one member of a dual stack [21]. Appropriate transgene stacks should delay resistance longer than approach, which use each component separately and sequentially because each weed resistant to either herbicide will be killed by the other herbicide in the stack. However, that stacking multiple HR into crops may or may not delay the evolution of herbicide resistance because effectiveness of the transgene stacks depends on the management decisions

Herbicide types	Crops
Glyphosate and glufosinate	Soybean, maize, cotton
Glyphosate and ALS inhibitors	Soybean, maize
Glyphosate, glufosinate and 2,4-D analogs	Soybean, cotton
Glyphosate, glufosinate and dicamba	Soybean, cotton
Glyphosate, glufosinate and HPPD inhibitors	Soybean, cotton
Glyphosate, glufosinate, 2,4-D and ACCase inhibitors	Maize

Table 2. Multiple HR crops under development [20].

and adoption of the accompanying stewardship programs [21]. Namely, it depends on the effectiveness of each included herbicide in control of each target weed species. Some soybean multiple resistant cultivars have recently been approved for commercial use, such as cultivars resistant to glyphosate, glufosinate, and 2,4-D, as well as resistant to glyphosate and dicamba [21]. Except that it is possible to develop stacks of transgenes for different traits. For example, maize containing transgenes for resistance to insects and to herbicides is commercialized [22].

### 3. Benefits and risks associated with growing of herbicide-resistance crops

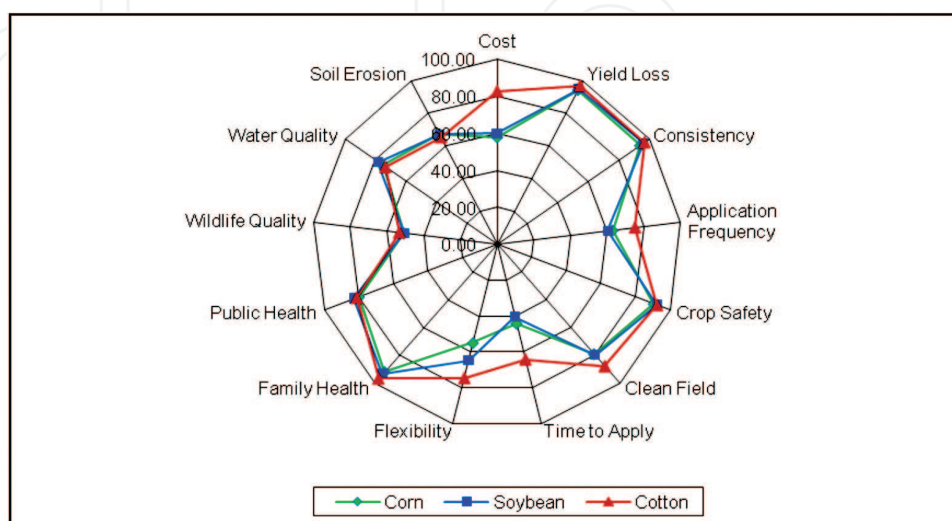
Cultivation of HR crops is associated not only with numerous benefits but also with various risk factors. The most important benefit is simplification of weed control using herbicides (including nonselective herbicides in many HR crops), in which some crops are able to control weeds that other herbicide that cannot control without concern for crop injury. Also, HR crops are good solution for control of parasitic weed species, in which control is more complex due to their attachment for host (mainly crop) plants [23]. Thanks to flexibility to the time of herbicide application, possible combination with other herbicides and integration with nonchemical methods, this weed control approach made weed management more effective and efficient, which results in higher and more profitable yields. For example, the average increase of yield of glyphosate-resistant soybean in developed countries was 7%, while in developing countries, it was 21% [24]. The higher yield with better quality of seed is not a direct result of HR crop traits *per se*, but it is the result of improved weed control, which is mainly more effective than the conventional weed management systems [20]. These approach to weed control also became popular thanks to the absence of new herbicides with novel sites of action during the last few decades and no prediction for its appearance in the near future [5, 6]. Also, weed resistance to herbicides becomes widely spread and still growing problem, which is difficult to manage. In conventional weed management systems control of weeds which are closely related to the crop is difficult or impossible. ALS-inhibiting resistant sunflower would allow to use this group of herbicides to control *Ambrosia trifida*, *Ambrosia artemisiifolia*, *Cirsium arvense*, *Xanthium strumarium*, weedy *Helianthus annuus*, and other weeds belonging Asteraceae family, without injuring the crop [25]. Although there are controversial views about HR crops impacts on environment, it is evident that this weed control system is more beneficial to the environment than conventional systems. Namely, herbicides used in HR crops are usually more environmentally friendly than herbicides used in conventional crops. ALS-inhibiting herbicides, which are used in many nontransgenic HR crops, are very effective with relatively low use rates and low mammalian toxicity [26]. Also, it is clearly shown that glyphosate-resistant crops are beneficial to the environment by reducing fuel use and soil erosion and residues of herbicides in ground waters [20] with the help of reduced tillage. Also, glyphosate-resistant crop cultivation has decreased herbicide use by 17 million kg per yr in the USA [27]. Except described, there are additional benefits growing HR crops to which farmers also give great importance. For example, in case of glyphosate-resistant maize, soybean and cotton, growers highlighted as



very important (**Figure 1**) consistency and protection from yield loss, application frequency and flexibility, a clean field, cost, crop safety, family and public health, water quality, etc. [28].

The cultivation of HR crops, whether they have been developed through genetic engineering or classical breeding methods, is fraught with risks, i.e., potential serious economic and ecological consequences. Unlike the HR crops, which have been obtained through conventional breeding methods, the cultivation of GM crops has been a cause of a number of debates, pertaining to the health safety of these products and the risks they present to the environment. The questions, which cause the greatest concern, are those which relate to: (1) direct and indirect toxic effects of products containing transgenes for nonspecific organisms; (2) the impact of modified genes and GM plants on biodiversity, ecosystems, and soil microorganisms; and (3) gene transfer from GM crops to their wild relatives and ecological consequences of this phenomenon [29]. Contrary to this, in the case of HR crops developed by conventional breeding methods, the greatest risk is transfer of genes responsible for resistance from those crops to related weed species, wild relatives, or conventional crops of the same species. Namely, described gene flow creating the hybrids between HR crops and weeds, the so-called “super weeds”, resistant to herbicides. Their eradication subsequently becomes one of the major problems in agriculture. Also, gene flow can change the fitness of recipient biotype/species, whereby increase of fitness resulting in greater weediness, while its decreases lead to extinction [30]. In addition, genes responsible for resistance can flow from HR crops to conventional varieties, which could be the source for resistant genes flow to wild or weedy relatives [31]. Gene flow from transgenic to nontransgenic crops of the same species has been a major controversy, the cause of law suits, and a factor influencing commercialization of some transgenic crops. Some authors [32] highlighted that the risks associated with transgenic crops cultivation may be more pronounced in the centers of origin of crops than in the other territories because of the presence of wild progenitors and other wild relatives in centers of origin.

The occurrences of volunteer populations of HR crops can also be leading to high risk. Namely, seed dissipation during harvest lead to the appearance of volunteer plants the next season



**Figure 1.** The important benefits growing of HR crops from the farmer point of view, made based on survey between 1176 glyphosate-resistant maize, soybean, and cotton growers [28].

generally in crop production of some crops. Negative consequences of volunteer plants are yield and quality reduction of the crop which they have invaded, contamination of harvested seeds, and maintenance of harmful insects and diseases. In the case of HR crops that volunteer plants basically represent resistant weed populations, which can be a source of pollen, which can contaminate the nonresistant crops or pass the resistance traits onto the related weed species. The control of volunteer plants, which has originated from HR crops, is impossible in the following cultures in which these herbicides are applied as a weed control measure. Therefore, volunteer plants of glyphosate-resistant cotton could be a problem in glyphosate-resistant soybean as subsequent crop [33] or volunteer glyphosate-resistant canola and wheat could be problem in weed control in conservation tillage system [34]. Also, seeds from volunteer plants of GM crop can contaminate harvest of conventional subsequent crop [35].

Intensive and repeated use of the same herbicides with the same mode of action in HR crops mainly increase selection pressure on weeds, which would most likely lead to an increase in the selection of HR weed populations. Today, at least 36 weed species have evolved resistance to glyphosate, EPSPS inhibitor (the main herbicide in transgenic HR crops), and at least 159 to ALS-inhibiting herbicides (the main group of herbicides in nontransgenic HR crops) [36]. In addition to these concerns, other negative effects are also possible: herbicide drift can damage conventional crops of the same species, the genes responsible for resistance can be transferred onto conventional crops, characteristics of nontarget plants can be modified, biodiversity may be damaged, and the environment and soil properties can be changed due to the changes in the crop production technologies.

Due to the dangers of the mentioned potential risks, the research into these issues, with the aim of developing suitable prevention strategies, as well as solutions to these problems, should they arise, has been intensified. Consequently, plenty have dealt with the issue of the gene transfer from HR crops to their relatives (wild/weedy forms or conventional crops) [37–42], the study of gene stability in recipients [43, 44], the study of crop-weed hybrid's fitness [41, 45–47] and the competition between crop-weed hybrids and sensitive weed plants of the same species [45, 46, 48].

#### 4. Gene flow from herbicide-resistant crops to wild or weedy relatives

Hybridization and introgression are normal processes, which have continuously occurred between crops and wild or weedy relatives [49, 50], as well as between relative populations of weedy and/or wild species [51, 52]. Even though the hybridization of crops and weeds has an important role in the evolution of many weed species [53], it can also result in the extinction of certain species related to the crops or the rise of new weed forms, which are more aggressive and better adapted to artificial habitats [30]. There are three types of gene flow: *vertical* (between sexually compatible individuals), *horizontal* (between distant related species), and *diagonal* (between related but incompletely incompatible species) [54], but introgression of genes from cultivated to wild or weedy forms of the same species is possible through *vertical* and *diagonal* gene flow.

The ecological consequences of gene transfer from crops to their wild relatives are determined by the quantity of genes, which are being transferred into the populations of wild plants and weeds and the phenotypic characteristics controlled by these genes. Some of the characteristics are insignificant for the fitness of wild relatives, while others (herbicide resistance, disease

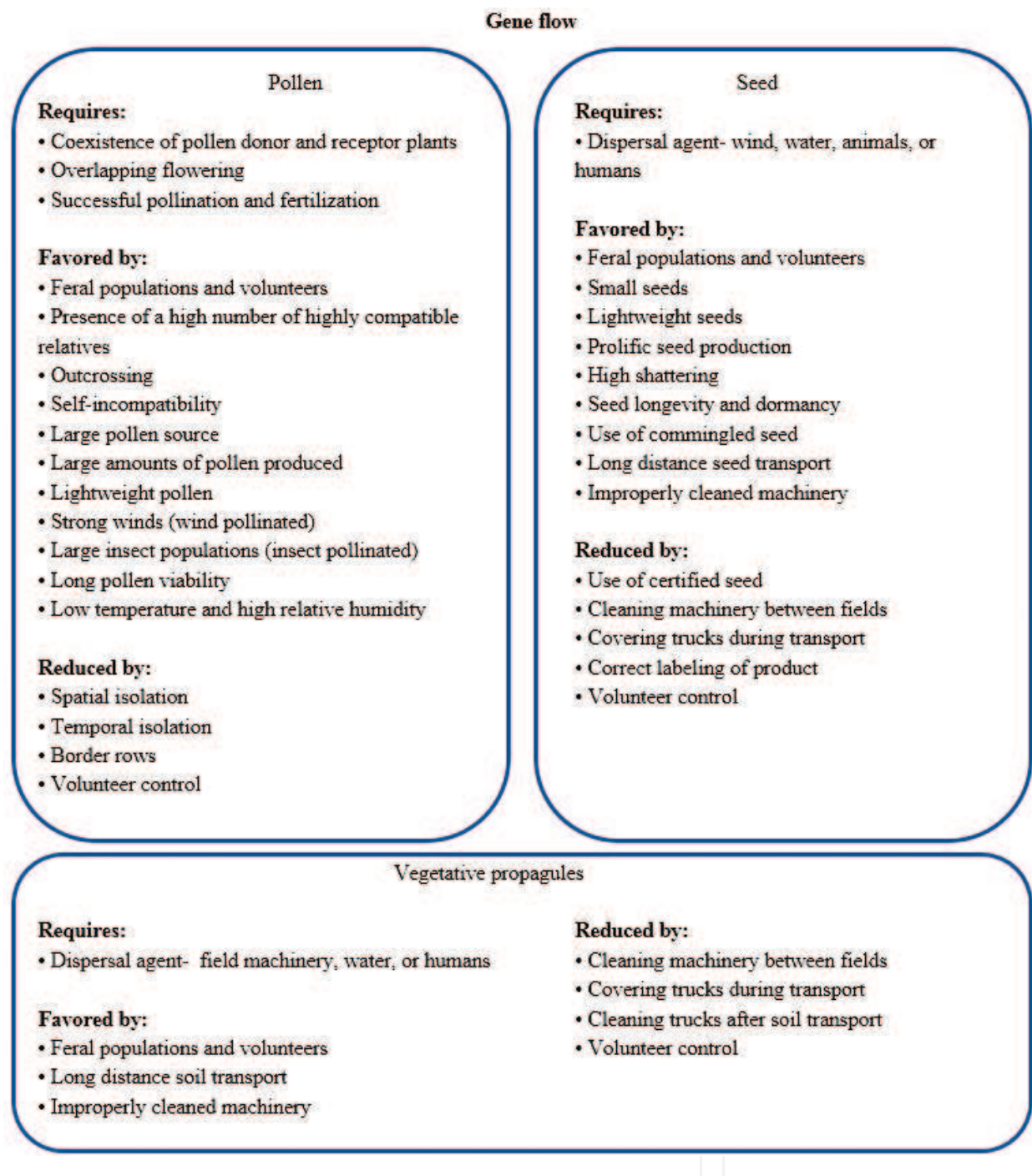


resistance, and tolerance to the environmental stress factors) mostly improve it. For example, the first generation crop-wild hybrids produced through hybridization between cultivated and wild radish populations [53, 55, 56] was relatively fecund, produced large quantities of seeds and rapidly evolved increased pollen fertility. Contrary to this, if the introduced genes weaken the fitness of their wild relatives, their invisibility will also decrease. This process can be accelerated by introgression and the introduction of new genes from neighboring crops, which ultimately leads to the extinction of the initial populations of wild relatives [57]. Except ecological consequences, gene flow from crops to weedy relatives is associated with many problems in crop production. Namely, the development of HR crops has given rise to the situation where the hybridization is often seen as a problem, particularly when it relates to the hybridization between GM crops and related species. Also, it is important to bear in mind that in some countries coexist different cropping systems, which cultivate conventional, organic, and GM crops. In that situation, there is risk for gene flow between GM and non-GM cultivars through cross-fertilization due to pollen flow between neighboring fields. Progeny of HR crops and weedy/wild relatives or volunteers will be resistant weeds, in which control is difficult.

Genes responsible for crop's herbicide resistance can be spread in the environment as a result of three mechanisms, including gene transfer across a pollen (as a result of allogamy), seeds (as a result of their dispersal) and for perennial species by the vegetative propagules. Potential for pollen-mediated gene flow is higher for both wind and insect pollinated out-crossing crops than for self-pollinated crops [58]. Although gene flow across a pollen is more studied, gene flow by seeds during commerce may be very important for the long-distance dispersal of genes responsible for resistance to herbicides [59]. The both ways of gene flow from HR crops including both GM and conventionally bred HR crops have been confirmed in many cases [37–40, 60, 61].

The transfer of genes from HR crops to their relatives is dependent on multiple factors (**Figure 2**), such as the coexistence and proximity of the crop and its close relatives, their biology and phenology, type of vector, development of F1 generation, which is fertile and capable of survival, the production of fertile subsequent generations, the potential for gene transmission, chromosome recombination and movement of genes of one species into the genome of another, due to introgressive hybridization and gene persistence in volunteer crop populations [58, 62]. Also, in study about gene flow from glufosinate-resistant rice to improved rice cultivars and weedy rice in China, the conclusion was that gene flow depends on the height of pollen recipient plants [63]. They found that the gene flow was lesser if recipients were taller than in situation when they were shorter.

Cross-pollination between HR crops and sexually compatible wild or conventional cultivated crops of the same species is the major pathway for gene escape. Therefore, transfer of genes responsible for HR between sexually compatible individuals is most often done through pollen, whether within the same population or between different populations [38, 64]. This occurrence is dependent on different factors of which autoincompatibility that enhances allogamy in wild forms, environmental conditions (wind speed and direction, temperature, light intensity, and humidity) as well as the type (wind and/or insect) of pollination vector [37, 38, 65, 66]. In addition to this, the crucial role in gene transfer through pollen lies in the coincidence of the flowering period between the HR crop and its wild relatives. Although experimental data suggest



**Figure 2.** Comparison of the requirements and factors affecting gene flow via pollen, seed, and vegetative propagules, modified figure from reference [58].

that the flowering period of wild populations is generally longer than the flowering period of crops, which makes the overlap highly likely [67], in some cases, gene flow between HR crops and relatives was disabled due to flowering period not overlapping or time of overlapping was short. For example, hybridization between imazamox-resistant and weedy sunflower was not confirmed in experiments in Serbia when period of flowering overlapping was short [42]. Also, it was confirmed that the gene transfer from the cultivated onto the wild sunflower in

Argentina depended on the overlap between the flowering period and the presence of common pollinators [68, 69]. Pollen dispersal from HR crops onto their wild relatives is also dependent on their mutual distance, the size of populations from which the pollen originates and where it is delivered, plant density, number of flowers per plant, and the location of wild relatives in relation to the crop [70].

Although numerous studies have confirmed the transfer of genes relevant for HR to their wild relatives, hybridization level mainly was low. Some authors [60] studied the transfer of genes responsible for imazethapyr-resistance, from the rice cultivars to the weedy rice species in 22 field sites. They confirmed that even though gene transfer occurs, in the majority of sites (18) less than 1% of hybrid progeny was present, while in the remaining four sites that percentage was somewhat higher (up to 3%). Also, low levels of hybridization (1–2%) were confirmed between rice and its wild congener *Oryza rufipogon* [71]. Similarly, Ref. [72] confirmed a low level of hybridization between HR rapeseed and related weed species *Raphanus raphanistrum*. Their research has shown that the proportion of crop-weed hybrids in the F1 generation was at the level of  $10^{-7}$  to  $3 \times 10^{-5}$ , depending on the geographic position of the weed species in the experimental plot. Gene flow from glyphosate-resistant canola to *B. rapa* in commercial fields was confirmed, but the genes were apparently not fully introgressed [73]. Contrary to that, in study of transgene escaping from canola to *B. rapa*, the gene frequency in the first backcross generation was 50%. But, in the fourth backcross generation, it was 0.1% in conditions without herbicide application, while in conditions with glyphosate application, gene frequency was about 5.5% within six successive backcross generations [44].

Despite the fact that the gene transfer from crops to their wild relatives is widely studied, there are no detailed data available on what happens with these genes, which have been introduced into wild populations after a longer period of time. Namely, the majority of this research concludes with the first generation of hybrids. However, genes originating from the cultivated sunflower can persist in wild populations over the five-year period, following the hybridization [43]. Some authors [74] have also studied the effects of a 40-year long gene transfer from the cultivated to the wild sunflower populations.

Importance of crop-weed hybrids produced as result of gene flow from HR crops to wild or weedy relatives for future crop production can be different depending on traits introduced into progeny. Therefore, assessment of gene flow occurrence requires not only estimating the degree of gene flow, but also evaluating the relative fitness of hybrids. It long dominated the view that crop-wild hybrids have a lower fitness than their wild parent [75, 76]. But, many studies confirmed that some hybrids display increased [47], while the other display reduced [77] fitness in comparison with their parents. Displayed fitness depends not only on the crop traits introduced to wild relatives, but also on environmental conditions. Namely, fitness of hybrids between crop and wild sunflower increases in stressful conditions common to conventional agroecosystem like competition and herbicide application [77].

The role of seeds in the transfer of HR genes from crops to their wild relatives is evident in their spread into new areas where volunteer populations are formed. After that HR genes can be transferred from these volunteer populations to their wild relatives through the pollen. Also, hybrids resulting from spontaneous crosses of HR crops and their wild relatives through seeds can be carried into new areas, where they subsequently present a source of pollen, which carries



the resistance genes. Unlike pollen, the seeds usually remain in the close proximity of the plants from which they have originated. But, as seeds are more persistent than pollen, movement of seeds is possible to further distances by human activities than pollen movement [59]. In general, seed dispersal of HR crops or progeny created through their spontaneous crossing with wild relatives, depends on the biological properties of the crop, the ecological conditions, crop production technology and the agrotechnical measures applied on these fields, following with harvest. Nevertheless, it is possible to monitor the dispersal of these seeds in space and time. Some authors [78] have confirmed the gene transfer of sugar beet to their wild relatives through the seeds whose dispersal resulted from soil transport. Namely, although spontaneous spatial dispersal is often considered as irrelevant since the seeds of a majority of crop cultures have lost this ability, seed dispersal is also possible as a result of spillage during the harvest and their transport and storage operations, which enables the spread to great distances. The dispersal of seeds containing the genes responsible for resistance over time depends on the dormancy characteristics and the seed's longevity in the soil, as well as the ecological requirements for its germination. Also, it should be considered that, in addition to pollen and seeds, soil seedbank has an important role in the plant dispersal [79]. Namely, when considering different life forms of sugar beet (cultivated, wild, and weedy), it is well known that they form long-term seedbanks [80], which, over a longer period of time, can provide the plants which are then a source of HR genes.

Gene flow by the vegetative propagules (stolons, rhizomes, roots, crowns, and bulbs) is possible on short distance via natural means or on equipment moved between fields, while long-distance movement could be possible only with human activities or through the waterways [58]. As HR crops are mainly annual species, gene flow via vegetative propagules can be interesting only for perennials like glyphosate-resistant alfalfa (commercially available) and creeping bentgrass (*Agrostis stolonifera*, under consideration) [58].

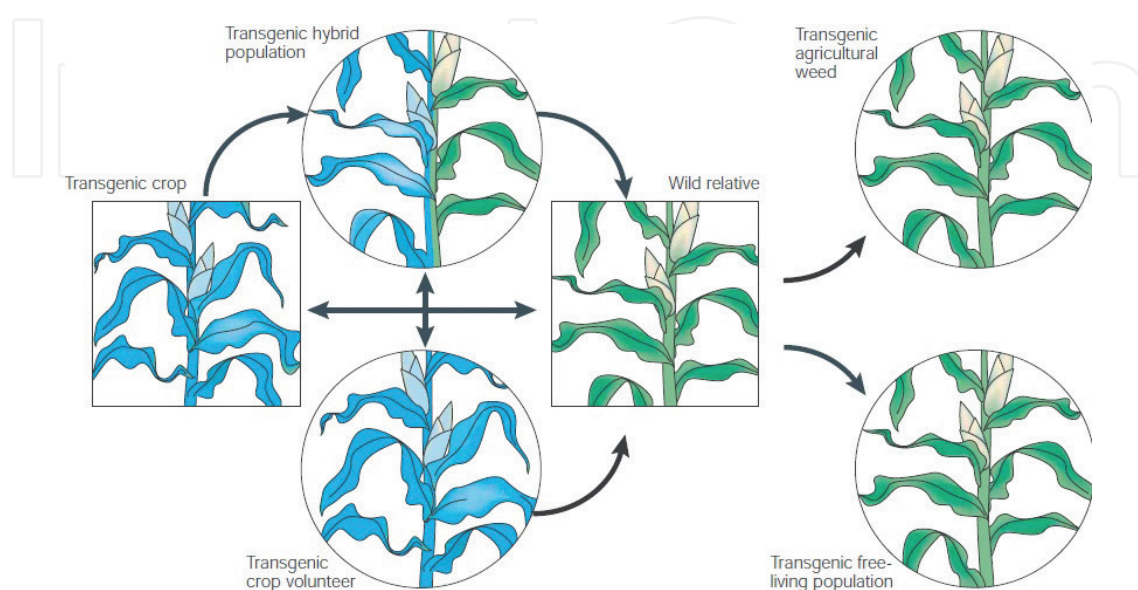
*Gene flow risk assessment* is a procedure, which helps determine whether the transfer will occur, and if it will, in which degree, with a goal to reduce such a risk to the minimal possible level. Furthermore, such estimates are also significant due to the possibility that the transfer of genes responsible for HR will lead to an increase in the survival and adaptability of the introduced weed species. Also, it is considered that certain plants can attain the traits of invasive species as a result of introduced genes, making the assessments of long-term consequences of gene transfer from crops to their wild relatives a necessity. There is no same potential for gene flow for all HR crops. For example gene flow from maize is theoretically possible to teosinte, but these species only exist in Mexico and Central America and not yet been reported for contamination with transgenes [81]. On the other hand, there is good potential for introgression from sunflower [74], sugarbeet [82] and rice [83] to wild relatives. Namely, the dangers of the transfer of genes responsible for HR and the ecological consequences of this must be evaluated individually for each specific case (herbicide, plant, wild relatives, etc.), regardless of the fact whether the crop resistance has been achieved through conventional breeding methods or genetic engineering. Crucial steps in the rational assessment of ecological consequences of this phenomenon include the understanding of the following: (1) gene transfer from crops to wild relatives; (2) gene expression and inheritance in hybrids, which have resulted from the gene transfer from crops to wild relatives; (3) changes in fitness in wild relatives caused by the introduction of genes; and (4) the dynamics of the gene transfer from crops to wild

populations. The invasibility of crop-weed hybrids, which have originated as a result of gene transfer from HR crops to wild relatives, is dependent on all of these aspects.

Pollen flow from crop to the relative seems as relatively simply process, but gene introgression is complex, occurring in several steps which mean several hybrid generations, which can exchange genes among themselves and coexist many years simultaneously (**Figure 3**). The likelihood of gene transfer from crops to their wild relatives depends on the genetic characteristics of crops and their wild relatives, as well as the homology of their genomes [62]. In the cases where the degree of the homology between the crops and their wild relatives is higher, as in the case of *Beta vulgaris* × *B. maritima* or *Raphanus sativus* × *R. raphanistrum*, the likelihood that the introduction of transferred genes will occur is higher. Additionally, the introduction of genes is dependent on the part of the genome, in which the gene is positioned. Some authors [30] found a possibility of gene introduction from 13 most important crops into wild relatives and determined that 12 of the studied crops can hybridize with their wild relatives. Of the 12 listed crops, cases of introduction have been confirmed for 7, while in the remaining five there is a possibility that the introduction will occur. Also, based on the potential danger of transgenic introgression into their wild relatives, some authors [76] have grouped GM crops based on their risk levels into four categories: high, middle, low, and very low (**Table 3**). A similar categorization pertaining to the risk assessment was also applied by other authors [84, 85].

In order to *prevent or reduce* the unwanted transfer of pollen from HR crops onto their relatives, different barriers can be used, although there is no absolute guarantee that the gene transfer can be prevented in this manner. The most often used barriers are isolation in space or time, protective vegetation barriers made up of one or more different species, male sterility as a genetic mechanism for the prevention of gene transfer, etc.

*Spatial (distance) isolation* means increasing the distance between fields sown by HR crops and populations of its relatives. Also, spatial isolation is applied as preventive measure in production of GM and non-GM crops in coexistence with the aim to avoid contamination products of non-GM crops. It has been known that by increasing the distance between crops



**Figure 3.** Gene flow and its potential resources, modified from reference [76].



Crop	Risk level	Wild relatives for which the introgression of gene has been confirmed
Johnson grass	High	<i>Sorghum halepense</i> , <i>S. alnum</i> , <i>S. propinquum</i>
Oilseed rape	Medium	<i>Brassica rapa</i> , <i>B. juncea</i> , <i>B. oleraceae</i> , <i>B. campestris</i> <i>Sinapis arvensis</i> <i>Raphanus raphanistrum</i>
Sugar-beet	Medium	<i>Beta vulgaris</i> ssp. <i>vulgaris</i> * <i>Beta vulgaris</i> ssp. <i>maritima</i>
Wheat	Medium	<i>Triticum turgidum</i> <i>Aegilops</i> sp. ( <i>Aegilops cylindrica</i> )
Sunflower	Medium	<i>Helianthus</i> sp. ( <i>H. annuus</i> * and <i>H. petiolaris</i> )
Alfalfa	Medium	<i>Medicago sativa</i> *
Rice	Low	<i>Oryza rufipogon</i>
Maize	Low	<i>Zea mexicana</i>
Potato	Very low	
Soybean	Very low	
Barley	Very low	
Common Bean	Very low	

\*Weedy crop forms.

**Table 3.** The risk level of the introgression of genes from crops to their wild relatives ([86] made based on data reviewed by [76]).

and its relatives, the dispersal of pollen is reduced, i.e., the level of hybridization is reduced. Thus, the frequency of pollen originating from a transgene oilseed rape decrease from 1.5 to 0.00033%, as distance increase from 1 to 47 m [87]. Also, frequency of crop-wild relative hybrid decreased from 0.156 to 0.0038% with increase in distance from pollen source between 200 and 400 m [88]. Distances between pollen source and gene occurrence can be very valuable in the planning of spatial isolation of HR varieties, in order to prevent the gene flow to their relatives. This distance depends on many factors such as the presence of local barriers, the local climate, and the topography of the area. In the case of sunflower, the isolation distance should be greater than 1000 m [89]. Also, maize pollen can be detected at distances greater than 800 m from the pollen source [90]. But, pollen of maize has short flight range [91], after which it settles to the ground rapidly [92] due to relatively heavy and large grains. Due to that cross-fertilization mainly occurs within 50 m of the pollen source [93]. Therefore, measure for keeping seed purity of non-GM maize, which coexists with GM maize, suggests isolation distance between 10 and 50 m to achieve EU admissible threshold of 0.9% in the harvest [37, 93, 94].

*Temporal isolation* is a measure, which should prevent overlapping flowering times of crop and wild relatives with the aim to avoid gene flow. About 5 days lag in flowering of imazamox-resistant in comparison with tribenuron-methyl resistant sunflower resulted in lack of gene flow to weedy sunflower probably due to the short period of overlapping flowering time between the resistant hybrid and the weedy sunflower [42]. Temporal isolation is very suitable to prevent non-GM crop contamination with GM when grow in coexistence. Study of maize

pollen mediated gene flow in Italy and showed that if time of flowering differs from 4 to 5 days the cross-pollination is reduced by 25%, while difference of 6 days provides 50% reduction [95]. Also, temporal separation in sowing days improves the coexistence of maize [96]. Contrary to that, temporal isolation based on selection of hybrid varieties in which flowering noncoincide achieves the same results although sowing date was the same [97]. Temporal separation and isolation distance together can be a good solution to minimize unwanted gene flow.

*Protective vegetation barriers*, made up of one or more different species, can prevent the gene flow by physically stopping pollen in the case of wind pollination. The sowing of conventional crops of the same species, also known as pollen traps, in the vicinity of HR hybrids is an efficient measure, as their role is to attract pollinating insects in order to leave pollen on these pollen traps. This type of barrier can be much more effective than isolation distance. Namely, the sowing of pollen traps between GM and conventional crops is the most efficient measure for the prevention of gene flow [98]. Also, gene flow through pollen from the HR oilseed rape decreases rapidly with the increase of pollen source distance, with the added necessity of a protective vegetation barrier or pollen traps [99]. Some authors [95] studied pollen-mediated gene flow between GM and non-GM maize and concluded that effect of two maize rows surrounding the recipient field in reduction of cross-fertilization is the same like effect of 12 maize rows surrounding the pollen donor.

The use of *biological barriers* achieves the best results in the prevention of gene flow, and so far the barriers based on cytoplasmic male sterility, maternal inheritance, and seed sterility have mostly been used. Cytoplasmic male sterility is based on the inability of plants to produce viable pollen. This type of barrier is suitable option to reduce gene flow in sunflower and maize [43, 66, 100]. Maternal inheritance is successfully used in the prevention of gene flow across the pollen, in the case of several species, including tobacco and tomato [101, 102]. The control of embryo and seed fertility is known as GURT (Gene Use Restriction Technology), i.e., terminator technology, which is considered to be a better control measure, in comparison with sterile pollen production. However, this strategy is seen as the most controversial control measure for limiting genes flow. Additionally, strategies, which include apomixis (vegetative reproduction and asexual seed formation), cleistogamy (self-fertilization without the opening of flowers), genome incompatibility, chemical induction/deletion, etc., are also used in limiting the gene flow [103]. None of these strategies can be applied in all crops, therefore using combinations of different approaches for the prevention of unwanted gene flow is recommended.

All mentioned measures for prevention and reduction of gene flow are important separately, but their integration and combination with stewardship production system could be the best solution.

## 5. Gene flow from herbicide-resistance sunflower to wild or weedy sunflower

Options for chemical control of broadleaf weed species, especially weeds belonging to Asteraceae family, without injuring the crop are quite limited in sunflower compared to most other row

crops [104]. Due to that, sunflower hybrids resistant to ALS-inhibiting herbicides, including imidazolinone (IMI) and sulfonylurea (SU), was developed by conventional breeding methods, with the aim to improve weed control. The Clearfield\_system [16] and the Clearfield-Plus\_system [105] have been developed with the aim to grow sunflower hybrids resistant to IMI herbicides. For development of those hybrids were used for subsequent crossings between cultivated sunflower and wild resistant sunflower [106] or seed mutagenesis [105]. Also, ExpresSun system has been developed as result of mutagenesis breeding [107] with the aim to grow sunflower hybrids resistant to tribenuron-methyl [17].

The breeding of sunflower hybrids resistant to herbicides belonging to IMI and SU groups in Serbia was started in 2000, and since 2003, this technology has been applied in the production. As a donor of imazamox-resistance gene, the wild sunflower originating from the USA was used, in which the resistance to herbicides of the imidazolinone group was developed following a seven-year consecutive application of imazethapyr [106]. The produced hybrid has shown a high level of resistance toward imazethapyr [108] and imazamox [109], not only regarding different vegetative parameters, but also considering the activity of ALS enzymes *in vivo*, and *in vitro*. Source populations SURES-1 and SURES-2 were used as a source of genes responsible for the resistance to tribenuron-methyl [1, 110], producing also a hybrid with a highly distinguished resistance for this herbicide [109, 111]. The introduction of such crops in the production in Serbian fields has enabled a more efficient control of economic harmful weed species, such as *Sorghum halepense*, *A. trifida*, *A. artemisiifolia*, *C. arvensis*, *X. strumarium* and weedy forms of *Helianthus annuus*, their cultivation is also linked with a very high risk of herbicide-resistance gene flow, from these hybrids onto the weedy form of *H. annuus*. Although the presence of four species from the genus *Helianthus* (*H. annuus*, *Helianthus tuberosus*, *Helianthus decapetalus*, *Helianthus scaberimus*) has been confirmed for Serbia, in both crop fields and nonarable lands [112], weedy populations of *H. annuus* occupy the biggest areas, which according to some estimates reach up to 1000 ha in Southern Srem and around 7–8000 ha in Southern Banat [113]. The origin of these populations is not known, but it is possible to determine. For example, origin of French and Spanish weedy populations was determined based on molecular analysis, which has shown that these populations originated from the unintentional introduction of crop-wild hybrids through contaminated seed lots [114]. Difficult eradication of weedy populations due to a high population variability [113, 115, 116] and pronounced invasibility caused by strong vegetative and generative potential [117, 118] presents an additional problem. Besides a reduced sensitivity of this species to nicosulfuron, which is often used as a weed control measure in maize fields where weedy sunflower is present in high densities, has also been detected [119, 120]. Therefore, even though the research into the transfer of HR genes from HR sunflower hybrids to weedy sunflower is in initial stages in Serbia [42, 121], there is high potential for its risk.

The main concern associated with cultivation of HR sunflower is potential gene flow from crop to weedy or wild relatives. Although wild sunflower populations are self-incompatible [122], new crop sunflower varieties are about 65% autogamous [123] and weedy population as a result of their hybridization are self-incompatible. Therefore, there is great potential for pollen-mediated gene flow. For example, seed-mediated gene flow from cultivated sunflowers

to wild sunflowers may be common [124]. Also, it has been known that there are inter- and intraspecific hybridization between *H. annuus* and its close relatives including its related species [41, 64, 74, 125] or its volunteer plants [65, 69]. In case of gene flow between cultivated sunflower and *Helianthus petiolaris*, the proportion of crop-weed hybrids in the F1 generation varies between 0.3 and 0.5%, depending on flowering period and the presence of common pollinators [68], while in case of gene flow between cultivated and wild sunflower, it was reached as high as 33% [69].

Gene flow from sunflower crops onto their wild relatives mediated by pollen is dependent on different factors. The overlap of flowering periods of cultivated sunflower and its wild relatives, the pollinators which they share, self-incompatibility of the wild species, diploidy, and high levels of cross-fertilization are all factors which contribute to the spontaneous hybridization [66]. However, the hybridization between the sunflower and its relatives can be absent due to the mismatch of the flowering periods, incompatibility, physical distance, differences in the genetic structure between the species and interspecific competition of pollen [89, 125]. Many studies [42, 70, 121] confirmed that the pollen transfer from the resistant crops to their relatives primarily depends on their distance to the pollen source and the plot size. Consequently, some authors [64] have confirmed, when studying gene flow from sunflower imidazolinones-resistant hybrids to their wild relatives, that the HR gene was transported to a distance greater than 30 m from the pollen source, while the percentage of the surviving offspring of wild relatives was reduced with the increase in the distance from the HR hybrid. Also, it has been confirmed that the gene flow from the crop sunflower to its wild form is reduced with an increase in their mutual distance, with it being 27% at a 3 m distance. However, gene flow has also been confirmed at a distance of over 1000 m from the pollen sources [89]. Additionally, it was determined that 42% of the wild offspring sunflower at a 3 m distance from the crop sunflower represented its hybrids, while at a distance of 200 m, this percentage was 10%, and 4% at a distance of 400 m [43]. Several authors [42, 45, 64] indicate that the wind direction affects the gene flow, which is ascribed to its influence on the flight of bees.

The main consequence of gene flow between crop and their wild relatives is the increasing of wild relative fitness as a consequence of introgressed genes, which can lead to the development of invasive weeds. Some studies confirmed fitness increase of hybrids between sunflower crop and their relatives [47], while the other [77] confirmed hybrids in the first generation after crossing had lower fitness than wild parent in natural habitats, but in the following generations, fitness of hybrid was recovered. Also, hybrids between crop and wild populations of sunflower express lower fertility than their wild counterparts [75]. Although, crop hybridization can reduce dormancy in a wild species, hybridization IMI-resistant hybrid and wild sunflower in Argentina did not alter seed dormancy [41], while F1 germination was greater in wild sunflower populations [126].

Strategies for prevention or reduction of gene flow between crop sunflower and its relatives can be developed based on understanding seed and pollen dispersal and influence of different factors on that processes. The biological barriers based on cytoplasmic male sterility, which disable of plants to produce viable pollen, could be good option to reduce gene flow in sunflower.

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