
Risk Assessment of Herbicide Resistant Crops with Special Reference to Pollen Mediated Gene Flow

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1. Introduction

The world population is projected to become a staggering 8.3 billion by 2030 from about 6 billion today, which will aggravate food insecurity especially in developing countries [31]. By 2050, developing countries will account for 93% of cereal and 85% of meat demand growth [73]. In agricultural crop production systems, insects, diseases and weeds continue to threaten sustainability and account for ~40% loss in crop production. Availability of farm land and productivity is decreasing because of soil erosion, degradation and annexation of farm land for alternative uses. The availability of water for agricultural crops is also decreasing. Drought, storm, flood and heat waves are predicted to occur more frequently and would have a large impact on crop productivity [19]. Since atmospheric concentrations of greenhouse gases continue to rise at rates that are both unprecedented and alarming, efforts have been made to understand their implications on crop production [2]. Higher growing season temperatures can have dramatic impacts on agricultural productivity, farm incomes and food security [8]. Salinity and other soil toxicities are likely to be much more problematic in some areas. In semi-arid regions, reduction in production of primary crops including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) are predicted in the next two decades [58].

Ability of the agriculture sector to support a growing population has been a concern and continues to be on high priority on the global policy agenda [5]. Simultaneous demands for replacement of oil-based fuels and plant-based bio-products, the desire for reduction in pesticide usage and crops to tolerate abiotic stress will place pressure on agricultural production systems. New agricultural technologies are required to ensure global food security and support conservation of water and land. Crop cultivars with higher yields and resistivity are required to meet food requirements in a sustainable manner without causing disruption to the environment. In her book "Silent Spring", Carson R. suggested finding a biological solution

of pest control in agriculture as an alternate of using chemical pesticides [18]. One approach is to develop genetically engineered crops. Technological responses to the need for bio-products, new crops and stress-tolerant crops have been predicated on the use of genetic engineering.

In addition to traditional uses of crops for food, feed and fiber, agricultural crops are being utilized for biodiesel, bio-products, industrial chemicals, bio-degradable plastics and plant-based pharmaceuticals [14]. Genetic engineering is increasingly used to accomplish this goal and viewed by researchers as a key technology to provide innovations. Long-term sustainability of technical applications has become quite important during the last two decades resulting from ecological concern, environmental awareness and new rules and regulations.

Introduction of herbicide-resistant crops at a commercial scale was controversial; however, this new technology has provided many benefits to growers, consumers, and environment [3]. In many parts of the world, agricultural pesticides have been used in excess of requirements, leaving residue in food and soil resulting in environmental pollution. Agro-chemicals are ineffective against viruses and only partly effective against many plant pathogens. The most cost effective and environmentally friendly method would be to deploy cultivars that have been developed for resistance to various agents causing biotic stress. For example, transgenic cotton in India, which is genetically engineered to contain the *Bt* toxin, has led to decrease use of insecticides and improved yields [12]. China is the major producer of cotton and Chinese growers are amongst the largest users of pesticides in cotton. A survey suggests that following introduction of *Bt* cotton, reduction in pesticide use was from 55 to 16 kg formulated product per hectare and the reduction in number of pesticide application was from 20 to 7 [46]. The other benefits of herbicide-resistant crops have been described in [9, 25].

Besides the benefits of herbicide-resistant crops, their commercialization has created great controversy among government agencies, business consortia, researchers and certain non-profit organizations about the consequences of herbicide-resistant crops and their negative impact on food, feed and environment [3, 27, 80]. The relevance of assessing weedy characteristics when considering the invasiveness of herbicide-resistant crops has been the subject of much debate [90]. Gene flow from transgenic plants to wild relatives may cause wild plants to acquire traits that improve their fitness [29]. Mere presence of wild relatives in a given area does not necessarily imply interspecific gene flow would happen; however, a long term co-existence in a given habitat may signal the need to assess the likelihood of spontaneous gene transfer from GE crops to their wild relatives [68]. For example, jointed goatgrass (*Aegilops cylindrica*), a wild relative of wheat can acquire the herbicide tolerant trait of wheat, and can therefore, thrive in crop fields unless applications of other herbicides are made [41, 42]. Similarly, the survey work in Africa suggested that wild and weedy sorghum occurred intermixed with and adjacent to cultivated sorghum and may pose risk of inter-specific transgene movement [64]. Weedy rice is an important weed in rice growing regions in more than 50 countries [57]. The concern for the introgression of the transgene into the weedy rice may result in more difficult to manage hybrid is a serious consideration for commercial production of novel rice [21, 93].

Plant-derived pharmaceuticals and industrial compounds may have an impact on human and animal health or public perception if they are found in the food or feed systems [38]. For example, the “Starlink” maize incident illustrates how genetically-engineered crop cultivars intended for special purposes may mix with commodity crops. The maize was grown exclusively for animal consumption before determination of whether it was suitable for human consumption. Within a single year, it entered the commodity corn grain supply of the USA [43]. Lack of a channelized production system left growers to decide whether to sell the grains for human or animal consumption. Management of externalities and of the possible unintended economic effects that arise in this context is critical and poses different concerns. The regulatory agencies worldwide are struggling to develop a risk assessment procedure prior to commercial release of engineered crops intended for plant molecular farming and for specialty chemicals [4, 83].

Adventitious presence (AP) is the low level presence of genetically engineered seeds in conventional and organic seeds, in addition to other unwanted materials [53]. Commingling has long been acknowledged and thresholds for AP have been established in conventionally grown crops. However, more recently AP became an issue after commercialization of transgenic crops. With respect to approved crops, the issue is not agronomic performance, food safety, environmental protection, or animal or human health; however, AP is more related with economic concerns, market access, contract specifications, and consumer preferences [53]. Pollen-mediated gene flow from herbicide-resistant to conventional crops and their crop volunteers are the major source of AP in subsequent crops and may create the problems in trade, especially with the European Union (EU) where strict regulations are prevailing for growing, importing and regulating herbicide-resistant crops [11]. These issues related with market and trade will become more complicated as the number of herbicide-resistant crops and traits increase in the future and there is little harmony in regulation of novel crops or their threshold levels internationally.

The aim of the research described in this chapter was to test the following objectives:

1. To discuss risk assessment of herbicide-resistant crops with flax as a model species
2. To determine the occurrence and distribution of weedy and wild relatives of flax and their potential hybridization with commodity flax to predict the risk of inter-specific transgene movement
3. To determine intra-specific pollen-mediated gene flow in flax under natural field conditions and to evaluate the potential for co-existence of herbicide-resistant and organic flax

2. Risk assessment of herbicide-resistant crops with flax as a model species

The cultivation of flax dates back to more than 6,000 years mainly for seed oil and fiber [1]. In addition to the traditional industrial and non-food uses of flax, with the increasing information on molecular biology derived from identification and expression of genes, the potential for the production of novel flax for quality traits has been developed [58, 62]. With the introduction

of high α -linolenic acid (ALA) flax cultivars [73], the world market is increasing dramatically for the flax based products [61].

Flax is a poor competitor and thus, flax fields should be kept free from weeds [89]. Herbicide resistant flax was registered and withdrawn due to market considerations. Few effective herbicides have been registered for controlling weeds in flax [15]. There is an opportunity to develop flax cultivars with enhanced agronomic traits including reduced maturity period and better nitrogen use efficiency. Considering the utility of flax or flax based products for various purposes, genetically engineered flax is under development in Canada. Before genetically engineered flax is commercialized; however, environmental biosafety assessment must be quantified. The following are five considerations of environmental risk assessment of genetically engineered crops adopted by the Canadian Food Inspection Agency (CFIA):

- Potential of the plant with novel trait (PNT) to become a weed of agriculture or be invasive of natural habitats
- Gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive
- Potential for the PNT to become a plant pest
- Impact of the PNT or its gene products on non-target species, including humans
- Impact on biodiversity

3. Inter-specific pollen-mediated gene flow in flax

If the transgene(s) move by pollen-mediated gene flow (*transfer of genetic information between sexually compatible plant populations via cross-pollination*) from herbicide-resistant crops to wild relatives and if it has effect on fitness, weediness, diversity or population size, it is considered as one of the consequences of genetically engineered crops [65]. Gene flow is a natural, biological process which occurs to some degree in all flowering plant species [28]. There is a possibility that generating novel crops for food quality, better weed control or insect control may have effects on other plant populations, especially to closely related species of crops [26, 27]. Some traits may provide a possible benefit to wild or weedy relatives [68, 69]. Therefore, to evaluate the potential introgression of herbicide-resistant flax with its closely related species, a meta-analysis to study the occurrence of wild relatives of flax, their hybridization with cultivated flax and the possibility of transgene movement was quantified.

The evolution of many crop plants has been the result of recurrent cycles of hybridization from wild and weedy relatives [69]. In plant breeding and cultivar development programs, crop wild relatives have been used as a source of gene pool which can be transferred into crop cultivars to expand genetic basis by transferring desirable traits to increase resistivity to biotic and abiotic stresses. However, after commercial production of herbicide-resistant crops, concerns have been raised about the possibility of the sexual transfer of crops' genes to wild relatives through recurrent back crossing [27]. Natural hybridization is known to occur in

many cultivated crops and the potential for transgene movement from herbicide-resistant crops to closely related species has been documented [17].

Flax belongs to family Linaceae which is composed of 22 genera [88] and approximately 300 species [45]. Linaceae is placed in the order Malpighiales [6]. It has been estimated that about 63 wild and weedy species of flax occur in North America [47]. The North American species of *Linum* were grouped into two sections, *Linum* (Eulinum) and *Cathartolinum*. Based on petal color, North American *Linum* includes two groups: the blue flowered and yellow-flowered [87, 71]. Twenty five yellow-flowered species of *Linum* are found in western North America and Central America. The greatest density of this segment of the genus is in east-central Mexico and point to that region as the probable area of origin and establishment of the yellow flowered *Linum* species in North America [71]. *Linum rigidum* Pursh occurs in extreme southern Florida and also has a vast range in the Great Plains extending from the northern Mexico to western Canada [63].

Interspecific hybridization has been attempted in many crops to improve germplasm, however, in *Linum*, such achievements have been very limited because of the difficulties in the successful hybridization between different species of the genus [47]. Attempts have been made to transfer the rust resistance from a wild relative to cultivated flax [23]. We hypothesize that cultivated flax is more likely to hybridize with closely related species having a similar ploidy level based on reported work [7, 36, 37, 75, 92]. Our objective was to predict the potential risk of inter-specific transgene movement from herbicide-resistant flax prior to commercial release.

Interspecific hybridization in *Linum* was first reported between flax and *L. narbonense* [101]. Later, successful hybridization of *L. usitatissimum* with *L. africanum*, *L. angustifolium*, *L. corymbiferum*, *L. floccosum*, *L. pallescens* and *L. tenue* were reported [7, 36, 54, 66, 85]. All crosses produced fertile F₁ hybrids in at least one direction, presumably due to their similarity in ploidy levels and size of chromosomes (Fig. 1). Crosses among five taxa, *L. africanum*, *L. angustifolium*, *L. corymbiferum*, *L. decumbens* and *L. usitatissimum* were highly successful in at least one direction with hybrid progeny exhibiting 80 to 90% germination [44]. Inter specific hybridization between species other than cultivated flax are also successful. When *L. strictum* was used as a male parent, it successfully hybridized with *L. africanum*, *L. angustifolium*, and *L. floccosum* [75]. When *Linum crepetans* and *L. humile* crossed with *L. hirsutum* and *L. hispanicum*, respectively, they produced fertile plants [36, 37, 75] (Fig. 2).

In summary, interspecific hybridization studies indicate that cultivated flax has the potential to hybridize with at least nine wild relatives with a haploid chromosome number of 15 (Fig. 1). *Linum africanum*, *L. angustifolium*, and *L. pallescens* were crossed with *L. usitatissimum* and all reciprocal crosses were successful (Fig. 1). Therefore, further studies should be conducted to determine if hybrids between these three species occur and retain transgenes from herbicide-resistant flax in the natural ecosystem through hybridization and introgression with other wild relatives (Fig. 1).

There have been studies of successful hybridization among taxa other than n=15 (Fig. 2). The species with a haploid chromosome number of 9, constitute the largest group in the genus *Linum* [34]. Some crosses between species of taxa n=9, *L. alpinum*, *L. altaicum*, *L. austriacum*, *L.*

julicum, *L. narbonense* and *L. perenne* produced hybrid plants [36, 37]. The pairing of chromosomes of this species revealed that *L. altaicum* differs by one reciprocal translocation from *L. alpinum*, *L. austriacum*, *L. julicum*, *L. narbonense*, and *L. perenne* [37].

Interspecific hybridization between *Linum* species with different chromosome numbers was also studied. Crosses between *L. alpinum* (n=9, 18), *L. austriacum* (n=9), *L. vulgare* (n=9) and *L. usitatissimum*; as well as crosses between other species with n=15 (i.e., *L. crepetans*, *L. hirsutum*, *L. strictum*, *L. usitatissimum*) with *L. grandiflorum* (n=8), either did not produce any seeds, or failed to produce fertile plants [7, 54, 66, 75, 76] (Fig. 2). These results suggest that similarity in chromosome numbers play an important role in successful inter-specific hybridization in *Linum* species. Therefore, species with different chromosome numbers have no or minimal risk of transgene movement.

Three closely related species of flax are reported to occur in western Canada. Two of them, *L. rigidum* and *L. sulcatum*, have the same chromosome numbers as cultivated flax (n=15). While inter-specific hybridization has not been documented for these species, hybridization of flax with other n=15 species suggests outcrossing may occur. Further research on selected *Linum* species, including a greenhouse study to quantify distribution, flowering time, preferred habit or population and hybridization potential with cultivated flax is warranted to determine whether introgression of the transgene occurs.

4. Intra-specific pollen-mediated gene flow in flax

Intra-specific gene flow is important as it is one of the main factors, along with seed impurity, that could contribute to adventitious presence by cross pollination with conventional cultivars and may cause problems during trade if the herbicide-resistant trait is not approved in the other country. Herbicide-resistant crops may cross pollinate with conventional crops and may introduce transgenes in conventional or organic crops [22]. In Canada and the USA, herbicide-resistant crops are not segregated from conventional or organic crops once the introduced trait has been unconfined released by government agencies [16]. Pollen-mediated gene flow or other sources of adventitious presence, however, may pose problems for the export of herbicide-resistant crop seeds to the countries where the novel trait has not been approved or deregulated [59]. In anticipation of the commercialization of herbicide-resistant crops, field trials have been conducted in many crops to determine distribution of gene flow and the appropriate isolation distances required between herbicide-resistant and conventional crop cultivars to meet AP thresholds.

There were two crop production systems (conventional and organic) prior to introduction of herbicide-resistant crops. However, after the commercial production of herbicide-resistant crops in 1996, the area, crop species, and number of countries growing herbicide-resistant crops have been increasing rapidly [32]. In addition to increase in the herbicide-resistant crop acreages, the concerns are also increasing about the co-existence of three production systems, adventitious presence, and transgene movement from herbicide-resistant crops to conventional and organic crops [40, 67]. Some studies reported that crop-to-crop gene flow can cause

more ecological and economical concerns than crop-to-wild gene flow because in any crop, when an herbicide-resistant cultivar would be grown commercially, gene flow to a conventional cultivar of the same crop may occur more frequently than to wild relatives [27, 28].

A major concern with the commercial cultivation of herbicide-resistant crops is the possibility of introduction of unwanted traits into conventional crop production systems. Adventitious presence of genetically engineered crop seeds in conventional crops may occur by means of pollen-mediated gene flow, admixture in seed lots and also by crop volunteers. Canadian flaxseed is being exported (> 80%) mainly to the EU, the USA, Japan and South Korea Flax Council of Canada, 2007). Growing of agricultural crops represents an open system, and thus a complete separation of plant material or crop production systems is not possible. Considering this fact and to protect the consumer's choice, the EU has defined a 0.9% labeling threshold for the adventitious presence of genetically engineered seeds in conventional products. In 1996, the herbicide (sulfonylurea) resistant flax was registered in Canada [60], but very soon it was de-registered at the request of the Flax Council of Canada, primarily to avoid the trade issue with the EU. This decision has halted further genetic modification in flax.

Currently, flax has been re-discovered as a source of functional foods [33]. In 2005, approximately 200 new flax based food and personal care products were introduced in the US market. This suggests that flax has the highest growth potential in functional food industry because of high level of omega-3 fatty acids [61]. Few studies have suggested that consuming flaxseeds in daily diet can reduce the risk of cardiovascular diseases [14, 61, 94], and certain cancers for example, breast cancer [61, 86]. Flax fibers are also becoming an integral part of new composite materials utilized in automobile and construction industry [58]. Therefore, considering the market demand for various applications, flax is required to be genetically modified. Considering this fact, Canada has decided to move forward for re-visiting genetic engineering of flax, but after risk assessment prior to commercialization by conducting research on environmental biosafety for science based decision making.

The future situation of co-existence of conventional, organic and novel flax cultivars and concern over potential movement of herbicide-resistant traits into commodity crops have illustrated the shortage of information on the gene flow among flax cultivars at intermediate distances. Therefore, the objective of this study was to determine the crop-to-crop gene flow in flax under the field conditions from high α -linolenic acid cultivar (high ALA 18:3 *Cis* $\Delta^{9,12,15}$) to low α -linolenic acid cultivar (low ALA 18:3 *Cis* $\Delta^{9,12,15}$) under the western Canadian climatic conditions.

Field experiments were conducted at two locations, Edmonton Research Station (EdRS) and Ellerslie Research Station (EIRS), University of Alberta in 2006 and 2007 to determine pollen-mediated gene flow in flax at an inter-mediate distance up to 50 m in western Canada. Flax cultivar "AC McDuff" was used as a pollen source (20x20 m in the center, Fig.3) and "Solin" cultivar "SP 2047" was used as a pollen receptor (120x120 m, Fig.3). Both the flax cultivars flowered synchronously. Prior to harvesting, the entire field was divided in eight arms or replicates (Fig. 3). A binder was used to cut all flax plants in the first 3.0 m receptor area to avoid contamination. After 3.0 m, samples were harvested by combine in 1.5 m block distance up to 12.5 m and then in 5.0 m blocks up to 50 m in all 8 replicates. Thiobarbituric acid (TBA)

test determines relative linolenic fatty acid content in oil extruded from flax seed [13]. This method was used for screening flax seeds from receptor populations collected from the field experiments from different distances.

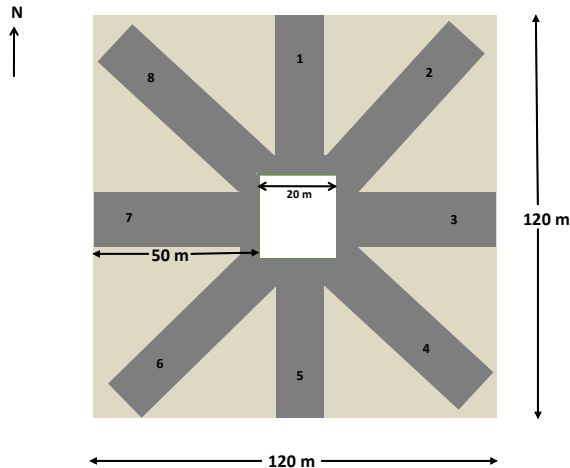


Figure 3. Design of pollen-mediated gene flow experiment in flax at all the locations and years. The pollen source flax cultivar (AC MCDuff) was seeded in the area of 20x20 m in the center of the field. The pollen receptor solin flax cultivar (SP 2090) was grown in surrounding 120x120 m area. After flowering but before harvesting, the pollen receptor area was divided in eight blocks and flax seed samples were collected at specific distances.

Gene flow was tested at various distances (up to 50 m) at each location. At EdRS research site, maximum frequency of gene flow (0.0166) was observed at the minimum mean distance of 0.1 m from the pollen source (Fig. 3). At EIRS site in 2006, 748,800 seeds were screened and the frequency of gene flow was 0.0145 and 0.00011, respectively at 0.1 and 35 m distance from the pollen source. In 2007, maximum frequency of gene flow (0.0242) was recorded at the mean distance of 0.1 m from the pollen source at EdRS site in 2007. Gene flow was quantified up to 25 m distance at EdRS site in 2007 with 13 seeds with high ALA. Some rare gene flow was observed (0.000028) after screening 476,800 seeds at 35 m distance from the pollen source at EIRS site in 2007.

The frequency of gene flow was highest in the recipient plants closest to the source, 0.0166 at EdRS, 2006; 0.0144 at EIRS, 2006; 0.0242 at EdRS, 2007 and 0.0186 at EIRS, 2007 (Fig. 4). At all four locations, gene flow ranged from 0.0001 to 0.001 at 7 m and from 0 to 0.0001 at 25 m from the mean distance from the pollen source. Some rare gene flow events were observed at 35 m mean distance from the pollen source, however at low frequency (0.00109). Several thousand seeds were analyzed at 45 m distance but no gene flow was detected at any location, however, unless all available seeds did not tested, zero gene flow cannot be interpreted but the number of seeds sampled suggest the maximum power value of test which increases the validity of the results obtained in this study.

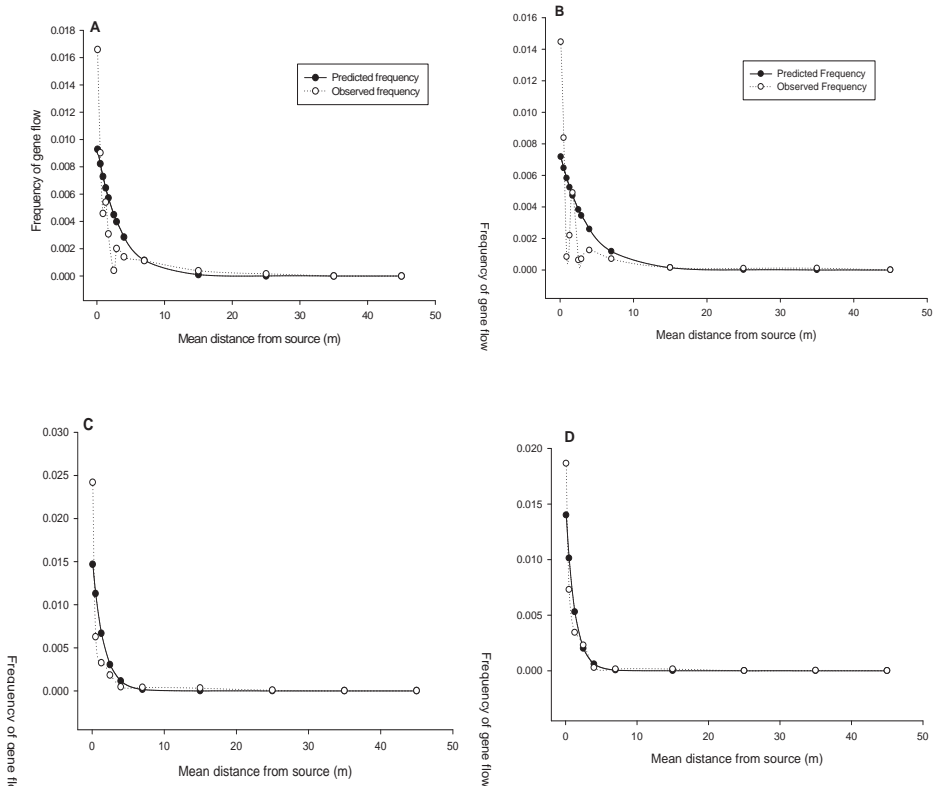


Figure 4. Pollen-mediated gene flow in flax at various locations and years (A) Edmonton Research Station (EdRS), 2006; (B) Ellerslie Research Station (EIRS), 2006; (C) Edmonton Research Station (EdRS), 2007; and (D) Ellerslie Research Station (EIRS), 2007. The triangle indicates the distance, where 50% (O₅₀) reduction in the frequency of gene flow and the arrow indicates the distance where 90% (O₉₀) reduction in gene flow from the pollen source (m) [48].

The frequency of gene flow in this study was almost equal to the other reported work, especially for samples, adjacent to the pollen source [24, 44, 52]. At 0.5 m, average gene flow was reduced to 0.0077; and at 1.0 m, 0.0027. Frequency of gene flow was reduced from 0.0126 to 0.0033, when flax cultivars were grown 1.25 to 0.25 m apart, respectively. In a similar experiment, [70] reported that frequency of gene flow in flax varied from 0 to 0.003, depending on the spacing between plants and climatic conditions.

Flax cultivars AC McDuff and SP 2047 were used in this experiment because they have similar flowering periods, which provides better chances of gene flow to occur. But for the flax cultivars with different flowering periods, the gene flow may be more or less than observed in this study. Environmental conditions and wind direction or speed did not appear to influence the amount of pollen-mediated gene flow in this study over two growing seasons. Dillman reported that sticky flax pollen was primarily disseminated by honey bees and thrips [24]. Subsequent experiments suggest that honey bee was the most frequent visitor of flax

flowers followed by bumble bees and thrips [79]. In this study, pollinators may have played a major role in gene flow between two flax cultivars.

5. Conclusion

Flax is the second most important oilseed crop in western Canada. Because flax has unique oil and fiber attributes, it is being evaluated as a model plant species for bio-industrial and nutraceutical products. The development of flax with novel traits has raised concerns regarding the pollen-mediated gene flow to conventional flax and closely related species. As a part of pre-commercialization risk assessment of herbicide-resistant flax, intra and inter-specific pollen-mediated gene flow were quantified. Results from the average value of all location-year suggest that the frequency of gene flow at 0.1 m distance was in the range from 0.0185 to 0.01 and declined rapidly with distance from the pollen source. The minimum frequency of gene flow was recorded at 35 m distance from the pollen source. Thus, pollen-mediated gene-flow from herbicide-resistant flax to commodity flax may occur at short distances but would be rare beyond 35 m.

The occurrence of flax wild relatives and reported inter-specific hybridization was reviewed to initiate the evaluation of environmental risk of inter-specific transgene movement from herbicide-resistant flax. Inter-specific hybridization and cytogenetic studies between flax and congeneric species demonstrated that cultivated flax has ability to hybridize and form viable F_1 plants with at least nine species of *Linum*, which are reported to occur in Asia and Europe indicates that inter-specific gene flow will be negligible in North America. Hybridization of flax with many other wild relatives reported to occur in North America has either not been studied or reported. More research is required to study species distribution, sympatry, concurrent flowering, ploidy level and sexual compatibility of flax wild relatives.

In summary, the results suggest that pollen-mediated gene flow in flax will be a minor contributor to adventitious presence of herbicide-resistant flax. Minimum threshold levels will have to be established based on the impurities arising from various transgene contributors such as gene flow from other flax cultivars, occurrence of gene introgression from closely related species, seed-and pollen-mediated gene flow from herbicide-resistant flax volunteers, and admixture during production, transportation or trade.

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