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Environmental biosafety of genetically engineered crops: Flax (*Linum usitatissimum* L.) as a model system

by

Amitkumar Jayendrasinh Jhala

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Examining Committee

Dr. Linda Hall, Agricultural, Food and Nutritional Science

Dr. Randall Weselake, Agricultural, Food and Nutritional Science

Dr. Lloyd Dosdall, Agricultural, Food and Nutritional Science

Dr. Jocelyn Hall, Biological Sciences

Dr. Robert Blackshaw, Agriculture and Agri-Food Canada, Lethbridge

Dedicated to my Madam for her continuous love, support and encourangement

Abstract

Flax (*Linum usitatissimum* L.) is considered as a model plant species for multipurpose uses with whole plant utilization for several purposes including industril, food, animal feed, fiber, nutraceutical, pharmaceutical, and bioproduct markets. Therefore, flax is in the process of genetic engineering to meet the market requirements. Prior to commercial release of genetically engineered (GE) flax, a risk assessment was conducted to determine intra- and inter-specific pollen-mediated gene flow and for quantifing and mitigating the adventitious presence (AP) of volunteer flax in canola (*Brassica napus* L.). The results of pollen-mediated gene flow study (crop-to-crop) suggest that about 1.85% outcrossing would occur in adjunct area, when two flax cultivars were grown in close proximity of 0.1 m apart. Some rare gene flow events were recorded maximum up to 35 m distance from the pollen source but at a very low frequency.

The genus *Linum* has several wild and weedy species, distributed in many parts of the world. A meta-analysis was conducted to determine the potential for gene introgression from GE flax to wild relatives, the occurrence, the phylogeny of flax wild relatives and reported interspecific hybridization. The results demonstrated that cultivated flax has ability to hybridize and form viable F_1 plants with at least nine species of *Linum*; however, none of these species have been reported to occur in Canada. Hybridization of flax with many other wild relatives has either not been studied or reported. However,

based on the evidence of reported work, gene flow from GE flax to wild or weedy relatives may occur elsewhere depending on species distribution, sympatry, concurrent flowering, ploidy level and sexual compatibility.

The results of the experiments to mitigate the adventitious presence of flax volunteers in canola suggest that combinations of pre-plant followed by post-emergence herbicides were most effective for reducing volunteer flax density and AP in glufosinate-resistant canola. Post-emergence application of imazamox+imazethapyr, however, was not effective for controlling volunteer flax in imidazolinone-resistant canola. Best management practices were developed to mitigate transgene movement from GE flax to ensure co-existance of GE, conventional and organic flax without market harm.

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Arachidonic acid	AA
Agriculture and Agri-Food Canada	AAFC
Adventitous presence	AP
Animal and Plant Health Inspection Service	APHIS
Amplified fragment length polymorphism	AFLP
Alpha-linolenic acid	ALA
Analysis of variance	ANOVA
Angiosperm Phylogeny Group	APG
Atherosclerotic cardiovascular disease	ASCVD
Bacillus thuringiensis	*Bt*
Canadian Food Inspection Agency	CFIA
C-reactive protein	CRP
Commission of the European Communities	EC
Docosahexaenoic acid	DHA
Edmonton Research Station	EdRS
Eicosapentaenoic acid	EPA
Ellerslie Research Station	ElRS
European Molecular Biology Organization	EMBO
Enolpyruvylshikimate-3-phosphate synthase	EPSPS
Environmental Protection Agency	EPA
European Union	EU

Economic Research Service	ERS
Food and Agriculture Organization	FAO
Food and Drug Administration	FDA
Fischer's Protected Least Significant Difference	LSD
Generally recognized as safe	GRAS
Genetically engineered	GE
Genetic Engineering Approval Committee	GEAC
Genetically engineered	GM
Germplasm Resources Information Network	GRIN
Gibberellic acid	GA_3
Genetically engineered organisms	GMOs
Genetic Use Restriction Technology	GURT
Glufosinate-resistant	GR
High-density lipoprotein	HDL
Imidazolinone-resistant	IR
International Service for the Acquisition of Agri-biotech Applications	IAAA
Ketoacyl-CoA synthase	KCS
Linoleic acid	LA
Linseed oil meal	LSOM
Low-density lipoprotein	LDL
2-methyl-4-chlorophenoxyacetic acid	MCPA
Mixed model	PROC

MIXED

neomycin phosphotransferase	*npt*
North Central Regional Plant Introduction Station	NCRPIS
Organization for Economic Co-operation and Development	OECD
Phosphonothricin acetyl transferase	PAT
Phosphomannose isomerase	PMI
Plant Biosafety Office	PBO
Plant Gene Resources of Canada	PGRC
Plant with Novel Trait	PNT
Polyhydroxybutyrate	PHB
Poly unsaturated fatty acids	PUFA
Random amplification of polymorphic DNA	RAPD
recombinant deoxyribonucleic acid	rDNA
Ribonucleic acid interference	RNAi
Serum amyloid A	SAA
Statisical Analysis Software	SAS
Stearoyl-ACP desaturase II	*sad2*
Thiobarbituric acid	TBA
Triacylglycerol	TAG
United States Department of Agriculture	USDA
Very long polyunsaturated fatty acids	VLCPUFA
World Health Organization	WHO

Chapter 1

Environmental biosafety of genetically engineered crops: Flax (*Linum usitatissimum* L.) as a model system[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 the developing countries (FAO, 2009). By 2050, developing countries will account for 93% of cereal and 85% of meat demand growth (Rosegrant and Cline, 2003). The ability of agriculture to support a growing population has been a concern and continues to be on high priority on the global policy agenda. Eradication of poverty and hunger was included as one of the United Nations Millennium Development Goals (Anonymous, 2000).

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, heat waves and rises in sea-levels are predicted to occur more

[1]*A version of this chapter will be submitted as a book chapter: Jhala A.J. and Hall L.M. Environmental biosafety of genetically engineered crops: Flax (Linum usitatissimum L.) as a model system. In "Plant Biotechnology and Transgenic Research" edited by Thangadurai D., Othman R.Y. and Biradar D.P., Bentham Science Publisher, Oak Park, IL, USA.*

frequently and would have a large impact on crop productivity (Challinor et al., 2009). 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 (Anderson and Bows, 2008). Higher growing season temperatures can have dramatic impacts on agricultural productivity, farm incomes and food security (Battisti and Nylor, 2009). 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 (Lobell et al., 2008).

New agricultural technologies will be needed to ensure global food security and support conservation of water and lands. Crop cultivars with higher yields and resistivity are required to meet the food requirements in a sustainable manner without causing disruption to the environment. In her book “Silent Spring”, Carson (1962) suggested finding a biological solution of pest control in agriculture as an alternate of using chemical pesticides. One approach is to develop genetically engineered (GE) crops.

Research and development of GE crops were started in 1980s. Several experiments have been conducted in laboratories and greenhouses to develop plants which can produce better yields under natural ecosystems by resisting insects, pests and diseases. GE crops were first grown at a commercial scale in

1995-96 in the USA and Canada (James and Krattiger, 1996). Manipulations of resistance to herbicides or insects were the first traits to be commercially released. Starting from few thousand hectares in 1996, planting area and number of crops and countries have been rapidly increased to >110 million hectares (>280 million acres) in 2007, including 60% in industrial countries and 40% in the developing world (James, 2007). From 2002 onwards, adoption of GE crops in the developing world increased rapidly. In 2007, GE soybean (*Glycine max* L.) was grown on about 60 million hectares, followed by maize (*Zea mays* L., 35 million hectares), cotton (*Gossypum hirsutum* L., 15 million hectares), and canola (*Brassica napus* L., 5 million hectares) (James, 2007). Out of 110 million hectares of total GE crops in 2007, about 70 million hectares have been herbicide resistant (HR) and nearly 20 million hectares have been insect resistant crops (James, 2007).

Global status of GE crops

Genetically engineered (GE) crops, when introduced commercially in 1996, growers in six countries (USA, China, Argentina, Canada, Australia and Mexico) grew GE crops on 2.8 million hectares (James, 1997). By 2008, twenty five countries had adopted commercial production of GE crops on an estimated 125 million hectares (James, 2008) (Table 1-1). The area and number of countries adopting GE crops can be expected to increase. It has been estimated that 63 countries now carry out research on > 50 agricultural

crops for genetic modification for various traits (Runge, 2004). Currently, five major GE crop producing countries are the USA, Argentina, Brazil, India and Canada (Figure 1-1).

Europe has the strictest regulations for growing, importing and labeling GE crops (Johnson et al., 2007). The only GE crop, *Bt* maize (*Zea mays* L.) expressing the insecticidal protein Cry1Ab from *Bacillus thuringiensis* has been commercially grown in the European Union (EU) (Devos et al., 2008). However, the EU might import GE seeds or GE crop based products for animal feed or other non-food applications (Devos et al., 2008). For example, the EU has authorised import and processing of three herbicide resistant canola cultivars for animal feed (Anonymous, 2007b). In 2007, *Bt* maize was grown on about 110,000 hectares in Spain, France, Czech Republic, Portugal, Germany and Slovakia (Anonymous, 2007a). In 2007, Spain was the largest producer of GE maize among the EU countries, comprising of ~75,000 hectares (Figure 1-2).

Producers, government, regulators and growers in the USA have adopted GE crops widely since their introduction. Herbicide resistant soybean (*Glycine max* L.) and cotton (*Gossypium hirsutum* L.) have been the most widely and rapidly adopted GE crops in the USA, followed by insect-resistant cotton and maize. Based on the United States Department of Agriculture (USDA) survey, plantings of GE soybean went from 17% of total US soybean acreage in 1997

to 68% in 2001, and as high as 92% in 2008 (ERS-USDA, 2008) (Figure 1-3). The USDA/APHIS (Animal and Plant health Inspection Service) is the major regulating agency for growing or importing GE crops in the USA. Number of petitions per year received by the USDA/APHIS for regulating GE crops in the USA varies considerably (Figure 1-4). GE crops field trial permits and notifications approved by USDA/APHIS generally have increased since 1995 (Figure 1-5). The adoption of herbicide resistant maize has accelerated, reaching 63 percent of US maize acreage in 2008. Planting of Bt maize grew from 8% of US maize acreage in 1997 to 57% in 2008 (ERS-USDA, 2008).

Argentina ranks second after the USA in terms of the largest area (21 million hectares) under GE crops in 2008 (Figure 1-1). The first GE crop, glyphosate-resistance soybean was introduced in Argentina in 1996, the first year of global commercialization of GE crops. Later on, GE maize and cotton resistant to herbicides or insects were approved by the local regulatory authorities in 1997. In Argentina, the area planted to HR soybean was <1.0% of the total soybean area in 1997, which was then extended to 98% in 2006 (14.5 million hectares); and in 2007, up to 99% with an annual growing area of about 16 million hectares, equivalent to an annual growth rate of 6%. The area of GE maize also extended to 2.8 million hectares in 2007-08, and approximately 40,000 hectares of GE cotton. It was estimated that the direct benefits for Argentina in the first decade, 1996 to 2005, would be a total of US

$ 20.2 billion by adopting GE soybean, maize and cotton.

Brazil is the third largest adopter of GE crops, estimated at 15.8 million hectares, of which 14.5 million hectares were grown to glyphosate resistant soybean, and the remaining area was grown to *Bt* cotton in 2007 (James, 2007). In 2006, the total area under GE crops was 11.5 million hectares in Brazil. Thus, considering the year-over-year growth of 30% was the second highest in the world, after India.

India has the fourth largest area under GE crops in 2008. In 2002, Bt cotton was permitted for commercial cultivation in India by the Genetic Engineering Approval Committee (GEAC). India is the third largest producer of cotton (after China and the USA) with an annual production area of about 9 million hectares, which accounts for approximately 25% of the world's total cotton area and 16% of the global cotton production (Bennett et al., 2004). In 2008, *Bt* cotton was grown on about 7.6 million hectares primarily in six states including Andhra Pradesh, Gujarat, Madhya Pradesh, Karnataka, Maharashtra and Tamil Nadu (APCoAB, 2006). The results of a study conducted on the economic impact of growing *Bt* cotton in India suggest that it has a significant positive impact on crop yields and economic performance for cotton growers (Bennett et al., 2004). Considering the experience and benefits of growing *Bt* cotton, the cultivation area is projected to increase in coming years in India (Barwale et al., 2004).

Canada was among the first countries to grow GE crops at a commercial scale with the introduction of glyphosate and glufosinate resistant canola in 1995-96 with the growing area of about 0.1 million hectares (James C., 1997). Total area under GE crops in 2008 in Canada was ~7.6 million hectares, mainly canola, maize and soybean (James, 2008). Canola cultivars resistant to glyphosate (Roundup Ready®), glufosinate (Liberty Link®) or imidazolinone (Clearfield®) herbicides were granted unconfined release in 1995. In 2008, planting of canola in Canada was 15.6 million acres. Among them, 45% were glyphosate resistant, 41% glufosinate resistant, 13% imidazolinone resistant and only 1% were conventional cultivars (Figure 1-6).

Despite the concerns raised by the EU, Australia and elsewhere, following introduction of GE canola, Canada's canola seed exports increased its share of world exports to 77%. Canada also dominates the canola oil export market. In 2005-06, Canada exported 1.09 million tonnes of canola oil, which equates to around 65% of total world canola oil exports (Anonymous, 2007c). Overall, the canola industry adds $13.8 billion to the Canadian economy (Canola Council of Canada, 2009).

The second most important GE crop in Canada is soybean, including glyphosate-, glufosinate- and sulfonylurea-resistant soybean. Glufosinate resistant soybean, however, has never been marketed in Canada (Sikkema and Soltani, 2007). In 2007, farmers planted > 1.35 million acres of glyphosate

resistance soybean, representing approximately 65% of the market share. Currently GE and non-GE soybeans co-exist to satisfy divergent market and domestic crush needs. In Canada, glyphosate- and glufosinate-resistant maize was introduced in 2001 and 2002, respectively. By 2005, 21% of the maize growing area in eastern Canada was planted to glyphosate resistant maize (Sikkema and Soltani, 2007). Studies have shown that yield, weed control, and net returns with herbicide resistant crops were generally higher than with conventional crops (Beckie et al., 2006b; Harker et al., 2007).

Environmental biosafety and risk assessment of GE crops

As the application of biotechnology to agricultural products evolved, research to define and observe effects of these products derived from recombinant DNA technology in the environment has been critical. It has also emphasized the need for a discipline in making a distinction between evidence critical for quantifying hazards or risks from that which is spurious or aberrant and does not contribute to the weight of evidence necessary to make an appropriate decision (Kok et al., 2008).

Risk assessors and decision makers have worked with the scientific community since the first GE crop was proposed for field testing in 1980s. The scientific community provided leadership in framing the approach to risk assessment of GE crops that includes risk assessment, risk management, risk communication and risk monitoring (Corbet et al., 2007). The scientific

community introduced the concept of familiarity, emphasizing the need for understanding the unmodified organism, the trait and the organism that donated the genetic material, as well as recipient environment into which the modified organism is to be used in order to identify potential hazards on a case-by-case basis (Ramessar et al., 2007). These concepts allowed a framework to evaluate GE crops to be constructed and elaborated internationally.

The Organization for Economic Co-operation and Development (OECD) played an important role in development of national and international risk assessment frameworks of GE crops by focusing on practical and scientific issues related with commercialization of GE crops (Gaugitsch, 2006). The OECD Consensus Documents as well as the OECD Product Database have provided good basis for biosafety assessment framework and their implementation (Schiemann, 2006). The book entitled “Recombinant DNA Safety Considerations”, also known as “Blue Book”, published by the OECD in 1986, is the resource document most frequently cited at the international level (Balazs, 2006). International and national guidance on environmental risk assessment provide critical direction about the types of scientific data needed to evaluate GE crops. This guidance by governments on risk assessment is strongly influenced by the results of biosafety research. In turn, national and regional research priority areas have been identified based upon

close collaboration with risk assessors as their needs evolve (Bergmans, 2006).

Tiered testing is one of the approaches suggested by researchers and regulatory authorities for the environmental risk assessment of chemical substances and GE crops (Garcia-Alonso et al., 2006). Experiments start with the worst case scenario in lower tiers, which directs the extent and nature of the experiments to be conducted in higher tiers (Raybould and Cooper, 2005). If the risks evaluated at any stage are shown to be negligible or acceptable with reasonable certainty, or it is considered that sufficient information to make a regulatory decision has been collected, then assessment is concluded. If unacceptable risks are identified or unacceptable uncertainty remains, the assessment is refined in more environmentally realistic conditions at higher tiers. The tiered approach is consistent with iterative or recursive nature of risk assessment where conclusions are reviewed when new knowledge is obtained (Raybould, 2006). Post-market monitoring is also a tool available to address uncertainty and allow collection of additional information following a commercialization of GE crops (Mauro and McLachlan, 2008).

Major benefits of GE crops

Introduction of GE crops at a commercial scale was controversial; but, this new technology has also provided many benefits to growers, consumers and environment. On a global basis, GE technology has reduced pesticide use, with the level of reduction varying between crops and the introduced trait. It

has been estimated that use of GE soybean, canola, cotton and maize modified for herbicide and insect resistant cotton reduced pesticide use by a total of 22.3 million kg of formulated product in the year 2000 (Phipps and Park, 2002). Since that time, GE crop production area has increased rapidly with continued reductions expected in pesticide usage.

In the 1970's, the World Health Organization (WHO) estimated that there were globally 500,000 pesticide poisonings per year, resulting in 5000 deaths. The Environmental Protection Agency (EPA) estimates that pesticide poisoning occur between 10,000 and 20,000 agricultural workers in the USA (Phipps and Park, 2002). There are no data available for pesticide toxicity in developing countries; but, it may be worse due to poor education level and lack of awareness of the inherent dangers of pesticides, inadequate protective clothing and lack of appropriate training. The average number of insecticide applications to cotton in India has been reduced from about 8 to 3.5 following the introduction of *Bt* cotton (Barwale et al., 2004). Among rice growers in Philippines, over half of the farmers claimed sickness due to pesticide use (Rola and Pingali, 1993). Thus, the reduction in pesticide application may reduce the incidence of pesticide toxicity to humans as well as the environmental damage.

Weeds compete with crops for moisture, nutrients and light and their presence greatly reduce crop yields (Blackshaw et al., 2005). Introduction of

glyphosate and glufosinate resistant crops have enabled farmers to select the most suitable and environmentally-friendly herbicides from a range of compounds (Duke, 2005). For example, in the United States, glyphosate resistant cotton, maize and soybeans have increased weed management levels in all three crops (Askew and Wilcut, 1999; Faircloth et al., 2001; Johnson et al., 2000). Similar results have been reported in glyphosate resistant canola in Canada (Harker et al., 2007). For example, in contrast to conventional canola, the introduction of herbicide resistant canola has increased yields by 10%, reduced herbicide use by 40% (g/g) and also reduced fuel use for tillage and pesticide applications in Canada (Breithaupt, 2004).

In many parts of the world, agricultural pesticides have been used in excess of requirements, leaving residue in food and 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. China is the major producer of cotton and the Chinese growers are amongst the largest users of pesticides in cotton fields. A survey conducted by Huang et al. (2001) suggest that following introduction of Bt cotton, pesticide use was reduced from 55 to 16 kg formulated product per hectare and pesticide applications were reduced from 20 to 7.

Crop improvements that confer tolerance to environmental stresses and soil toxicity and high yields and biomass are under development (Warwick et al., 2009). First generation GE traits focused on herbicides and insects resistant have been exploited from the last decade. Relatively few traits in limited crops have been released for commercial production despite extensive research efforts in several crops including in minor crops, vegetables, fruits and ornaments. This is in part because of the cost associated with the regulatory hurdles and in part due to the risk of market harm from GE crops in some parts of the world. Subsequent traits now in development will focus of abiotic stress resistance (second generation GE crops) (Warwick et al., 2009) and for plant molecular farming and specialty industrial products (third generation GE crops) (Fischer et al., 2004; Ma et al., 2005).

Modification of fatty acids is a prime target for modification of oilseed crops (Cahoon, 2003; Kinney and Clemente, 2005; Taylor et al., 2008). Many of the enzymes involved in fatty acid biosynthesis and degradation have been characterized and much research has been carried out on GE approaches for the modification of oil and fat content in plants (Weselake, 2005). This can be applied to enhance levels of the essential fatty acids, linoleic acid and α-linolenic acid and to synthesize very long chain polyunsaturated fatty acids (VLCPUFAs): arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are usually sourced from fish oils

(Burdge, 2005). Recent progress has also been made in the reconstitution of the DHA biosynthetic pathway in GE plants. For example, GE *Arabidopsis* seeds with total fatty acids containing up to 0.5% DHA were produced. Another significant achievement was the production of DHA in soybean seeds up to 3% of total fatty acids (Damude and Kinney, 2008). Current technology will allow for these contents to be increased threefold while maintaining EPA abundance in 10 to 15% range and may provide an effective marine oils substitute (Hoffman et al., 2009).

Genetic transformation techniques can also be used to introduce plants DNA that code for the expression of novel proteins of direct interest. This allows host plant to be used in molecular farming where intended harvest products are high value recombinant proteins and organic molecules that can be used in the production of pharmaceutical compounds (Fischer et al., 2004), as well as recombinant enzymes and polymers with industrial applications (Howard, 2005). For example, safflower (*Carthamus tinctorius* L.) has been transformed with the intention of field scale production of high value proteins for use as pharmaceuticals and industrial enzymes (McPherson et al., 2009). There are several biotechnology derived drugs have been approved by the United States Food and Drug Administration (FDA) with more than 300 new biopharmaceutical drug products and vaccines currently under clinical trials (US Food and Drug Administration, 2002). The other benefits of GE crops

have been described in Beckie et al. (2006b) and Duke (2005).

Major consequences of GE crops

Development and commercialization of GE crops have caused great controversy among government agencies, business consortia, researchers and certain non-profit organizations about the consequences of GE crops and their negative impact on food, feed and environment. However, many of these focus on unintended effects that cannot be predicted from the trait, crop or environmental interaction. In this thesis, I will focus on environmental biosafety issues and market implication of gene flow with the agro-environment.

The following are five considerations of environmental risk assessment of GE crops, outlined by the OECD and followed by CFIA for regulating GE crops in Canada,

- 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

The relevance of assessing weedy characteristics when considering the invasiveness of GE crops has been the subject of much debate (Warwick et al., 2009). Gene flow from GE plants to wild relatives may cause wild plants to acquire traits that improve their fitness (Ellstrand and Schierenbeck, 2006). The mere presence of wild relatives in a given area does not necessarily imply interspecific gene flow would happen; however, 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 (Rieseberg et al., 1999). For example, jointed goatgrass (*Aegilops cylindrica* H.), 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 (Hanson et al., 2005; Hanson et al., 2002). Similarly, the survey work has been conducted for wild and weedy sorghum (*Sorghum bicolor* L.) in Africa that was inter-fertile with the crop and constitutes a crop-wild-weed complex (Snow et al., 2005). Weedy rice is an important weed in rice growing regions in more than 50 countries (Kumar et al., 2008). Introgression of transgene into weedy rice may result in a more difficult to manage hybrid and thus is a serious consideration for commercial production of GE rice (Chen et al., 2004; Zhang et al., 2006).

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 (Graff, 2006). For example, the “Starlink” GE maize

incident illustrates how GE crop cultivars intended for special purposes may become mix with commodity crops. The GE maize was grown exclusively for animal consumption before determination was made of whether it was suitable for human consumption. Within a single year, it entered the commodity maize grain supply of the USA (Haslberger, 2001). Lack of a channelized production system left growers to decide whether to sell the grain 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 different risk assessment procedure prior to commercial release of GE crops intended for plant molecular farming and for specialty chemicals (Andow, 2004; Stewart and Knight, 2005).

Adventitious presence (AP) is the low level presence of GE seeds in conventional and organic seeds, in addition to other unwanted materials (Kershen and McHughen, 2005). Commingling has long been acknowledged and thresholds established in conventionally grown crops. However, more recently AP became an issue after commercialization of GE crops. With respect to approved GE crops, the issue is not agronomic performance, food safety, environmental protection, or animal or human health; however, AP is more related to economic concerns, market access, contract specifications and consumer preferences (Kershen and McHughen, 2005). GE crop volunteers

are the major source of AP in subsequent crops and may create the problems in trade, especially with the European countries where very strict regulations are prevailing with the growing, importing and regulating GE crops (Beckie and Owen, 2007). These issues related with market and trade will become more complicated as number of GE crops and traits increases in future and there is little harmony in regulation of GE crops or their threshold levels internationally.

The argument on consequences of GE crops is multi-faceted and complex involving diverse issues including sustainability of modern agriculture, ethics and concern that multi-national corporations dominate agriculture industry. However, risk assessment studies carried out to identify hazard and exposure and experience of past 12 years of commercialization of GE crops on several million hectares in many countries have not documented any major risk or direct consequences caused by GE crops. Quite the reverse, substantial benefits to the environment have been accrued (Beckie et al., 2006a; James, 2003).

Flax- A model crop

Flax (*Linum usitatissimum* L.) is an annual, eudicot, oilseed species. The cultivation of flax dates back to more than 6,000 years mainly for seed oil and fiber (Gill, 1987). Flax is a poor competitor and thus, flax fields should be kept free from weeds (Wall, 1994). Herbicide resistant flax was released and withdrawn due to market considerations. Few effective herbicides have been registered for controlling weeds in flax (Brook, 2007).

In addition to the traditional industrial and non-food uses of flax (Vaisey-Genser and Morris, 2003), with the increasing information on molecular biology derived from identification and expression of genes, the potential for the production of GE flax for quality traits has been developed (Kymäläinen and Sjöberg, 2008; Moryganov et al., 2008). With the introduction of high α-linolenic acid (ALA) flax cultivars (Rowland, 1991), the world market is increasing dramatically for the flax based products (Morris, 2007). The current use of flax oil and fiber and their future applications in functional food and nutraceutical markets are discussed in **chapter 2**.

Considering the utility of flax or flax based products for various purposes, GE cultivars of flax are under development in Canada. Before GE flax is commercialized, however, environmental biosafety assessment must be quantified. Pollen-mediated gene flow (transfer of genetic information between sexually compatible plant populations via cross-pollination) is

considered as one of the consequences of GE crops (Poppy, 2000). Gene flow is a natural, biological process which occurs to some degree in all flowering plant species (Ellstrand et al., 1999). There is a possibility that generating GE crops for food quality, better weed control or insect control may have effects on other plant populations, especially to closely related species of crops (Ellstrand, 2006). Some traits may provide a possible benefit to weeds that introgress the genes (Snow and Jorgensen, 1999). Therefore, to evaluate the potential introgression of GE flax with its closely related species, a meta-analysis to study the taxonomy, phylogeny, distribution and occurrence of wild relatives of flax, their hybridization with cultivated flax and the possibility of transgene movement was conducted and will be discussed in **chapter 3**.

Pollen-mediated gene flow is not unique to GE crops, however, the process has received much attention with the need to ensure co-existence of GE, conventional and organic crops (Poppy and Wilkinson, 2005). GE crops may cross pollinate with conventional crops and may introduce transgenes in conventional or organic crops (Colbach et al., 2009). In Canada and the USA, GE crops are not separated from conventional or organic crops once the introduced trait has been approved by government agencies (Brookes and Barfoot, 2004). Pollen-mediated gene flow or other sources of adventitious presence, however, may pose problems for export of GE crops seeds to the

countries where GE trait has been approved or deregulated (Mallory-Smith and Zapiola, 2008). In anticipation of the commercialization of GE crops, field trials have been conducted in many crops to determine intra-specific gene flow and the distance up to which pollen can travel to determine the isolation distances between GE and conventional crop cultivars. In allogamous species (e.g. maize), pollen-mediated gene flow is generally important component of transgene dissemination (Goggi et al., 2007). Frequency of gene flow varies from species to species and environment. For example, in canola, gene flow has been reported up to 3,000 m in Australia (Rieger et al., 2002). Although wheat is a highly self pollinated species, gene flow has been reported to occur up to 2.75 km from the source population (Matus-Cadiz et al., 2007). Intra-specific pollen-mediated gene flow in flax is evaluated experimentally and discussed in **chapter 4**.

Seed-mediated gene flow is the dissemination of transgenes via GE crop seeds during seed shattering, harvest loss, seed production by crop volunteers, movement by birds and predators, or by humans during transportation, handling, grading and other operations including shipment for trading (Kershen and McHughen, 2005). Seed-mediated gene flow from GE crops has received less consideration especially for minor crops (Gruber et al., 2008). Seed admixtures of GE and conventional crops is a routine concern for the seed trade because of the issues related to adventitious presence (AP)

[unintentional commingling of trace amounts of genetically engineered crop seeds (in addition to other unwanted materials) in conventional or organic crop seeds] (Demeke et al., 2006). For autogamous species like soybean and flax, dissemination of transgenes via seed would be a primary source compared to pollen-mediated gene flow. Therefore, quantification and modeling seed-mediated gene flow is an important aspect of risk assessment of GE crops to predict the ability to segregate GE crops from conventional crops and to reduce the adventitious presence (Christianson et al., 2008). Herbicide resistant crop volunteers may be a concern in subsequent crops, if they are resistant to herbicide that would be used for control of other weeds and there is no another option available as effective or economical (Beckie and Owen, 2007). Volunteer crops are common weeds in western Canada (Leeson et al., 2005) and require management strategies to control them. Volunteer crops perpetuate seed and pollen-mediated gene flow.

Crop rotation of at least three years between a flax crop is recommended for controlling volunteer flax, and various soil-borne or stubble-borne diseases and pests of flax (Anonymous, 2006). Cereals after flax may also have options for controlling volunteer flax by pre- and post-emergence herbicides (Brook, 2007). Rotation to canola from flax is less frequent but may occur on the Canadian Prairies. Limited information is available on controlling volunteer flax in herbicide resistant canola. Thus,

multi-year-location experiments were conducted to determine the amount of AP of volunteer flax in canola and a strategy to control them **(chapter 5)**.

Best management practices and stewardship programs have been introduced to mitigate transgene movement from GE crops. Agronomic measures to minimize gene flow may rely on spatial isolation (e.g. distances between GE and convention crops fields), temporal isolation (planting period and coordination of crop rotation with neighbor growers) and on GE crops free zones (Devos et al., 2008). To reduce the seed-mediated gene flow, the harvest loss of GE crop seeds should be reduced by proper adjustment of combine settings and by controlling GE crop volunteers by herbicides. Education of growers, cleaning of farm equipments and separate production and supply chains of GE and conventional crops are additional management practices to minimize transgene movement. In addition, genetic use restriction (GURTs) technologies have been an interested field of study aimed to reduce transgene movement from GE crops (for more details on GURT's see Hills et al., 2007; Van Acker et al., 2007). The registration and regulation of GE flax in Canada and determination of the best management practices to mitigate transgene movement under co-existence of GE and organic flax are discussed in **chapter 6.**

Research objectives

This research project was a component of a larger program to develop flax as a functional food and bioindustrial crop in Canada. The aim of the research described in this dissertation was to test the following objectives:

1. To describe current uses and future applications of flax in nutraceutical, industrial and functional food markets
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 GE and organic flax
4. To quantify and mitigate volunteer flax in herbicide resistant canola by using pre- and post-emergence herbicides
5. To describe the potential benefits, risks, regulations and mitigation of transgene movement from GE flax

Table 1-1 Global area of GE crops grown in individual country in 2008*

Rank	Country	Area (million hectares)	GE Crop Grown
1	USA	62.5	Soybean, maize, cotton, canola, squash, papaya, alfalfa, sugarbeet
2	Argentina	21	Soybean, maize, cotton
3	Brazil	15.8	Soybean, cotton
4	India	7.6	Cotton
5	Canada	7.6	Canola, maize, soybean
6	China	3.8	Cotton, tomato, poplar, petunia, papaya, sweet papper
7	Paraguay	2.7	Soybean
8	South Africa	1.8	Maize, soybean, cotton
9	Uruguay	0.7	Soybean, maize
10	Bolivia	0.6	Soybean
11	Phillipines	0.4	Maize
12	Australia	0.2	Cotton
13	Spain	0.1	Maize
14	Mexico	0.1	Cotton, soybean
15	Colombia	< 0.1	Cotton, carnation
16	Chile	< 0.1	Maize, soybean, canola
17	France	< 0.1	Maize
18	Honduras	< 0.1	Maize
19	Czech Republic	< 0.1	Maize
20	Portugal	< 0.1	Maize
21	Germany	< 0.1	Maize
22	Slovakia	< 0.1	Maize
23	Romania	< 0.1	Maize
24	Poland	< 0.1	Maize
25	Burkina Faso	< 0.1	Cotton

*Source: (James, 2008)

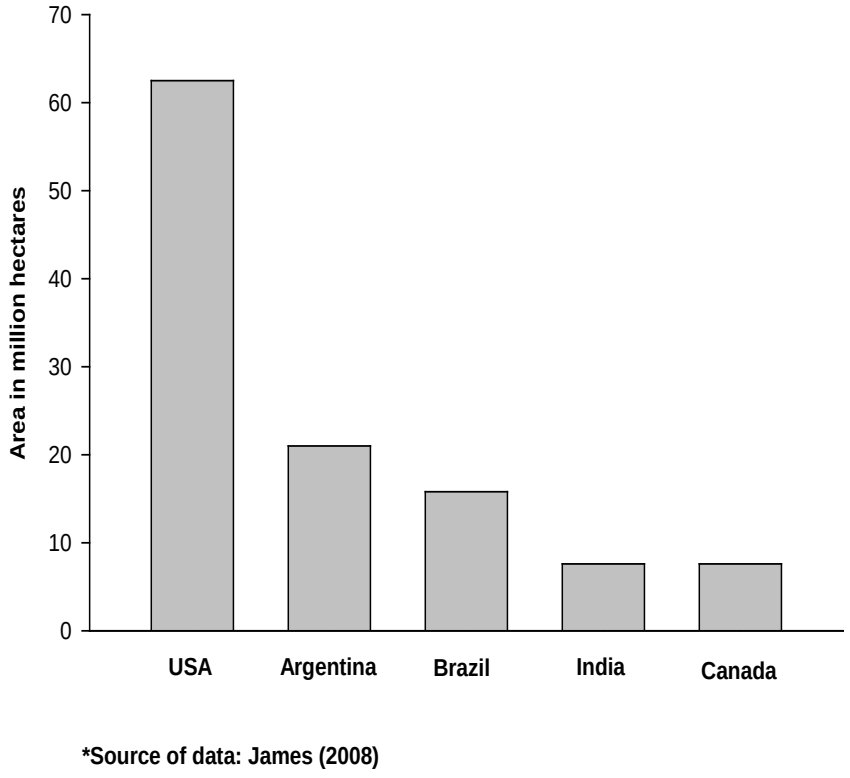


Figure 1-1 Growing area of GE crops in five major GE crops growing countries in 2008*

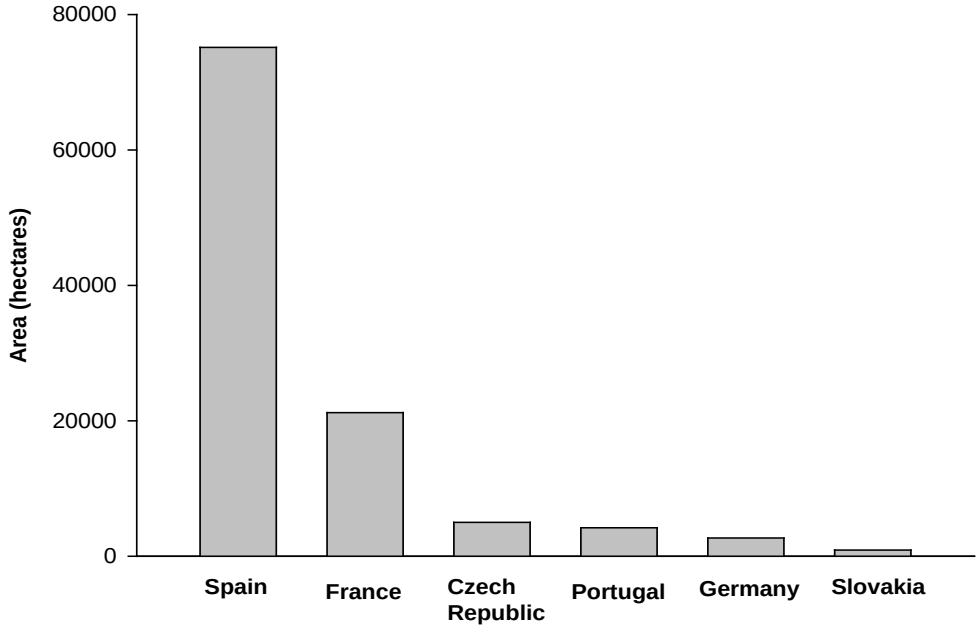
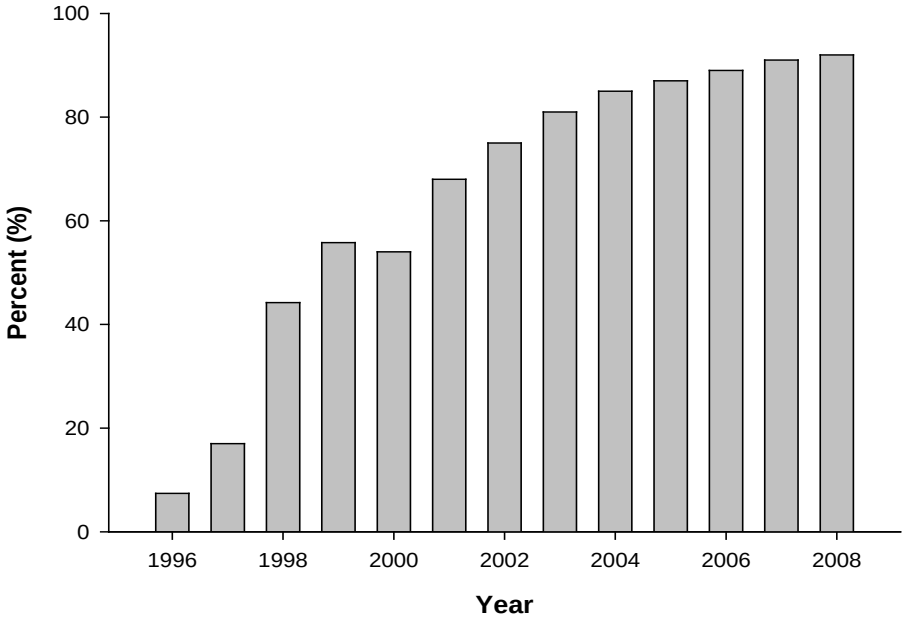


Figure 1-2 Major GE maize growing countries and area in the EU in 2007*.



***Souce of data: Economic Research Service (ERS-USDA, 2008)**

Figure 1-3 Percent share of herbicide-resistant soybean annually in the USA*

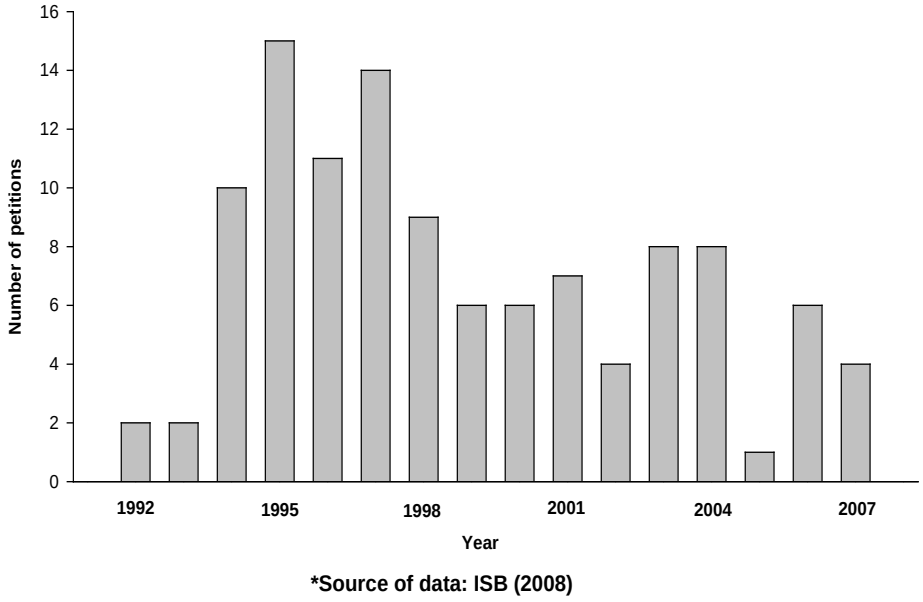
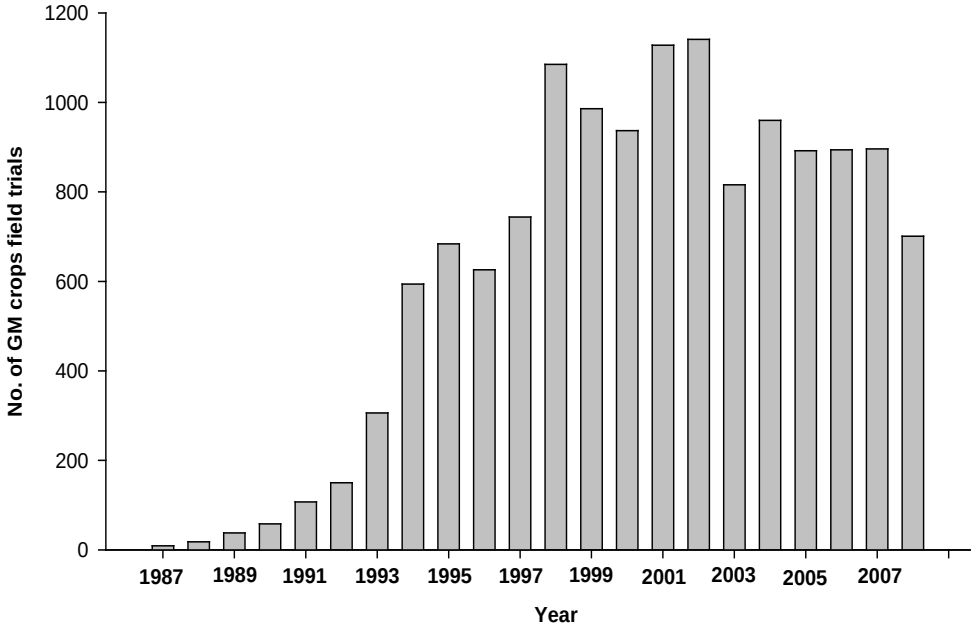
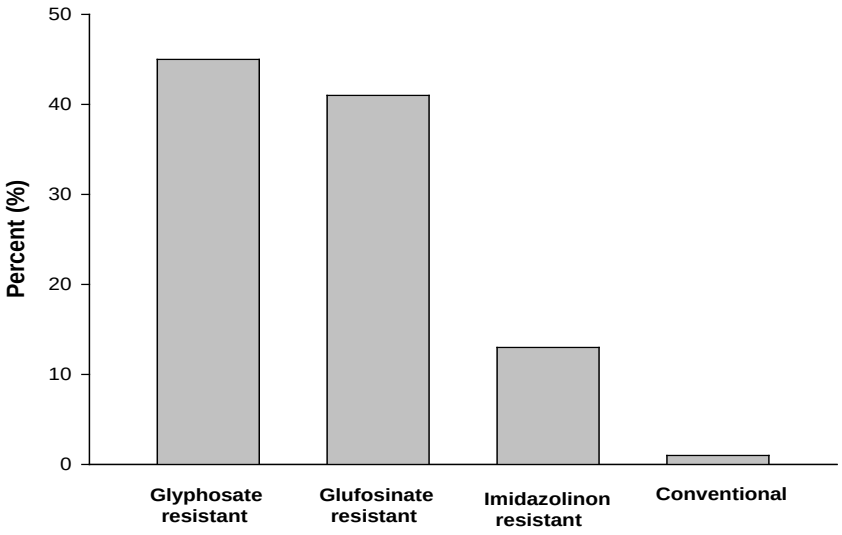


Figure 1-4 Number of petitions received by the United States Department of Agriculture/ Animal and Plant Health Inspection Service (USDA/APHIS) annually for regulating GE crops in the USA*



***Source of data: ISB (2008)**

Figure 1-5 Field trial permits and notification of GE crops approved annually by the United States Department of Agriculture/ Animal and Plant Health Inspection Service (USDA/APHIS)*



*Source: Canola Council of Canada (2009)

Figure 1-6 Percent share of GE canola traits planted in 2008 in Canada*.

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Chapter 2

Flax (*Linum usitatissimum* L.): Current uses and future applications[2]

Introduction

Flax or linseed is among the oldest crop plants cultivated for the purpose of oil and fiber. It belongs to the genus *Linum* and family Linaceae. The botanical name, *Linum usitatissimum* was given by Linnaeus in his book "Species Plantarum" (Linnaeus, 1857). It is an annual herbaceous plant with a shallow root system. The common names flax and linseed are used in North America and Asia, respectively, for *L. usitatissimum*. Oilseed varieties and fiber varieties are a specialized development of this species (Millam et al., 2005). The cultivars grown primarily for seed/oil purpose are relatively short in height and possess more secondary branches and seed bolls (seed capsule). The cultivars grown for fiber are tall growing with straight culms and have fewer secondary branches.

The Mediterranean and Southwest Asia have both been proposed as the center of origin of flax (Millam et al., 2005); but the exact location is uncertain (Lay and Dybing, 1989). The initial use of flax has also been debated. Based on archeological evidence, it was proposed that flax was used first for fiber. However, a more recent comparative study of genetic diversity of the

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stearoyl-ACP desaturase II (*sad2*) locus from flax and pale flax (*L. angustifolium*) showed reduced diversity in the cultivated species. This suggests that flax may have been initially selected as an oilseed crop (Allaby et al., 2005).

Flax, while a minor crop, is grown in a wide range of countries, climates and for many different products (Figure 2-1). Because of its adaptability and product diversity, it is being considered as a platform for the development of novel bioproducts. Research on use of flax for bioproduct production is being conducted in Australia, North America, Europe and Asia. The objective of this paper is to discuss the various applications, demands and developments of an under utilized oilseed crop, flax.

Area and production of flax

Flax was grown in 47 countries in 2004 with the seed production of 1.903 million metric tonnes (Smith and Jimmerson, 2005). Canada has the highest area and production of flax in the world followed by China, USA, India and EU. In 2006, Canada produced 1.014 million tonnes of flax seed from an area of about 800 thousand hectares (Statistics Canada, 2006). The Canadian prairie (Manitoba, Saskatchewan and Alberta) is the major flax growing area in Canada. In the USA, flax has been grown primarily in North Dakota, South Dakota, Minnesota and Montana (Berglund, 2002). In India, flax is grown mainly in Uttar Pradesh, Madhya Pradesh and Maharashtra, with three states

accounting for about 74% of the linseed output in India (Gill, 1987). In India, flax is grown primarily for oil, although in temperate hill regions like Himachal Pradesh, it is grown for fiber on a small scale (Richharia, 1962). Flax for fiber purpose is grown primarily in China, Russia, Egypt, and near the northwestern European coast for the production of high quality linen and several other products (Vromans, 2006).

Flax for human consumption

Flax was used as a food source and natural laxative dating back as far as the ancient Greeks and Egyptians. It was also used as a food in Asia and Africa (Berglund, 2002). The unique and diverse properties of flax are reviving interest in this crop. In 2005, approximately 200 new food and personal care products were introduced in the US market containing flax or flax ingredients (Morris, 2007), which suggests that flax based products have the highest growth potentials in functional food industry.

Conventional flax seed, containing a mixture of the fatty acids, is rich in two essential fatty acids, alpha-linolenic acid (ALA; C18:3$\Delta^{9,12,15}$) (ω-3) and linoleic acid (LA; C18:2$\Delta^{6, 9, 12}$) (ω-6) (Table 1). In an average Canadian flax cultivar, ALA comprises about 57% of the total fatty acids in flaxseed, whereas ω-6 comprises about 16%, giving ω-6/ω-3 ratio of 0.3:1.0. The typical Western diet is high in ω-6 and low in ω-3. Current dietary ω-6/ω-3 ratio ranges from 10:1 to 25:1 while Health Canada recommends a ratio of 4:1

to 10:1, especially for pregnant women and infants (Scientific Review Committee, 1990). Consuming flax or other food rich in alpha linolenic acid like fish oil, ω-3 enriched eggs, increases ω-3 family intake, which would improve ω-6/ ω-3 ratio.

Flax is a rich source of ALA, a precursor for the synthesis of very long chain polyunsaturated fatty acids (VLCPUFA), eicosapentaenoic acid (EPA, C20:5 $\Delta^{5, 8, 11, 14, 17}$) and docosahexaenoic acid (DHA, C22:6$\Delta^{4, 7, 10, 13, 16, 19}$). Metabolism of ALA in animals by a series of alternating desaturations and elongations, converts it into very long chain polyunsaturated fatty acids (VLCPUFA), EPA, and DHA. Conversion of ALA to VLCPUFA in humans is affected by various hormonal changes and dietary factors (Yamazaki et al., 1992). High levels of ω-6 fatty acids in the food supply interfere with the conversion of ALA to EPA and DHA because the ω-3 and ω-6 family compete for the same desaturase enzymes.

The ω-3 fatty acids, particularly DHA, are required for the optimal development of nervous system and maturation of visual acuity (retina) in preterm and term infants (Neuringer and Connor, 1986; Uauy et al., 1996). EPA and AA (arachidonic acid; C20:$\Delta^{5, 8, 11, 14}$) are the precursors of eicosanoids and also components of mammalian cell membranes, including the prostaglandins, blood clotting, cell signaling and blood pressure regulation (Kinsella et al., 1990). Deficiency in ω-3 increases the chances of diabetes,

cancer, arthritis, inflammatory diseases, depression, heart disease, hypertension, memory problems, weight gain and some allergies (Morris, 2007).

In leafy green plants, fatty acids are usually in the form of ALA alone; however, their over all lipid content is very low, so they can not meet the total requirement of ALA alone. Most fish contain only trace amount of ALA, although a few species of fish such as salmon are rich in EPA and DHA (Nelson and Chamberlain, 1995). However, the consumption of fish oil is predicted to continue to decrease because of diminished global fish stocks and heavy metals contamination of oils derived from fish which may affect neuropsychological function in adults (Yokoo et al., 2003). For vegetarian diets, flax is the richest plant source of ALA.

Flax for edible oil

The direct use of unprocessed conventional flax oil in the human diet is limited by product stability. Linseed oil with high ALA is highly susceptible to oxidation and polymerization. While these properties make it suitable for other industrial applications (discussed below), it limits the direct substitution of flax oil in place of canola (*Brassica napus* L.) or maize (*Zea mays* L.) oil. The oil properties of flax are so unique that considerable effort is being expended to emulate the fatty acid profile. Modification of soybean oil (*Glycin max* L.) and canola oil using conventional and molecular approaches to enhance the

ALA content and therefore the health benefits and to replace fish oils in the diet are an extremely active area of research (for recent reviews, see Cahoon, 2003; Scarth and Tang, 2006).

To use flax oil in food applications where stability is essential Green and Marshall (1984) isolated mutants with as low as 1-3% α-linolenic acid, a level which is considered suitable with self stability for traditional edible oil applications (Rowland, 1991). Solin is the name given by the Flax Council of Canada to describe the flax cultivars with less than 5 % ALA for use in the food industry. A domestic source of a vegetable oil high in palmitic acid also has potential in Canada for the manufacture of high quality margarines. Edible oil of linseed also provides an opportunity to produce cocoa-butter replacement oil (Rowland et al., 1995). However, this oil has reduced health benefits due to the reduction in (<5%) ALA content.

Flax for functional food

Functional or nutraceuticals are foods that are claimed to have health-promoting or disease-prevention properties in addition to basic nutritional properties in the food. Many health-claims have been made for whole flax seed, flax meal and milled flax. While a complete assessment of the research on flax as a functional food is beyond the scope of this article, readers are directed to Bloedon and Szapary (2004) and Fitzpatrick (2007)

A recent study in Europe indicates that the consumption of flax oil for 12

weeks (one tablespoon, providing 8 g ALA/day) in daily diet lowered blood pressure significantly in middle aged men with high blood cholesterol levels (Paschos et al., 2007). A role of the flax oil in preventing thrombosis has been reported in a study showing a 40% increase in the activated protein ratio in a population who consumed flax oil diet for six weeks (Richard and Thompson, 1997; Allman-Farinelli et al., 1999). In a study of 50 men with high blood cholesterol levels who consumed one table spoon of flax oil daily for 12 weeks, reduced 48% C-reactive protein (CRP) and 32% serum amyloid A (SAA) levels (Paschos et al., 2005).

Clinical studies on rats and other animals reported that flax has antioxidant effects and decreases blood lipids and inflammation (Prasad, 1997; Bhathena et al., 2002). Many studies revealed that consuming traditional milled flax or partially defatted flax decreased total cholesterol, low-density lipoprotein (LDL) cholesterol without a significant decrease in high-density lipoprotein (HDL) cholesterol (Chan et al., 1991; Jenkins et al., 1999). Cardiovascular disease which includes several diseases (like coronary heart disease, stroke) is one of the leading causes of death in North America (Heart and Stroke Foundation of Canada, 2003).

Experiments revealed no effect of linseed oil on blood total cholesterol and LDL-cholesterol levels (Sanders and Roshanai, 1983; Layne et al., 1996). However, studies conducted on rats suggest that the diets rich in ALA from

flax seed have been shown to decrease blood cholesterol and triacylglycerol levels (Kim and Choi, 2005; Vijaimohan et al., 2006) and also in some human populations (Chan et al., 1991). The high mucilaginous soluble fiber content of flaxseed has been utilized in the cure of hyperglycemia and hypercholesterolemia in humans (Richard and Thompson, 1997).

Flax helps reduce cardiovascular diseases by altering the ω-3 fatty acid content of cell membranes by improving blood lipids and endothelial function and also by exerting antioxidant effects (Bloedon and Szapary, 2004).The advantageous effects of flax in human health cited in previous studies were achieved with intakes of 2-5 tablespoon of milled flax which provides 4-11 g of ALA or 1-3 tablespoon of flax oil which provides 3-20 g of ALA in the daily diet.

Data derived from animal trials on the effects of flax on breast cancer suggest that the main nutritional components of flax interfere with tumor initiation and promotion. By altering estrogen metabolism and decreasing cell proliferation, flax favorably affected breast cancer risk (Hutchins et al., 2000; Thompson et al., 2005). Some studies have also suggested that flax may reduce prostate cancer risk by dampening inflammatory reactions (Zhao et al., 2004; Zhao et al., 2007). However, there is no direct evidence indicating role of flax contribution to prostate cancer and the data are mainly from animal studies. Indeed, more studies are required in humans.

Lignan acts as an antioxidant in humans. Flax seed provides 800 times more lignans than any other plant seed (except sesame seeds which has 47 times less lignan than flax seed), thus it is considered as one of the richest sources of plant lignans. On consumption of flax, lignans are converted into phytoestrogenic compounds. Studies have revealed that the chemical release of phytoestrogenic compounds is believed to block the action of hormone sensitive cancers (Morris, 2007). However, it is reported that the activity of flax lignans depends on the presence of specific bacteria (Clavel et al., 1991). It is recommended that eating 2-4 table spoon of flaxseed in daily diet help in preventing the formation of the cancerous tumors.

Flaxseed is also an important source of both soluble and insoluble fibers, which is important for the efficient digestive system. Most of the soluble fiber in flax is mucilage, which serves as an effective cholesterol-lowering agent. It is utilized by including flaxseed in muffins, bread or juice. Studies have reported that insoluble fiber is also helpful in preventing constipation and regulating bowel movements. In Germany, the government has authorized use of linseed for constipation, irritable bowel syndrome and general stomach discomfort (Blumenthal et al., 2000).

Investigation of the functionality of flax is an exciting field of research that holds promise for additional flax products and health benefits (Fitzpatrick, 2007).

Flax fiber and its uses

For centuries, flax fiber has occupied a prominent place in textile industry. Flax fiber was used by the pre-historic Lake Dwellers of Switzerland for the production of linen > 5000 years BP. The art of weaving flax fiber to linen may have originated in Egypt because winding-clothes for the bodies of the Pharaohs of Egypt were composed of flax fiber. It was then introduced in India, where, before the use of cotton, linen was worn by many tribes (Richharia, 1962). The early colonists brought flax for fiber to the United States. With the increased area and production of cotton and invention of cotton gin in USA, the use of flax linen for textile declined.

Flax bast fibers are primarily phloem cells, in which cell wall thickness can reach 10 μm and more (10 to 100 times thicker than other cell types). One of the limitations of flax is the separation of bast fiber from other stem fibers. This was traditionally done by retting; two traditional methods were used commercially to ret flax for industrial grade fibers, water- and dew-retting (Sharma and Van Sumere, 1992). Water retting method was discontinued because of the high cost of drying and the pollution from the anaerobic decomposition of flax stem in lakes and rivers. Dew-retting has also limitations including poor quality fiber and is restricted to regions which have appropriate moisture and temperature ranges suitable for retting (Foulk et al., 2002).

In the 1980s, several efforts were made to overcome these limitations and to develop a new method known as enzyme-retting, replacing the anaerobic bacteria with enzymes (Van Sumere, 1992). Attempts were also being made by United States Department of Agriculture (USDA) to develop an enzyme-retting pilot plant method to replace traditional methods of retting, thus producing flax fibers with specific properties for industrial uses (Foulk et al., 2002). Advantages of this new method include reduced retting time, increased yield and fiber consistency and consistency of supply (Foulk et al., 2002).

Flax fiber is soft, lustrous and flexible. It is stronger than cotton fiber but less elastic. Fiber obtained from flax is known for its length, strength and fineness; but chemical composition and diameter are also important (Smeder and Liljedahl, 1996). In comparison to industrial wood particles, flax particles were characterized by higher length to thickness and length to width ratios and lower bulk density (Papadopoulos and Hague, 2003). The best grades are used for linen fabrics such as damasks, lace and sheeting. Coarser grades are used for the manufacturing of twine and rope.

Flax is a source of industrial fibers and, as currently processed, results in long-line and short fibers (Van Sumere, 1992). Long line fiber is used in manufacturing high value linen products, while short staple fiber has historically been the waste from long line fiber and used for lower value

products like blankets, mats, mattresses and carpets. Flax fiber threads are strong enough for preparation of sewing threads, button threads and shoe threads. Linen is also used in making the highest quality handkerchiefs, bedding, curtains, drapery, cushion covers, wall coverings, towels, other decorative materials and materials for suits and traditional dresses in Asia (Gill, 1987). It can also be used for manufacturing composites such as particleboard (Papadopoulos and Hague, 2003). Flax fibers are also becoming an integral part of new composite materials utilized in automobile and constructive industry. Biocomposites made up from the flax fiber based on polyhydroxybutyrate (PHB) polymer could be an eco-friendly and biodegradable alternative to conventional plastics (Wrobel et al., 2004).

After extraction of bast fiber from flax stem, 80% of the remains fiber can be separated mechanically. This material can be converted into pulp and can be used for manufacturing papers. Flax fiber is also a raw material for the paper industry for the use of printed banknotes and paper for cigarettes. There are several advantages of using flax fibers for industrial applications. It is a biodegradable, renewable raw material, nonabrasive. However, for technical uses, the mechanical properties like tensile strength, elastic modules it may not be suitable (Wedler and Kohler, 1994; Smeder and Liljedahl, 1996). The relation between the cost of production and the comparative advantages of the fiber may limit the use of flax in large scale applications.

Flax as animal feed

Flax is integrated into animal rations in several forms; whole seed, oil supplements, hulls, or as meal. Meal, known as LSOM or linseed cake in Europe and Asia, respectively, is the residue after the extraction of oil from seeds. This valuable feed product can be used to supplement the diets of both ruminants and non-ruminants.

The quantity of hull in flax seed meal is about 38%, twice the level in canola or soybean meals (Agriculture and Agri-Food Canada, 1997). The fine fraction obtained as a byproduct of dehulling (a process of preparing flaxseed for value added industrial products) could be a potential ingredient in pet food; whereas the medium and mix fractions can be blended into poultry feed formulations (Oomah and Mazza, 1998).Flax seed oil is also used in mixed pet diets, including dogs, cats and horses. The essential fatty acids (ALA and LA) present in flax seed contribute to a lustrous coat, help prevent dry skin and dandruff, and also help in reducing digestive and skin problems in animals.

The ω-3 enriched eggs are produced by increasing ground flax seed to 10-20% of the diet of laying hens. Eggs produced from this diet formula would be ten times higher in ω-3 fatty acids than conventional eggs (Canadian Egg Marketing Agency, 2007) (Table 1). A single ω-3 enriched egg provides half of the optimal daily intake of ALA and about one quarter of EPA and DHA (de Lorgeril et al., 1999).

Flax for industrial uses

Industrial applications are possible because an average Canadian flax cultivar contains 57% α-linolenic acid (18:3 CΔ $^{9, 12, 15}$). When this flax oil is exposed to air, the double bonds of ALA react with oxygen and result in relatively soft, durable film. This property is known as “drying” quality of linseed oil and is responsible for extensive use in manufacturing varnishes, oilcloth, printer’s ink, imitation leather and also as an anti-spalling and curing agent for concrete surfaces on highways (Rowland et al., 1995). The drying quality of oil can be improved by the addition of a metal catalyst to promote oxidation and also by partially pre-oxidizing oil through exposure to the air. Along with the use of flax oil as an oil paint carrier, it is also used as a painting medium, making oil paints more fluid, transparent and glossy. Linseed oil can also be used as “finishing oil” for wooden furniture to prevent it from denting. It does not cover the surface of wood but soaks into the pores, leaving a shiny but not glossy surface. It is used by billiards/pool cue manufacturers on the shaft portion of the cue.

Linseed oil is the most important raw material used to make the flooring from linoleum. In the process of linoleum manufacturing, oxidized linseed oil is mixed with rosin and other raw material to form linoleum granules, which are pressed onto a jute backing, making linoleum sheets (Green floors linoleum flooring, 2008). This natural material made from a sustainable

resource is long lasting and attractive.

Flax seed mucilage has emulsifying properties better than Tween 80 and gum Arabic and has potential industrial uses (Minker et al., 1973). Dehulling of flax seed is also an important process for preparing value added industrial products. To obtain low and high protein products, attempts have been made to remove flax seed hydrocolloidal gum with dry dehulling of seeds (Dev and Quensel, 1988). The hull fraction obtained through this process can be used as a raw material for the extraction of phytochemicals (Oomah and Mazza, 1998).

Conclusion

An oilseed cum fiber crop, flax has been used by humans from more than 5000 years and it is among the first plants domesticated. The utilization of flax for various purposes including industry, nutraceutical, bio- pharmaceutical, fiber, animal feed and human food is continuing to develop. Increasing cost of artificial fibers and the advantages of natural flax fiber, new technology and equipments for growing, harvesting of flax is useful to make flax a model plant species. New improved methods of retting flax, more efficient processes at each stage of linen manufacture point towards a possible upturn of the utilization of flax fiber, especially in North America. There is a demand for alternative sources of VLCPUFA and the possibility of obtaining them from higher plants in commercial quantity is particularly attractive. As no oil-seed species produces such products naturally, genetically engineering would be required to synthesize these fatty acids. Because flax already contains the precursor to VLCPUFA and the highest value of ALA, it may be a choice platform species. Linoleum and other flax based materials such as linen will become increasingly popular as governments and consumers turn to products with smaller environmental footprints. There are also opportunities for production of sustainable bio-products and green building materials. The molecular and gene expression experiments are not widely studied in flax, which may also expand the applications and uses of flax in future.

Several constrain must be overcome to facilitate the further development of flax and flax bioproducts. Because flax is a minor crop, it has not received significant research resources compared to other North American oilseeds such as canola and soybean. While flax can be easily transformed (McHughen and Holm, 1995a; McHughen and Holm, 1995b; McHughen, 2002) and herbicide resistant traits have been developed to increase flax yield, constraints in the European market for GE LSO meal limits this method of crop improvement and economic enhancement (McHughen, 1995). The public sector remains the primary contributor to flax breeding and research because the lack of commercial prospects limits the interest of the private sector. Currently, a lack of basic knowledge of flax genomics, and a concerted effort in flax breeding limits rapid development of flax for bioproducts.

Table 2-1 Comparison of fat profile of ω-3 enriched eggs and conventional eggs[b] (Canadian Egg Marketing Agency, 2007)

Content	ω-3 enriched egg[a]	Conventional egg[a]
Total fatty acids	4.9 g	5.0 g
ω-6	0.7 g	0.7 g
ω-3	0.4 g	0.04 g
Monosaturated	1.6 g	2.0 g
Saturated	1.2 g	1.5 g
Cholesterol	185 mg	190 mg

[a]based on one whole large egg
[b]values are based on 10% flax in the diet

Table 2-2 Fatty acid composition in conventional and Solin flax (Morris, 2007)

Fatty acid	No.of double bonds	Omega family	Formula	Average % fatty acid
Conventional flax				
Saturated Stearic acid	0	-	18:0	9
Monounsaturated Oleic acid	1	ω-9	18:1 Δ^{9}	18
Palmitoleic acid	1	ω-7	16:1 Δ^{9}	
Polyunsaturated Linoleic acid (LA)	2	ω-6	18:2 $\Delta^{9,12}$	16
Alpha-linolenic acid (ALA)	3	ω-3	18:3 $\Delta^{9, 12, 15}$	57
Solin flax				
Saturated Stearic acid	0	-	18:0	9
Monounsaturated Oleic acid	1	ω-9	18:1 Δ^{9}	18
Palmitoleic acid	1	ω-7	16:1 Δ^{9}	
Polyunsaturated Linoleic acid (LA)	2	ω-6	18:2 $\Delta^{9,12}$	71
Alpha-linolenic acid (ALA)	3	ω-3	18:3 $\Delta^{9,12, 15}$	2 to 3

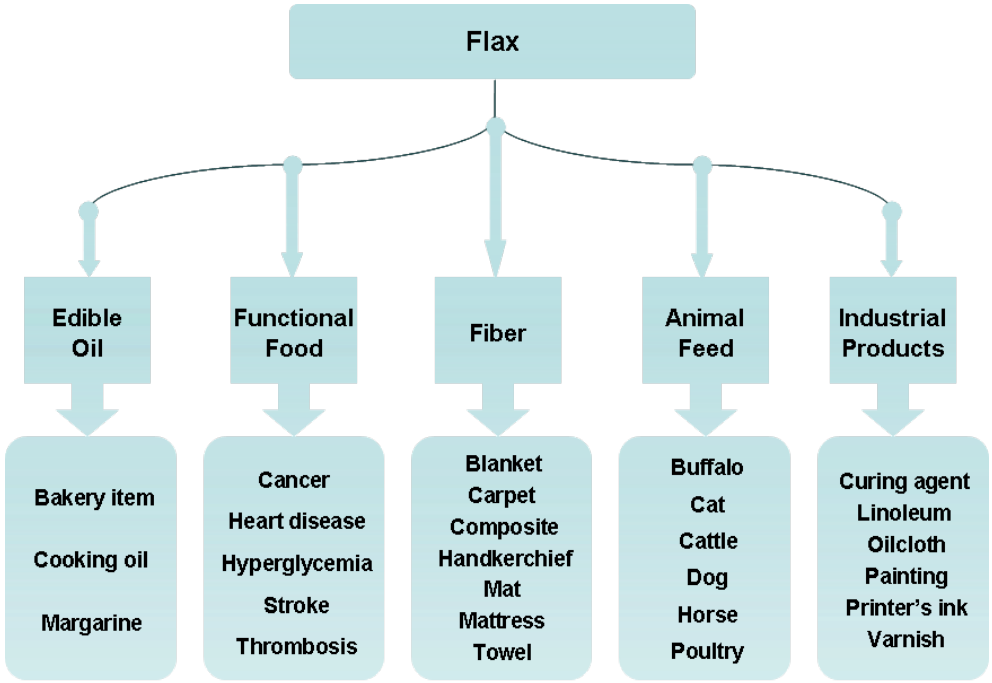

Figure 2-1 Schematic diagram of use of flax for various purposes.

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Chapter 3

Potential hybridization of flax (*Linum usitatissimum* L.) with weedy and wild relatives: An avenue for movement of engineered genes?[3]

Introduction

Flax (*Linum usitatissimum* L.) is the sixth largest oilseed crop in the world and is one of the oldest cultivated plants (Bhatty and Rowland, 1990). It is grown for linen fiber, the earliest vegetable fiber domesticated by mankind, and as an oilseed (Dillman, 1938; Richharia, 1962). The center of origin of flax has not been identified (Lay and Dybing, 1989), but it was reportedly disseminated from Egypt (Cooke, 1903) where it was in use during the time of the Pharaohs. Flax fabrics from Egyptian mummy-cloths were dated at > 4,500 years (De Candolle, 1904; Matthews, 1908). It is believed that Phoenicians were responsible for transporting flax into Europe from the Near East (Rosberg, 1996; Stephens, 1997) during the period from 2,500 to 1,200 B.C. Flax cultivation by Aryans extended north to Russia and Finland (De Candolle, 1904). During the Colonial era, European colonists transported flax to North America, New Zealand and Australia (Rosberg, 1996).

Fiber flax prospered in North America for many years as production

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followed settlers westward (Hammond and Miller, 1994). Fiber flax has been cultivated in the Netherlands and probably in Belgium and Northern France since ancient times. Today, fiber flax is grown primarily in China, Russia, Egypt, and near the northwestern European coast for the production of high quality linen (Vromans, 2006). In 2004, linseed was grown in 47 countries, and seed production was 1.903 million metric tonnes (Smith and Jimmerson, 2005). Canada, China and the United States together are responsible for 64% of the total world flax seed output. Canada is currently the world's leader in the production and export of flax seed, a position it has held since 1994. In 2006, Canada produced 1.041 million tonnes of flax seed (Statistics Canada, 2006) and exported 80-90% of the total production, mainly to Europe, the US, Japan and South Korea (Flax Council of Canada, 2007a).

Flax was among the first crop species to be both genetically engineered with *Agrobacterium* mediated transformation and transformed with genes of potential agronomic value (McHughen, 2002). Several novel traits have been expressed in flax including chlorsulfuron and metsulfuron resistance (McSheffrey et al., 1992), glufosinate-resistance (McHughen and Holm, 1995); and glyphosate resistance (Jordan and McHughen, 1988). Only one GE flax cultivar, 'CDC Triffid' (McHughen et al., 1997), was released in Canada in 1998 for unconfined use in fields with persistent herbicide residues (CFIA, 2004b), but it was deregistered almost immediately at the request of the flax industry (Flax Council of Canada, 2007b). Although GE flax may have been a

solution to significant agronomic issues such as weed control (McHughen, 1989) or disease resistance (Polyakov et al., 1998), a concern over the market's reaction to the import of genetically engineered material halted all genetic modification of the crop, even for primary use in paint and flooring industries and animal feed as a co-product. Since then, the EU is moving towards being more open to bioproducts and GE crops (Hricova, 2002; Breithaupt, 2004; see also Millam et al., 2005).

GE crops are now grown worldwide, and the number of species and area continues to increase (James, 2003; Nap et al., 2003). One of the critical concerns that must be addressed prior to the release of a novel crop is the potential movement of transgenes from GE crops to wild populations (Raybould and Gray, 1993; CFIA, 2004a). A better understanding of crop-to-wild gene flow is essential for ecological risk assessment of the potential for transgene spread (Dale, 1993; Conner et al., 2003). In addition, the potential impact on biodiversity (Wilkinson et al., 2003) and genetic resources must be evaluated (Ellstrand, 1988; Andow and Alstad, 1998). Risk assessment of GE flax including transgene movement from GE flax to its weedy relatives is in progress (Hall et al., 2006).

We hypothesize that *L. usitatissimum* is more likely to hybridize with closely related species having a similar ploidy level, genome and chromosome pairing. Our objective is to establish the potential risk of gene flow from GE flax (*L. usitatissimum*) prior to experimental testing, based on; a) biology,

distribution and flowering phenology of closely related species, b) relatedness and crossability, and c) probability of interspecific hybridization and introgression between GE flax and its wild or weedy species.

Taxonomy and Phylogeny

Flax is a member of the family Linaceae which is composed of 22 genera (Vromans, 2006) and approximately 300 species (Hickey, 1988; Heywood, 1993). Linaceae is placed in the order Linales by some taxonomists (Cronquist, 1981), but most recently the family has been placed in the order Malpighiales (APG II, 2003). Important genera in the family include: *Linum* (230 species), *Hugonia* (40 species), *Reinvardtia* (2-4 species), *Anisadenia* (2 species), *Roucheria* (8 species) and *Radiola* (Heywood, 1993).

The genus *Linum* is traditionally divided into five sections: *Linum, Linastrum, Cathartolinum, Dasylinum* and *Syllinum* (Winkler, 1931) with an additional section, *Cliococca,* added by Ockendon and Walters (1968). Cultivated flax, *Linum usitatissimum*, is placed in the section *Linum*. The taxonomy and classification of *Linum* has changed with increased knowledge. Many researchers classified *Linum* species either on the basis of morphological characters or center of origin (Linnaeus, 1857; De Candolle, 1904; Tammes, 1925; Vavilov, 1926; Winkler, 1931; Dillman, 1933; Dillman, 1953; Richharia, 1962). Alternatively, other researchers grouped *Linum* species based on chromosome number (Kikuchi, 1929; Nagao, 1941; Ray, 1944; Osborne and Lewis, 1962; Gill, 1966; Ockendon, 1971; Chennaveeraiah

and Joshi, 1983; Gill, 1987). However, there is no single prevailing classification scheme for this genus. The grouping of 41 *Linum* species proposed by Gill (1987), based on morphological, cytological and interspecific compatibility evidence, will be followed in this paper.

Phylogenetic studies based on molecular markers are limited. An amplified fragment length polymorphism (AFLP) based phylogeny of 17 species of *Linum* is not compatible with traditional sections of the species (e.g. Winkler, 1931; Ockendon and Walters, 1968; Diederichsen and Richards, 2003), although there is evidence of 5 species clusters (Vromans, 2006). McDill and Simpson (2005) conducted a more comprehensive phylogenetic study of *Linum* based on DNA sequence variation from multiple chloroplast markers and the nuclear encoded internal transcribed spacer region (ITS). Their analysis of ca. 70 species indicates that blue-flowered *Linum* species were sister to a predominantly yellow-flowered lineage. These lineages initially diversified in Eurasia and members of both the blue and yellow-flowered lineages appear to have independently colonized North America. The subsequent diversification of the yellow flowered *Linum* species in North America includes members previously classified as separate genera: *Hesperolinon*, *Sclerolinon digynum* and *Cliococca selaginoides*.

Karyotype number is not reflective of phylogenetic relationships among *Linum* species. For example, an analysis based on RAPD data indicate that *L. decumbens* (2n=30) is clustered with *L. grandiflorum* (2n=16), not with other

species that share the same chromosome number (e.g. *L. angustifolium* and *L. usitatissimum*; Fu et al., 2002). *Linum perenne* group can be easily distinguishable from other *Linum* species morphologically (Ockendon, 1968), but the molecular study of Vromans (2006) indicate that classification among the *L. perenne* group is still complicated. Neither *L. perenne* nor *L. austriacum* form a specific group, even though *L. austriacum* is considered a member of *L. perenne* group (Diederichsen, 2007) and they have the same haploid karyotype number of nine (Nagao, 1941; Gill, 1987).

Additional molecular studies have focused on within species variation of *L. usitatissimum* L. Mansby et al. (2000) used isozyme markers to study the genetic diversity in flax and defined five groups but with low variation within the groups. An unexpectedly high genetic diversity within accessions lead to the conclusion that the large heterozygosity found in *L. usitatissimum* may be the result of more outbreeding than earlier believed (Mansby et al., 2000). This finding was unexpected as flax is reported to be an obligate inbreeding species (Durrant, 1986). In a study on geographic patterns of flax variability, Fu (2005) pointed out that accessions from the East Asian and European regions were most diverse, whereas accessions from the Indian subcontinent and Africa were the most distinct. Overall, comparatively more variation existed in landraces than cultivars. Considerable difference within and among the four groups of cultivated flax cultivars were observed in quantitative traits; however, RAPD and two qualitative characters did not show marked

differences (Diederichsen and Fu, 2006). A molecular study comparing fiber and oil flax indicated that fiber cultivars have a narrower and more homogenous genetic base than oil cultivars (Fu et al., 2002). Vromans' (2006) AFLP study supports this finding and he further speculated that linseed cultivars and a wild relative *L. bienne* could be important sources for the introduction of favorable traits to fiber flax.

Variability in chromosome numbers

Karyotypic analysis of *Linum* species began more than a half century ago, which has allowed several species to be recognized and differentiated (Tutin et al., 1968). The genus *Linum* has a large number of diploid species that exhibit a remarkable diversity in chromosome number including n=8, 9, 10, 12, 14, 15, 16, 18, 30 and > 30 (Darlington and Wylie, 1955; Gill, 1987). Diversity in chromosome numbers may be due to polyploidy and aneuploidy (Chennaveeraiah and Joshi, 1983). Initial studies of the chromosome number of cultivated flax estimated the chromosome number to be 2n=32 (Martzenitzin, 1927; Lutkov, 1939). However, later cytogenetic and interspecific hybridization studies confirm the chromosome number to be 2n=30 (Kikuchi, 1929; Dillman, 1938; Nagao, 1941; Ray, 1944; Richharia, 1962; Gill, 1966; Chennaveeraiah and Joshi, 1983). The reasons for the conflicting results were the small size of the chromosomes in *Linum*, the tendency of the observed fragments to retain some stain (Ray, 1944; Gill, 1966) and an accidental segmentation in the somatic mitosis (Martzenitzin, 1927).

There were some disagreements among various researchers regarding the chromosome numbers of other *Linum* species (Table 3-1). For example, Kikuchi (1929) classified *L. alpinum* as a member of group III with chromosome number n=18, whereas, Ray (1944) and Nagao (1941) have grouped this species as n= 9 (Table 3-1). Gill (1966) indicated uncertainty in the chromosome number of this species (Table 3-1). The *Linum alpinum* specimen from which Kikuchi (1929) counted chromosomes may be a Japanese tetraploid (Simonet and Chopinet, 1939), which could account for the variability in the results. *Linum narbonense* was grouped as n=14 (Ray, 1944), but Kikuchi (1929) and Nagao (1941) observed n=9, and 2n=18 and/or 36 (Gill, 1966). *Linum monogynum* has been reported, with qualification, as n=43 and 2n=86 (Kikuchi, 1929). *Linum hirsutum* has a variable reported chromosome count of n=8 (Ray, 1944), n=9 (Nagao, 1941), n=15 (Seetaram, 1972) and n=16 or 18 (Gill, 1966; Table 3-1).

Center of origin and evolution of *L. usitatissimum*

The center of origin of cultivated flax is uncertain (Lay and Dybing, 1989) with many existing theories. Among the eight independent centers of origin of the world's most important cultivated plants (Vavilov, 1926), *Linum* species were reported to have originated in four; the Central Asiatic, the Near Eastern, the Mediterranean and the Abyssinian Center. Gill (1987) and Richharia (1962) have also discussed these four probable centers of flax origin. Alternatively, other researchers believe that Egypt could be a center of dissemination (De

Candolle, 1904). Finally, an area east of the Mediterranean towards India has been suggested as another center of origin because a diverse form of flax is found in the area (De Candolle, 1904; Zeven, 1982).

The progenitor of cultivated flax is also uncertain (Gill, 1987). Many authors reported that cultivated flax is derived from two or more ancestral forms (De Candolle, 1904; Vavilov, 1926; Richharia, 1962). The species cultivated by ancient Egyptians were believed to be different from those indigenous to Russia and Siberia. Alternatively, it was suggested that cultivated flax originated from a single wild species, *L. angustifolium* (Heer, 1872). This hypothesis is supported by morphological (Dillman, 1936; Diederichsen and Fu, 2006) and cytological studies (Kikuchi, 1929; Ray, 1944; Gill and Yermanos, 1967a; Gill and Yermanos, 1967b). A RAPD analysis of seven *Linum* species revealed that *L. angustifolium* and *L. usitatissimum* have a high RAPD similarity and these two species consistently clustered in the same group (Fu et al., 2002). A different AFLP study indicates that *L. bienne* is the sister species to *L. usitatissimum* (Vromans, 2006), although some consider *L. angustifolium* and *L. bienne* to be the same species (Tutin et al., 1968; Zohary and Hopf, 2000). However, genome comparisons with molecular markers of these three species (*L. angustifolium*, *L. bienne* and *L. usitatissimum*) confirm that they are very closely related genetically and *L. bienne* can be considered as a subspecies of *L. usitatissimum,* rather than a separate species (Muravenko et al., 2003).

The *sad* gene is responsible for converting stearoyl-ACP to oleoyl-ACP and, thus, has been used for manipulation of unsaturated fatty acids (Ohlrogge and Jaworski, 1997). In a molecular study it was estimated that the genetic diversity of the stearoyl-ACP desaturase II (*sad2*) locus in cultivated flax is low compared to pale flax (*L. angustifolium*) suggesting flax was first domesticated for oil, not for fiber (Allaby et al., 2005).

Interspecific hybridization in *Linum*

Hybridization between crop species and wild relatives has played a role in the evolution of many crop plants (Arnold, 1997) and is also responsible for the expression of new characters not found in either parent (Briggs and Knowles, 1967). Hybridization of several closely related species of *Linum* might have played a role in the evolution of *L. usitatissimum* in the Mediterranean and Southeast Asia where a diverse form of flax has been found (De Candolle, 1904; Richharia, 1962; Gill, 1966; Zeven, 1982). The studies of interspecific hybridization of *L. usitatissimum* with its wild relatives enable estimates of crossability and provide information to predict potential gene flow between *Linum* species (Kikuchi, 1929; Gill, 1966; Gill and Yermanos, 1967a).

Heterostyly must be taken into consideration when selecting *Linum* species for interspecific hybridization (Rogach, 1941). Heterostylous species have two (distyly) or three (tristyly) contrasting flower types. The plants that have flowers with long styles and short stamens are known as “pin” and vice a

versa called “thrum”. Several species of yellow flowered *Linum* were found to be heterostylous (Ockendon, 1968). Hand pollinations in *L. grandiflorum* of pin x thrum or thrum x pin were highly fertile (85-97%), but self pollination of pin or thrum flowers were only 3.0% successful (Kostopoulos, 1970).

***Linum* hybrids among taxa with n=15**

The first interspecific hybridization in *Linum* was reported by Kolreuter between *L. usitatissimum* and *L. narbonense* but later *L. narbonense* was considered to be synonymous with *L. angustifolium* (Tammes, 1928). There have been many reports of successful hybridization between *L. usitatissimum* and *L. africanum, L. angustifolium, L. corymbiferum, L. floccosum, L. pallescens* and *L. tenue* (Tammes, 1928; Kikuchi, 1929; Ray, 1944; Gill, 1966; Gill and Yermanos, 1967a; Bari and Godward, 1970; Seetaram, 1972). All these crosses produced fertile F_1 hybrids in at least one direction, presumably due to their similarity in ploidy levels and size of chromosomes (Bari and Godward, 1969; Seetaram, 1972). Crosses among five taxa, *L. africanum, L. angustifolium, L. corymbiferum, L. decumbens,* and *L. usitatissimum* were highly successful in at least one direction with F_1 progeny exhibiting 80 to 90% germination (Gill, 1966). In a cytogenetic study Gill (1966) reported that *L. usitatissimum* differs by one translocation from three closely related species, *L. africanum, L. angustifolium* and *L. decumbens*, but did not differ from *L. corymbiferum* in this respect. *Linum angustifolium* differs from other three wild species (*L. africanum, L. corymbiferum* and *L.*

decumbens) by two translocations, each involving two non-homologous chromosomes. Hybridization of *L. usitatissimum* with *L. decumbens, L. hirsutum* and *L. nervosum* were reported but with low F_1 fertility (Sharma and Khanna, 1964; Bari and Godward, 1970; Seetaram, 1972; Figure 3-1).

Hybridization events among 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* (Seetaram, 1972). *Linum crepetans* and *L. humile* pollen have produced fertile plants when crossed with *L. hirsutum* and *L. hispanicum,* respectively (Gill and Yermanos, 1967a; Seetaram, 1972; Figure 3-1).

In summary, interspecific hybridization studies indicate that cultivated flax has the potential to hybridize with at least nine wild relatives with karyotype n=15 (Figure 3-1). *Linum africanum, L. angustifolium,* and *L. pallescens* were crossed with *L. usitatissimum* and all reciprocal crosses produced fertile F_1 plants (Figure 3-1). Therefore, further studies should be conducted to determine if hybrids between these three species occur and retain transgenes from novel flax in the natural ecosystem, not only through back-crossing, but also by hybridization and introgression with other wild relatives (Figure 3-1). All three species have produced fertile F_1 seeds in crosses with at least two of the following species: *L. decumbens, L. floccosum, L. hirsutum, L. strictum* and *L. tenue* (Sharma and Khanna, 1964; Gill, 1966; Seetaram, 1972; Figure 3-1).

Linum hybrids among taxa other than (n=15)

There have been studies of successful hybridization among taxa other than n=15 (Figure 3-2). The taxa with n=9, constitute the largest group in the genus *Linum* (Gill, 1966). Some crosses between species of taxa n=9, *L. alpinum, L. altaicum, L. austriacum, L. julicum, L. narbonense* and *L. perenne*, produced fertile F_1 plants (Gill, 1966; Gill and Yermanos, 1967b). The pairing of chromosomes of these n=9 species revealed that *L. altaicum* differs by one reciprocal translocation from *L. alpinum, L. austriacum, L. julicum, L. narbonense,* and *L. perenne* (Gill and Yermanos, 1967b). They further speculated that *L. austriacum* and *L. narbonense,* and *L. julicum* and *L. narbonense* also apparently differ by one translocation, whereas *L. narbonense* and *L. perenne* differ by two translocations.

The chromosomes not involved in translocations formed normal bivalents indicating that the genomes of the six species were sufficiently homologous for normal pairing to occur. However, a difference of two translocations was discovered between *L. alpinum* and *L. perenne* involving three nonhomologous chromosomes (Gill, 1966). When *L. perenne* was crossed with *L. austriacum*, hybrids were produced but only by embryo culture (Laibach, 1929; Figure 3-2). The diploid *L. perenne* was successfully hybridized with autotetraploid *L. alpinum* (Kikuchi, 1929). Meiosis in this cross was studied and trivalents, bivalents and univalents were observed at metaphase I (Nagao, 1941).

Interspecific hybridization between *Linum* species with different chromosome numbers was also studied. Crosses between *L. alpinum* (n=9,18), *L. austriacum* (n=9), *L. vulgaricum* (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 F_1 plants (Kikuchi, 1929; Ray, 1944; Sharma and Khanna, 1964; Gill, 1966; Bari and Godward, 1970; Seetaram, 1972; Figure 3-2). These results suggest karyotype plays an important role in interspecific hybridization in *Linum* species (Gill, 1966; Bari and Godward, 1970; Figure 3-2).

Thus, only hybridization between species with equal chromosome numbers was successful in producing fertile F_1 plants (Rogach, 1941; Richharia, 1962; Gill, 1966; Bari and Godward, 1970; Seetaram, 1972; Figure 3-2). When cultivated flax is crossed with species having a different chromosome number, not a single cross has produced fertile plants. These greenhouse studies suggest that species with different chromosome numbers have no or minimal risk of gene flow to them.

Geographic distribution

Linum species distribution records are grouped into regions in accordance with the standard publication of Hollis and Brummitt (1992), which divides the terrestrial world into nine areas: Africa, Antarctic, Asia-Temperate, Asia-Tropical (in this manuscript, we have considered Asia as a single region),

Australia, Europe, North America, Pacific, and South America. However, a distributional report for a taxon in a geographical or political region does not necessarily imply widespread occurrence in that region, but indicates that a literature citation or other evidence (i.e. herbarium specimen) records the presence of the species (USDA NRCS, 2006). State or provincial distributions were not itemized for taxa widespread within countries, except in North America. Here we discuss the geographic distribution of 41 *Linum* species (Table 3-2).

Flax is cultivated in almost all continents with temperate climates (Gill, 1987). In Europe, it is grown primarily for fiber, except in Germany, Hungary, Poland and Romania where it is grown as an oilseed. *Linum angustifolium* Huds, a putative wild progenitor of flax, is a perennial species of the Mediterranean and sub Mediterranean area, Ireland and southern UK (Tammes, 1928). There are many other perennial species found in the Mediterranean extending up to Asia including *L. alpinum, L. campanulatum, L. capitatum, L. dolomiticum, L. hologynum, L. julicum* and *L. viscorum* (Tutin et al., 1980; Table 3-2). Many species such as *L. austriacum, L. flavum, L. grandiflorum, L. hirsutum, L. narbonense* and *L. perenne* have attractive flowers and so these species are frequently cultivated in European and Canadian botanical gardens and available in nurseries as ornamental plants.

There are many *Linum* species native to Asia (Zeven, 1982). Two species, *L. mysorense* and *L. usitatissimum* were recorded in many states of

India (Cooke, 1903). Hooker (1875) reported two additional species, *L. perenne* and *L. strictum*. *Linum angustifolium* and *L. grandiflorum* were introduced in India as ornamental plants (Richharia, 1962). *Linum perenne* was reported in escaped clusters and this species might have been in cultivation in India during the Dravidian period (around 2000-1500 BC; Richharia, 1962). Many species including *L. altaicum, L. angustifolium, L. flavum, L. nervosum, L. pallescense, L. perenne* and *L. tenuifolium* are native to the Russian Federation and distributed extensively within that region (Greuter et al., 1984; Table 3-2). *Linum marginale* and *L. monogynum* are distributed in Australia and New Zealand (Willis, 1972; Hnatiuk, 1990; Table 3-3). Detailed information on geographical ranges of individual species is given in Table 3-2.

Linum species in the New World

There are more than 63 *Linum* species distributed in the New World throughout the USA, Canada and Mexico (Small, 1907; Budd, 1987; Diederichsen, 2007; Table 3-3). Flax is grown primarily for seed in the Canadian prairies, Kansas, Minnesota, Montana, Nebraska, North Dakota, and Wisconsin (Scoggan, 1993; USDA, 2007). Rogers (1963; 1968), who developed an extensive classification and distribution of *Linum* species in North America, reported that *L. rigidum* and closely related species are believed to be the most primitive in North America. These species are

distributed in southern Florida and also have a vast range in the Great Plains extending from northern Mexico to western Canada (Mosquin and Hayley, 1967). There are eight species of *Linum* distributed in Canada (Scoggan, 1993). Plant Gene Resources of Canada (PGRC) has a germplasm collection of 5296 accessions of *L. usitatissimum* and 76 identified flax wild relatives (Diederichsen, 2007). *Linum sulcatum* (n=15) is extensively distributed in several states of the United States and provinces of Canada (Table 3-3).

Linum species in North America can be divided into three groups: blue, white and yellow flowered species (see Rogers, 1969 for relationships among these three groups). There are three basic karyotypes in the North American species, each representing an invasion from the Old World: i) n= 8 (*Linum catharticum*), ii) n=9 (blue flowered species) and iii) n=18 (yellow flowered species) (Harris, 1968) as well as many species with n=15 (Figure 3-3). A large number of interspecific crosses of two Florida tetraploids (n=30) *Linum rigidum* var. *rigidum* and *L. rigidum* var. *carteri*, were attempted with two diploid species (n=15) of the Great plains, *L. alatum* and *L. aristatum*, but all of the crosses either failed to produce seed or the seeds failed to develop into mature plants (Mosquin and Hayley, 1967). However, crosses of both the Florida tetraploids have resulted in successful hybridization with the diploids of the Great Plains: *L. rigidum* var. *berlandieri, L. rigidum* var. *rigidum* and *L. elongatum*.

Based on the results of interspecific hybridization experiments, we

conclude that chromosome number is an important factor in hybridization and introgression between *Linum* species (Figure 3-1 and 3-2). With the one exception of *L. corymbiferum*, none of the native North American species presented in Figure 3-3 have been included in reports of hybridization studies with cultivated flax. Because all these species have the same chromosome number (n=15), there may be the potential for transgene introgression from GE flax to them (Figure 3-3). However, hybridization of crop-weed complexes can be influenced by environmental, temporal and spatial variables (Ellstrand et al., 1999; Hall et al., 2006). Hybridization is also influenced by many other factors including sympatry of crop and weedy species, availability of pollinators, duration of pollen viability, synchronicity of flowering, floral morphology, genetic relatedness, direction of hybridization, heterostyly and sexual compatibility (Kostopoulos, 1970; Govindaraju, 1988; Ellstrand and Hoffman, 1990; Rieseberg and Wendel, 1993). The geographic distribution of North American wild relatives with n=15 is given in Table 3-3.

Biology and ecology

Limited information is available on the biology and ecology of *Linum* species. Almost all *Linum* species are noted for their value in mixes for erosion control and in beautification. A long period of flowering makes the plant more aesthetically appealing (USDA, 2007) but also increases the potential flowering synchronicity with cultivated flax. Most of the species are fire resistant due to the leaves and stems staying green with relatively high

moisture content during most of the fire season (USDA, 2007). The following is information on specific *Linum* species.

***Linum usitatissimum* L. (2n=30)**

Linum usitatissimum (cultivated flax) is grown for seed oil and fiber. Linseed type flax is a relatively short plant which produces many more secondary branches compared to the fiber type (Gill, 1987). The flowers are hermaphroditic, hypogynous and slightly protandrous (Eyre and Smith, 1916) with five sepals, five petals, five stamens and a compound pistil of five carpels in a radially symmetrical arrangement (Dillman, 1938). The fruit is a capsule, containing 8-10 seeds. Flax is predominantly a self pollinated species but cross pollination rates have been reported in the range of 1-5% (Eyre and Smith, 1916; Robinson, 1937; Dillman, 1938; Gill, 1987), with important pollinators being honeybees, bumble bees and butterflies (Dillman, 1938; Gubin, 1945). The life cycle of a flax plant consists of a 45-60 day vegetative period, 15-25 day flowering period and a fruit maturation period of 30-40 days (Anonymous, 2006). In addition to being cultivated, *L. usitatissimum* is found as an escape in waste places, along roadsides (Richharia, 1962), in disturbed land habitats and in un-managed ecosystems (CFIA, 1994; Thomas et al., 1997). The establishment and spread of flax in disturbed habitats warrants further study.

Flax grows best on soils with high water holding capacity and good inherent fertility. It does not thrive on sandy soils unless a large supply of moisture is available (Anonymous, 2006). Although flax is considered to be a

cool season crop, air temperature below 10 °C in the spring may inhibit growth and development, which can delay flowering (Gusta et al., 1997). In a recent study on seed color, seed weight and seed oil content in several flax accessions, Diederichsen and Raney (2006) revealed that yellow seeded flax had a higher seed weight and oil concentration than brown seeded flax. In vigour tests, yellow seed had lower seed vigour than brown seed (Saeidi and Rowland, 1999).

Poor management practices may result in large numbers of seeds being returned to the soil during harvest. This can result in an increase in the flax seed bank and resulting in volunteer weed problems in succeeding crops (Leeson et al., 2003). Flax is a poor competitor (Friesen, 1988; Wall, 1994), and volunteer flax does not usually result in yield losses in crops like cereals and canola (*Brassica napus* L.). However, it can cause considerable difficulty at harvest time (Anonymous, 2006). Thomas et al. (1997) reported that volunteer flax was present in twice as many fields under zero tillage, but at lower densities when compared to conventional tillage systems. A recent survey on volunteer flax emergence indicated that it varied throughout the growing season from 0-189 plants m^{-2} in the direct seeded plots to 1-1510 plants m^{-2} in the conventional seeded plots (Dexter et al., 2006). These data infer that volunteer flax can also contribute to substantial gene flow if not controlled. The distribution of small clumps of volunteer flax seedlings in a field indicates that many seeds germinate within seed bolls, rather than as

single dispersed seeds (personal observation).

***Linum perenne* L. (2n=18)**

Linum perenne is a perennial that grows 20 to 80 cm in height with stems arising from the cotyledonary node, and linear to lanceolate-linear leaves. Inflorescences are loose cymes containing white to blue flowers. The flowers are heterostylous, as in many of its species group (Gill, 1966). It is known as blue flax (USDA, 2007) or perennial flax (Scoggan, 1993). It is commonly found in hills and eroded banks over the northern plains, prairies and in open fields in moist, well-drained, calcareous soils (Scoggan, 1993).

Blue flax is noted to have forage value for livestock and wildlife because plants stay green throughout the growing season. Birds use seeds and capsules in the fall and winter. It is also considered desirable for deer (*Odocoileus hemionus*, *O. virginianus*), antelope (*Antilocapra americana*) and birds, either as herbage or seed (USDA, 2007). Blue flax is a native to Eurasia and has been distributed not only in the United States (USDA, 2007), but also in some provinces of Canada (Scoggan, 1993).

Linum perenne is cultivated for horticultural or re-vegetation purposes in Iowa, Oregon and North Dakota. Seed yields of 600-700 pounds per acre of blue flax can be expected under irrigated conditions and 200-300 pounds per acre under dry land conditions (USDA, 2007). Flowering is indeterminate and there is the possibility of some flowers present at harvest. Some seeds will shatter once capsules open. Seed retains viability for several years under 15%

moisture conditions (USDA, NRCS, 2006).

***Linum sulcatum* Riddell (2n=30)**

Linum sulcatum, known as grooved flax, is generally considered as an annual plant species, but has also been recorded as a biennial in North Carolina (Radford et al., 1968). Leaves are alternate, sessile, about 2 cm long and 2 mm wide, with a single midrib. Plants vary in height from 20 to 70 cm and inflorescences are axillary loose panicles or racemes (Gill, 1966). Flowers have five yellow petals that are rounded at the apex. Grooved flax is insect pollinated and probably self-compatible (Zaremba, 2003). Rogers (1963) has divided *L. sulcatum* in two cultivars, *L. sulcatum* var. *harperi* (Small), distributed in Southern America; and *L. sulcatum* var. *sulcatum*, widely distributed from Manitoba to Texas and east to Georgia and New Hampshire. Like cultivated flax, *L. sulcatum* is n=15 (Dillman, 1933), so it may have potential to hybridize with flax. Rogers (1963) has classified *L. sulcatum* as an intermediate form between two complexes in the genus *Linum*, which was supported by Giannasi and Rogers (1970) and by a cytogenetic study of Harris (1968). Attempts to hybridize *L. sulcatum* with its near relatives to assess chromosome similarities were unsuccessful (Harris, 1968).

Grooved flax can be considered a weed (Stevens, 1932). The seeds of *L. sulcatum* are known to persist in the soil seed bank (Blake, 1935). Zaremba (2003) observed that plants are first evident in early June and begin to flower by late June continuing through the early fall, and that even small plants (4 cm)

can produce flowers and seeds. It can also colonize new sites easily (Cunningham, 1997). There is no detailed information available on pollination mechanisms, but it is believed to be insect pollinated (Zaremba, 2003). Robertson (1971) reported that many species of genus *Linum* are homostylous, so *L. sulcatum* may be self-compatible.

Soil persistence of flaxseed

Flax seed and seed boll (capsule) losses occur prior to and during harvest and they enter the soil seed bank. With small seeded crops such as flax, small yield losses at harvest can be a large input to the seed bank. Seed losses have not been quantified in flax as they have been in other oilseed crops like in canola (Gulden et al., 2003). In areas where flax is grown, volunteer flax is a significant component of weed populations (Wall, 1994). The relative abundance of volunteer flax, a composite index of species frequency, field uniformity and field density, has increased relative to other species across western Canada over the past 30 years (Leeson et al., 2005). Averaged across Alberta, Saskatchewan and Manitoba, volunteer flax ranked as the 32nd most abundant weed in the 1970s and as the 26th most abundant in the early 2000s (Leeson et al., 2005). Weed populations reflects changes in crop management practices including crop rotations, reduction in use of tillage, altered herbicide usage and the introduction of herbicide resistant crops (Thomas et al., 1997). Recent studies conducted at the University of Alberta suggest that flaxseed did not persist more than three years in artificial seed banks. Flax populations did

not persist greater than three years under conventional managemntTherefore, it is unlikely that GE flax would be a weed of agriculture or be an invasive in nature.

Conclusion

Most of the cultivated crop plants diverged from their wild relatives less than few thousand generations ago, and it is unlikely that complete isolation halting the flow of domesticated alleles from crop species to progenitors has occurred (Ellstrand et al., 1999). Whereas flax has been cultivated for > 5000 years (De Candolle, 1904; Dillman, 1938; Richharia, 1962; van Zeist and Bakker, 1975; Gill, 1987; Fu et al., 2002) it can be artificially crossed with several wild relatives and produce fertile progeny (Gill and Yermanos, 1967a; Yermanos and Gill, 1967; Bari and Godward, 1970; Seetaram, 1972; Figures 3-1 and 3-2). If fertile hybrids can be produced from crosses between *L. usitatissimum* and closely related species, a transgene may be able to transfer to these wild species. This is particularly noteworthy since cultivated flax and wild relatives may grow in sympatry in several locations.

Although artificial hybridization of *Linum* species under controlled conditions does not predict the success of hybridization in the natural ecosystem, it can establish potential cross compatibility between those species (Ellstrand et al., 1999). In nature, the hybridization rate is predicted to be lower than that of greenhouse studies. However, the number of plants in the environment increases the chance that a successful cross will eventually occur.

A limited distribution of wild populations and weed control practices would also reduce the population size of wild plants and volunteer flax and thus minimize gene flow.

Western Canada is the largest flax growing region in the world and only three wild species are distributed in this area. Two of them, *L. rigidum* and *L. sulcatum*, have the same karyotype as cultivated flax. While interspecific outcrossing has not been documented for these species (Figure 3-3), hybridization of flax with other n=15 species suggests outcrossing may occur (Figure 3-1). *Linum lewisii,* n=9, seems less likely to outcross with cultivated flax (Gill, 1987). Among the *Linum* species in the United States, *L. corymbiferum* is the only species where successful hybridization with cultivated flax has been documented but there are several other species for which outcrossing with flax has not been reported (see Figure 3-3). Little is known about the distribution, flowering time, preferred habitat or population size of these species. Further research on species, including a greenhouse study to quantify outcrossing potential with cultivated flax is warranted to determine whether introgression of a transgene can occur. The assessment of the potential for gene flow to wild relatives is one of many components of an environmental risk assessment prior to the release of a new GE crop. Other concerns are the movement of transgenes via pollen to conventional crops, seed and volunteer mediated gene flow, influences on non-target species and the potential harm to biodiversity. Details on the specific trait and the

consequence of its expression are required prior to determining whether the transgene could have an impact on wild species, should introgression occur.

Table 3-1 Comparison of various groupings of *Linum* species based on chromosome numbers, including only those species for which cytological information is available.

Kikuchi (1929)	**Ray (1944)**	**Nagao (1941)**	**Gill (1987)**
Group I (n=9)	**Group I (n=8)**	**Group I (n=8)**	**Group I (2n=18)**
L. altaicum Fisch.	*L. grandiflorum* Desf.	*L. grandiflorum* Desf.	*L. alpinum* Jacq. (2n=18,36)
L. austriacum L.	*L. hirsutum* L.		*L. altaicum* Ldb.
L. extraaxillare Kit.			*L. anglicum* Mill.
L. hologynum Reichb.			*L. austriacum* L.
L. lewisii Pursh			*L. grandiflorum* Desf. (2n=16,18)
L. muilleri Moris.			*L. tenuifolium* L.
L. narbonense L.			*L. hologynum* Reichb.
L. perenne L.			*L. julicum* Hayek.
L. sibiricum DC			*L. lewisii* Pursh.
			L. narbonense L. (2n=18,28)
			L. perenne L.
			L. strictum L.
Group II. (n=15)	**Group II (n=9)**	**Group II (n=15,16)**	**Group II (2n=30)**
L. americanum L.	*L. alpinum* Jacq.	*L. angustifolium* Huds.	*L. africanum* L. (2n=30,32)
L. angustifolium Huds.	*L. altaicum* Fisch.	*L. crepitans* Dum.	*L. album* Kotschy

L. corymbiferum Desf.	*L. austriacum* L.	*L. usitatissimum* L.	*L. angustifolium* Huds. (2n=30,32)
L. flavum L.	*L. collinum* Guss.		*L. bienne* Mill. (2n=30,32)
L. usitatissimum L.	*L. hologynum* Reichenb.		*L. corymbiferum* Desf. (2n=18,30)
	L. lewisii Pursh.		*L. decumbens* Desf.
	L. loreyi Jord.		*L. flavum* L. (2n=28, 30)
	L. perenne L.		*L. hispanicum* Mill.
	L. strictum L.		*L. humile* Mill
	L. tommasinii Nym.		*L. medium* Planch. (2n=30,36) var. *texanum*
			L. nervosum Waldst.
			L. pallescens Ldb.
			L. rigidum Pursh. var. *filifolium* Rog.
			L. rigidum Pursh. var. *rigidum* Rog.
			L. sulcatum Riddell.
			L. tenue Desf.
			L. usitatissimum L.

Group III (n=18)	**Group III (n=10)**	**Group III (n=14)**	**Group III (2n=28)**
L. alpinum Jacq.	*L. gallicum* L.	*L. campanulatum* L.	*L. campanulatum* L.
		L. flavum L.	*L. capitatum* L. (2n=24,28)
			L. dolomiticum Borb.
Group IV (n=43)	**Group IV (n=14)**	**Group IV (n=9)**	**Group IV (2n=16)**
L. monogynum Forst.	*L. capitatum* Kit.	*L. hirsutum* L.	*L. catharticum* L. (2n=16,57)
	L. narbonense L.	*L. maritimum* L.	*L. hirsutum* L. (2n=16,18)
			L. viscorum L.
	Group V (n=15)	**Group V (n=9)**	**Group V Others**
	L. angustifolium Huds.	*L. alpinum* Jacq. (n=18)	*L. gallicum* L. (2n=20)
	L. flavum L.	*L. altaicum* Ldb.	*L. marginale* A. Cunn. (2n=80)
	L. medium Britton.	*L. austriacum* L.	*L. maritimum* L. (2n=20)
	L. usitatissimum L.	*L. extraaxillare* Kit.	*L. monogynum* Forst. (2n=86)
		L. hologynum Reichb.	*L. rupestra* Engelm. (2n=36)
		L. lewisii Pursh.	*L. schiedeanum* S. & C. (2n=36)
		L. montanum Schleich	
		L. muilleri Moris.	
		L. narbonense L.	

		L. perenne L.	
		L. sibiricum DC.	
		L. tenuifolium L.	

Table 3-2 Geographical distribution of *Linum* species classified by Gill (1987).

Taxon	Location (endemic and /or naturalized)	Reference
Group I (2n=18)		
L. alpinum Jacq. (*L. perenne* subsp. *alpinum* (Jacq.) Stoj.& Stef.) (2n=18, 36)	**Europe:** Austria, Bulgaria, France, Greece, Italy, Spain, Switzerland, Yugoslavia	Tutin et al. (1980); Greuter et al. (1984); Huxley (1992)
L. altaicum Ldb.	**Asia:** Kazakhstan, Mongolia, Russian Federation	Czerepanov (1995)
L. anglicum Mill. (*L. perenne* subsp. *Anglicum* (Mill.) Ockendon	**Europe:** England, Scotland	Ockendon (1968)
L. austriacum L.	**Africa**: Algeria, Morocco	Tutin et al. (1980); Greuter et al. (1984)
	Asia: Albania, Armenia, Azerbaijan, , Bulgaria, Greece, Iran, Italy, Russian Federation, Turkey, Western Siberia	
	Europe: Austria, Czechoslovakia, France, Germany, Hungary, Poland, Romania, Spain Switzerland, Ukraine, Yugoslavia	
L. grandiflorum Desf.	**Africa:** Algeria	Greuter et al. (1984); Huxley (1992)
L. hologynum Reichb.	**Europe :** Albania, Bulgaria, Greece, Romania, Yugoslavia	Tutin et al. (1980); Greuter et al. (1984)
L. julicum Hayek (*L. perenne* subsp. *alpinum* (Jacq.). Stoj & Stef.)	**Europe :** Austria, Bulgaria, France, Greece, Italy, Spain, Switzerland, Yugoslavia	Tutin et al. (1980); Greuter et al. (1984)
L. lewisii Pursh	**North America: Canada:** AB, BC, MB, ON, QC, SK, YT	Hitchcock et al. (1969); Cody, (1996)
	USA: CA, CO, ID, MT, NV, OR, ND, SD, UT, WA, WV, WY	
L. narbonense L. (2n=18, 28)	**Africa:** Algeria, Morocco	Tutin et al. (1980); Greuter et al., (1984)
	Europe: Albania, France, Italy, Portugal, Spain, Yugoslavia	
L. perenne L.	**Asia:** India, Russian Federation	Richharia (1962); Komarov (1969); Tutin et al. (1980)
	Europe: Albania, Austria, Belarus, Bulgaria, Czechoslovakia, France, Germany, Greece, Hungary, Italy, Moldova, Poland, Romania, Russian Federation, Spain, Switzerland, Ukraine, United Kingdom, Yugoslavia	

	North America: Canada: MB, ON, SK, Victoria Island, YT	
	Mexico	
	USA: AK	
L. strictum L.	**Africa :** Algeria, Egypt, Ethiopia, Libya, Morocco, Tunisia	Rechinger (1963); Tutin et al. (1980); Meikle (1985); Hooker, (1875)
	Asia : Iran, Iraq, Israel, Jordan, Pakistan, Syria, Turkey	
	Europe : Albania, Bulgaria, France, Greece, Italy, Portugal, Spain, Yugoslavia	
L. tenuifolium L.	**Asia:** Armenia, Azerbaijan, Georgia, Iran, Russian Federation, Syria, Turkey	Komarov (1969); Tutin et al. (1980)
Group II (2n=30)		
L. africanum L. (2n=30, 32)	**Africa:** Cape Province	Bond and Goldblatt (1984); Arnold and DeWet (1993)
L. album Kotschy	**Asia:** Iran, Syria	Guest et al. (1966)
L. angustifolium Huds. (*L. bienne* Mill) (2n=30, 32)	**Asia:** Armenia, Azerbaijan, Cyprus, Georgia, India, Iran, Iraq, Israel, Lebanon, Russian Federation, Syria, Turkey	Guest et al. (1966); Tutin et al. (1980); Meikle (1985)
	Africa: Algeria, Libya, Morocco, Portugal, Tunisia	
	Europe : Albania, Bulgaria, France, Greece, Ireland, Italy, Portugal, Spain, UK, Ukraine, Yugoslavia	
L. corymbiferum Desf.	**Africa:** Algeria, Tunisia	Greuter et al. (1984); USDA, NCRPIS, personal communication (2006)
	North America: USA: IA	
L. decumbens Desf.	**Europe:** Germany	PGRC, personal communication (2006)
L. flavum L. (2n=28, 30)	**Asia:** Russian Federation, Turkey	Davis et al. (1988); Huxley (1992)
	Europe: Austria, Czechoslovakia, Germany, Hungary, Poland, Belarus, Moldova, Ukraine, Albania, Bulgaria, Italy, Romania, Yugoslavia	
L. hispanicum Mill.	**Europe:** Albania, Bulgaria, France, Greece, Ireland, Italy, Portugal, Spain, Ukraine, United Kingdom, Yugoslavia	Komarov (1969); Davis et al. (1988)
L. humile Mill (*L. usitatissimum* var. *humile* Mill Pers.)	**Africa:** Mediterranean	Komarov (1969); Tutin et al. (1980)
L. medium Planch. (2n=30, 36)	**North America: Canada**: ON	Rogers (1963); Scoggan

	USA: AL, AR, FL, GA, IA, KS, LA, OK, MO, NC, SC, TX, VA	(1993); Magee and Ahles (1999)
L. nervosum Waldst.	**Asia:** Armenia, Azerbaijan, Georgia, Iran, Russian Federation, Turkey	Tutin et al. (1980); Davis et al. (1988)
	Europe: Bulgaria, Ukraine, Romania, Yugoslavia,	
L. pallescens Ldb.	**Asia:** Kazakhstan, Kyrgyzstan, Russian Federation, Tajikistan,	Komarov (1969)
L. rigidum Pursh. var. *filifolium* Rog. var. *rigidum* Rog.	**North America: Canada:** AB, MB, ON, SK	Rogers (1963); Rogers (1968); Scoggan (1993)
	Mexico	
	USA: FL, TX	
L. sulcatum Riddel.	**North America: Canada:** MB, ON, QC	Rogers (1963); Scoggan (1993)
	USA: AL, AR, GA, IL, IA, KS, KY, LA, MD, MS, MN, MO, NE, NC, ND, OK, PA, TN, TX, VA, WI	
L. tenue Desf.	**Africa:** Algeria, Morocco	Tutin et al. (1980)
	Europe: Portugal, Spain	
L. usitatissimum L.	Cultivated species of *Linum*, grown in almost all continents	Richharia (1962); Gill (1966)
Group III (2n=28)		
L. campanulatum	**Europe:** Italy, France, Spain	Tutin et al. (1980)
L. capitatum L. (2n=24, 28)	**Europe:** Albania, Bulgaria, Greece, Italy, Yugoslavia	Tutin et al. (1980)
L. dolomiticum Borb.	**Europe:** Hungary	Tutin et al. (1980)
Group IV (2n=16)		
L. catharticum L. (2n=16, 57)	**Africa:** Morocco	Rogers (1963); Scoggan (1993)
	Asia: Azerbaijan, Georgia, Iran, Russian Federation, Turkey	
	Europe: Albania, Austria, Belarus, Belgium, Bulgaria, Czechoslovakia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Norway, Sweden, United Kingdom, Netherlands, Poland, Switzerland, Russian Federation, Ukraine, Portugal, Romania, Spain, Yugoslavia	
	North America: Canada: NF, NS, ON, QC	
	USA: MI, PA	
L. hirsutum (2n=16, 18)	**Europe:** Bulgaria, Czechoslovakia, Hungary, Yugoslavia	Greuter et al. (1984); Tutin et al. (1980)
L. viscorum L.	**Europe:** Austria, France, Germany, Italy, Spain, Yugoslavia	Tutin et al. (1980)
Group V Others		

L. gallicum L. (*L. trigynum* L.)	Cyprus, Lebanon, Syria, Turkey	Davis et al. (1988); Komarov (1969)
L. marginale A. Cunn. (2n=80)	**Australia:** New South Wales, Queensland, South Australia, Tasmania, Victoria, Western Australia	Willis (1972); Hnatiuk (1990)
L. maritimum L.	**Africa:** Algeria, Morocco	Tutin et al. (1980); Greuter et al. (1984)
L. monogynum Forst. (2n=86)	**Australia:** Australia and New Zealand	Allan (1961); Hnatiuk (1990)
L. rupestre (Gray) Engelm. Ex Gray	**North America: USA:** NM, TX	USDA, NRCS (2006)
L. schiedeanum S. & C.	**Mexico**	Rogers (1969)
	USA: FL, TX	

Table 3-3 Distribution of *Linum* species (n=15) in North America.

Species	Distribution	Reference
L. alatum (Small) Winkler	**USA:** TX	Rogers and Harris (1966)
L. aristatum Engelm.	**USA:** AZ, CO, NM, UT, TX	Rogers and Harris (1966)
L. australe Heller	**USA:** AZ, NM	Rogers (1963)
L. corymbiferum Desf.	**USA:** IA	USDA, NCRPIS, personal communication (2006)
L. hudsonioides Planch.	**USA:** KS, NM, OK, TX	Harris (1968); Correl and Johnston (1970)
L. imbricatum (Raf.) Shinners	**USA:** TX	Osborne and Lewis (1962)
L. puberulum (Engelm.) Heller	**USA:** FL, OK, TX	Mosquin and Hayley (1967)
L. rigidum Pursh var. *rigidum* var. *berlandieri* var. *compactum* var. *filifolium* var. *carteri*	**Canada:** AB, MB, ON, SK **USA:** FL, NM, TX	Mosquin and Hayley (1967); Rogers (1968); Scoggan (1993)
L. sulcatum Riddell	**Canada:** MB, ON, QC, SK. **USA:** AL, AR, CT, GA, IL, IN, IA, KS, KY, LA, MD, MI, MS, MN, MO, NE, NJ, NY, NC, ND, OH, PA, PA, TN, TX, VT, WV, WI	Rogers (1963); Rogers and Harris (1966); Scoggan (1993)

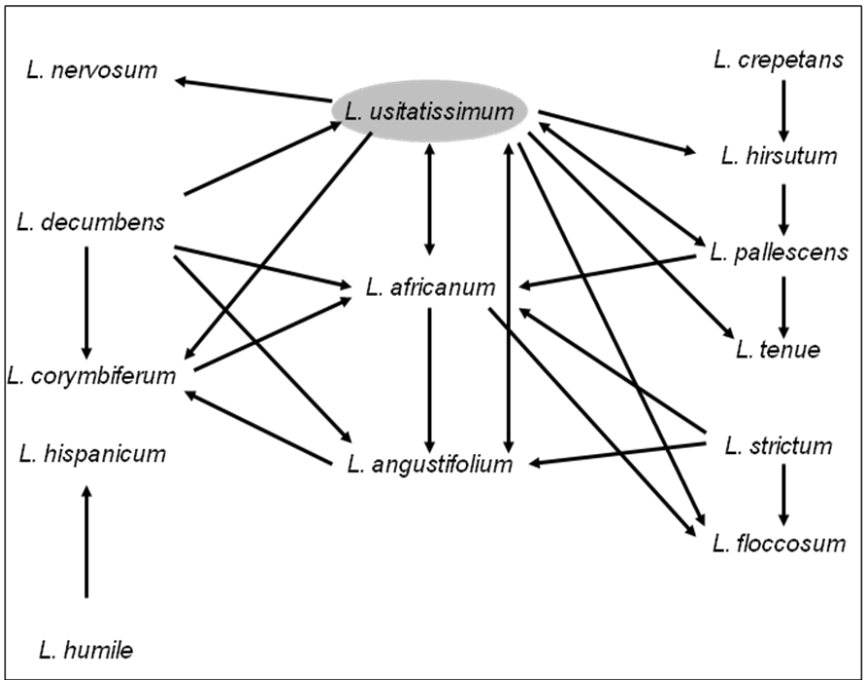

Figure 3-1 Artificial interspecific crosses among *Linum* species (n=15) that resulted in fertile progeny. Arrows indicate the direction of the cross (male to female). These are the related species with the greatest potential to hybridize with flax.

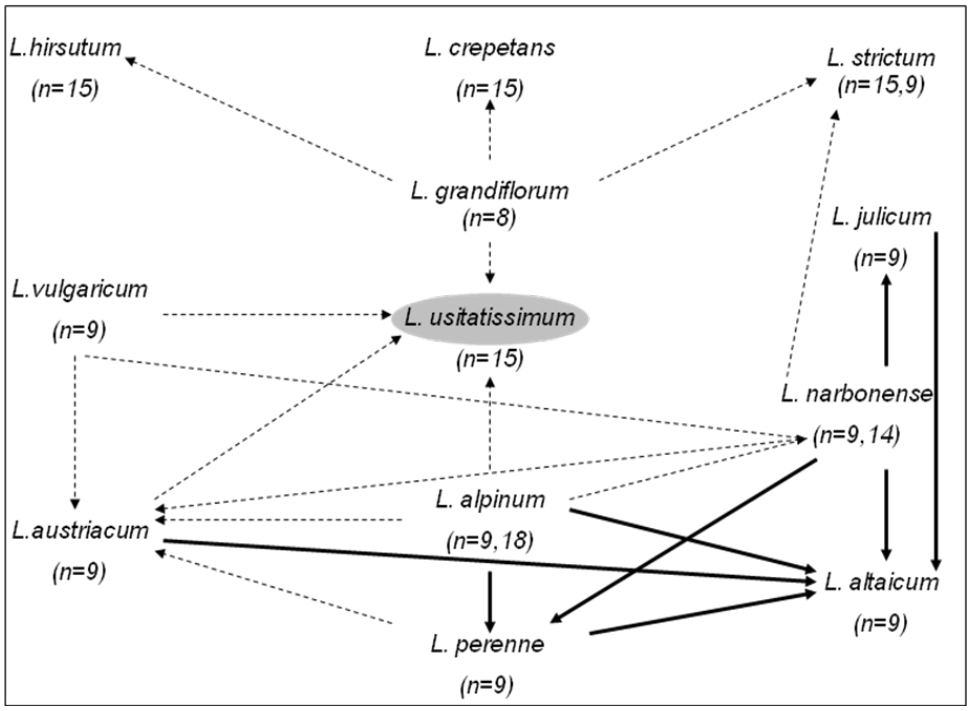

Figure 3-2 Interspecific hybridization in *Linum* (species with different chromosome numbers). Arrows indicate the direction of the cross (male to female). Solid lines indicate fertile F_1 hybrids were obtained with viable seed production. Dotted lines indicate hybridization occurred, but F_1 hybrids were not obtained with embryo rescue and/ or treatments with colchicine.

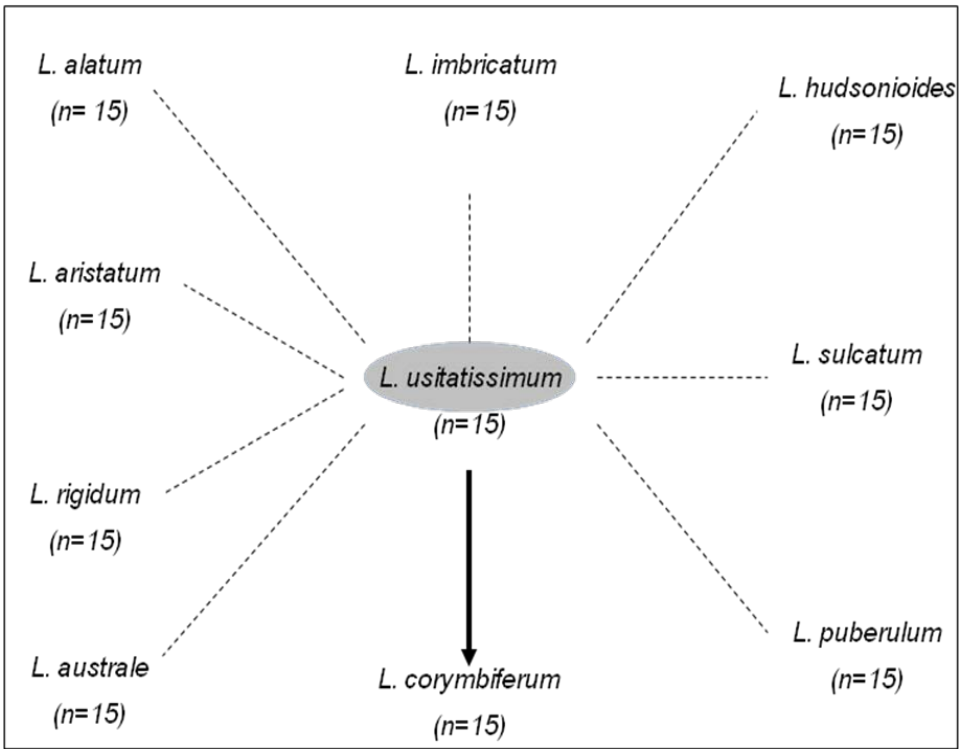

Figure 3-3 Potential hybridization of flax with closely related species (n=15) in the New World. A dotted line indicates species that may hybridize with flax, but no evidence of hybridization has been reported, while a solid arrow indicates a successful hybridization from male to female.

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Chapter 4

Pollen-mediated gene flow in flax (*Linum usitatissimum* L.) and strategy to confine transgene movement: Can genetically engineered and organic flax co-exist?[4]

Introduction

Since the introduction of genetically engineered (GE) crops, the potential for pollen-mediated gene flow has become easier to detect and more important to mitigate. Detection of adventitious presence (AP) of transgenes at low frequency using real time polymerase chain reaction (PCR) has the potential to become a routine testing procedure to detect GE seeds of major crops including canola (*Brassica napus* L.), maize (*Zea mays* L.) and soybean (*Glycine max* L.) and their products (Hubner et al., 2001; Pla et al., 2006). In addition, conventional products with minimal GE content are in demand in some regions, notably the European Union (EU), but worldwide, the AP thresholds either vary or have yet to be established (Demeke et al., 2006). Concurrent with the introduction of GE crops, there has been increased demand for organic products where there is a zero threshold for the presence of transgenes. Traditional isolation distance and other management practices designed to segregate crop varieties and production systems may be insufficient given these constraints. By quantifying pollen-mediated gene flow, we can develop practices for GE, conventional and organic crops to co-exist and maintain AP below the threshold levels to avoid constraints to international trade (Devos et al., 2004).

[4] *A version of this chapter will be submitted: Jhala A.J., Bhatt H., Topinka K.C. and Hall L.M. Pollen-mediated gene flow in flax (Linum usitatissimum* L.*) and strategy to confine transgene movement: Can genetically engineered and organic flax co-exist? Nature Heredity*

Flax, *Linum usitatissimum* L., an oilseed crop, is reported to be predominantly a self pollinated species (Gill, 1987). Flax has a perfect flower; stamens and pistils present in the same flower. As each flower opens, stamens bend inwards, discharging pollen on the stigma, usually resulting in self-pollination (Dillman, 1938). Gene flow in flax not only depends on the position of the anthers in relation to stigma, the receptivity of stigma, the viability of pollen, the availability of pollinators (Henry and Tu, 1928; Yermanos and Kostopoulos, 1970) but also may vary with genotype and environment (Dillman, 1938). Previous reports have indicated that gene flow in flax is in the range of 1 to 5% (Bolley, 1927; Dillman and Goar, 1937; Dillman and Stoa, 1935; Graham and Roy, 1924; Howard and Howard, 1919; Joshi, 1994). The occurrence of gene flow in flax and the tendency of some cultivars to possess higher gene flow rates than others when plants were grown in close proximity is well established (Robinson, 1937). The potential for pollen-mediated gene flow in flax, however, over distances beyond 7 m is undocumented.

Flax has been transformed with several novel genes (McHughen, 2002; Dong and McHughen, 1993; McHughen and Holm, 1995; McSheffrey et al., 1992; Wijayanto and McHughen, 1999), but currently there is no GE cultivar of flax available. After initial commercialization, sulfonylurea resistant flax was withdrawn at the request of the Flax Council of Canada, primarily to avoid trade issues with the European Union (EU). Over 80% of the Canadian flaxseed is exported, mainly to the EU, USA, Japan and South Korea (Flax Council of Canada, 2007), and the EU has the strict regulatory system for growing and

importing GE crops for food and feed. Genetically engineered cultivars of flax are under consideration in Canada and in several other parts of the world (Wrobel-Kwiatkowska et al., 2004; Wrobel-Kwiatkowska et al., 2007). However, the flax industry in Canada and the USA is facing questions regarding potential gene flow from GE flax to commodity and organic flax and whether GE flax can co-exist with conventional and organic flax production systems.

Determining pollen-mediated gene flow prior to commercialization of GE flax can be useful to allow developers and the flax industry to assess the risks (if any) of releasing a GE variety at a commercial scale. However, because pollen movement is dependent upon the size of the donor population, small scale experiments may underestimate the distance and frequency of pollen-mediated gene flow. For example, when the size of the pollen source was 45.7 m diameter in a pollen-mediated gene flow experiment in wheat (*Triticum aestivum* L.), the maximum distance that gene flow was detected was 42 m and thus, an isolation distance of 45 m was suggested (Hanson et al., 2005). In larger scale trials, when the size of pollen source was 33 ha low frequencies of gene flow were reported even up to 2.75 km (Matus-Cadiz et al., 2007).

Without a molecular marker, it is more difficult to accurately measure low frequency gene flow or rare events of gene flow specifically at longer distances from the pollen source. Several markers have been used to detect pollen-mediated gene flow in donor-receptor experiments including molecular markers based on quantitative PCR (Pla et al., 2006; Weber et al., 2007; Weekes et al., 2005; Weekes et al., 2007), micro-satellite markers (Chaix et al., 2003; Dje et al., 2004;

Isagi et al., 2004) and some other techniques like green fluorescent protein (GFP) (Halfhill et al., 2003) and blue aleurone seed color in wheat (Hanson et al., 2005) and triticale (Hills et al., 2007). There was no GE cultivar of flax available to be used as donor population in gene flow studies. The petal color of the flax flower has been used as a marker in a few studies (Dillman, 1938b), but it was not reliable and accurate. Flax cultivars with very high ALA (α-linolenic acid: 18:3$^{cis\Delta 9,12,15}$) (Kenaschuk, 1994) and "Solin" (*the name Solin was given by the Flax Council of Canada to define the flax cultivars with < 3% ALA*) cultivars with low ALA have been developed (Dribnenki et al., 2003). Two independently inherited genes, *LuFAD3A* and *LuFAD3B* control the low ALA trait in flax (Vrinten et al., 2005). Hybrids between conventional and solin flax cultivars express high levels of ALA.

A thiobarbituric acid (TBA) test has been described for rapid screening of individual seed of flax for the content of ALA (Bhatty and Rowland, 1990). Kohn and Liversed (Kohn and Liversedge, 1944) were the first to discover the thiobarbituric acid (TBA) test as a red color complex when observing the aerobic incubation of animal tissue. Bernheim et al. (1948) concluded that red color produced was the result of a complex formed from TBA and oxidation products of unsaturated fatty acids. Initially this method has been widely adopted as a sensitive assay method for lipid peroxidation in animal tissues (Ohkawa and Ohishi, 1978). However, (Kenaston et al., 1955) revealed that TBA test was also sensitive in determining peroxides of ALA and arachidonic acid (C20:4 $^{cis\Delta 5,8,11,14}$) because autoxidation of monoenes and dienes do not yield products that react with

TBA, only trienes and more highly unsaturated fatty acids yield such products (Dahle et al., 1962). This is due to the ability of polyunsaturated fatty acids (PUFA) to form upon oxidation, a cyclic five membered ring peroxide (McGregor, 1974). The TBA method is rapid and sensitive for ALA detection from the vegetable oils (Bhatty and Rowland, 1990). The dominant high ALA trait in combination with the sensitive TBA test was exploited to measure pollen-mediated gene flow between two flax cultivars.

An additional constraint to measure pollen-mediated gene flow is sample size. It is not possible to determine zero transgenes in a population (unless every seed is tested). Even in gene flow studies conducted in small plots, it is not economical and practical to screen all the collected seeds from the different distances and directions of pollen source. A strategy is required for testing the minimum number of seeds to provide a statistically meaningful conclusion. Power, broadly defined as the probability that a statistical significant test will reject null hypothesis for a specified value of an alternative hypothesis, provides a decision making framework (Bausell and Li, 2002). The sampling strategy was developed by using power analysis and binomial distribution to define the minimum number of seeds required to measure the pollen-mediated gene flow between two crop cultivars. A larger sample size generally leads to parameter estimates with smaller variances, giving a greater ability to detect a significant difference.

The objective of this study was to measure pollen mediate gene flow in flax under small scale field conditions using high and low ALA cultivars in western Canada. The frequency of gene flow and distance from the source were

quantified up to the distance of 50 m. We also determined the minimum number of seeds required determining gene flow at various distances by using the theoretical values of gene flow frequencies and various confidence intervals (α) to ensure that sufficient power (1-β) exist to measure pollen-mediated gene flow.

Materials and methods

Plant material

Breeder seed of two flax cultivars, "AC McDuff" and "SP 2047" were provided by Vittera (previously known as Agricore United), Canada. AC McDuff, a late maturing flax cultivar with very high oil content (~70% ALA), was released by Agriculture and Agri-Food Canada (AAFC), Morden Research Station (Kenaschuk, 1994). SP 2047, a solin flax cultivar with very low ALA (< 3%), was developed by Vittera and registered in 2002 (Dribnenki et al., 2003). Prior to planting, the seeds were tested for cross-contamination by screening more than 1.0 million seeds by thiobarbituric acid (TBA) test during both the years. The seeds were found to be true to its type with the purity of more than 99.9% (data not shown).

Field experiments

Field experiments were conducted at two locations, Edmonton Research Station (EdRS) and Ellerslie Research Station (ElRS), University of Alberta in 2006 and 2007 under western Canadian climatic conditions. Soil texture at EdRS was clay loam and consisted of 34% sand, 37% silt, and 28% clay with a pH 5.6 and 13% organic matter. Soil texture at ElRS was clay loam with 28% sand, 41% silt and 31% clay with a pH 6.5 and 11% organic matter. Flax cultivar AC McDuff was used as a pollen source (20x20 m in the center, Figure 4-1) and Solin cultivar

SP 2047 was used as a pollen receptor (120x120 m) in a Bulls-eye experimental design at both the locations and years (Figure 4-1). Seeding of flax was done on 16 and 18 May in 2006; and 28 and 26 May in 2007 at EdRS and ElRS, respectively. Fertilizers were applied as per the soil testing report of the individual site and the recommended rates for flax cultivation (data not shown). The seeder was thoroughly cleaned by vacuum cleaner after seeding the source cultivar (AC McDuff) to avoid seed contamination in the outer (recipient) area. Both the flax cultivars flowered synchronously at all the location-year. Prior to harvesting, the entire field was divided in eight blocks or replicates (Figure 4-1). Harvesting was done on Sep. 17 and 21, respectively at EdRS and ElRS in 2006; and on Sep. 23 and 27, respectively at EdRS and ElRS in 2007. Because of higher probability of gene flow in the area near the source, flax plants were harvested at every 20 cm (single raw of flax plant) distance up to 1 m and then at every 40 cm (two rows of flax plants) up to 3.0 m. A binder was used to cut flax plants in an individual raw in the first 3.0 m (pollen receptor) area to avoid contamination. After 3.0 m, samples were harvested by combine at every 1.5 m block up to 12.5 m, and then in 5.0 m blocks up to 50 m in all 8 blocks (replicates). Harvesting began distal to the source and continued inward in the clockwise direction to reduce cross contamination of samples. Seeds were cleaned and stored at room temperature until laboratory screening was done.

Seed screening

The TBA test determines relative ALA content from oil extruded from flax seed (Bhatty and Rowland, 1990). This method was used for screening of the

seeds from receptor populations. The procedure of the TBA test has been described in Bhatty and Rowland, (1990). Protocol of the TBA test was modified to accommodate about 500 flax seeds in a 13 x 30 cm rectangle. Nearly 20,000 seeds per day were screened by TBA test to reach the total of ~4.0 million flaxseeds by this method to detect the gene flow frequency. The chemistry of the TBA test has been investigated in a number of studies but it is not fully understood (Pryor, 1976).

Statistical analysis

A power analysis, using binomial probabilities was used to determine sample size required to detect at least one GE seed for samples with different frequencies (theoretical) at three different confidence intervals (α) and power (1-β) values as explained in Zar (1999). The following formula was used to determine the minimum sample size (number of seeds) required to detect gene flow at a given frequency. The null hypothesis that the frequency $\hat{p} \geq p_0$ could be rejected when zero GE seeds were found in a sample of seeds (n_p) with a confidence value related to the type I error alpha value by 1-α.

$$\beta = P\left(Z < \frac{p_0 - p}{\sqrt{\frac{pq}{n}}} - Z_\alpha \sqrt{\frac{p_0 q_0}{pq}} \right)$$

$$n = \frac{pq\left(\phi^{-1}(\beta) + Z_\alpha \sqrt{\frac{p_0 q_0}{pq}} \right)^2}{\left(p_0 - p \right)^2} = \frac{(p_0 - \Delta)(1 - p_0 + \Delta)\left[\phi^{-1}(\beta) + Z_\alpha \sqrt{\frac{p_0(1-p_0)}{(p_0 - \Delta)(1 - p_0 + \Delta)}} \right]^2}{\Delta^2}$$

---------- (1)

Where,

n: minimum number of seeds required

β : Power

Z : Random variable following N (0, 1);

q : $1 - p$; $q_0 = 1 - p_0$

p_0 : True value in Null hypothesis- hypothesized parameter

p: Theoretical frequency of outcrossing

Δ : Effect size ($p_0 - p$)

Z_a : Critical value for significant level *a*

Φ^{-1} = anti-function of the normal curve

In order to test the herogeneity (if any) in frequency of gene flow between the blocks for each experiment, a log-likelihood ratio test using the chi-square distribution was conducted using the -2 log-likelihood ratio *(used to compare the fit of two models one of which is nested within the other and the value of this ratio is used for making a decision between two hypotheses)* provided by regression analysis as explained in McPherson et al. (2008). Seeds harvested from the pollen recipient populations of low ALA flax cultivar were screened in laboratory by TBA test. Seeds with high ALA were considered as the product of gene flow from high ALA to low ALA flax cultivars. The percentage of gene flow was calculated as the ratio of number of high ALA seeds in a sample and the total number of seeds analyzed. The frequency of gene flow from high ALA to low ALA flax cultivar was expressed as the frequency of high ALA seeds in a sample of receptor population at various distances from the source. A nonlinear regression mixed model (PROC NLMIXED; SAS 2007) was used for

modeling the frequency of gene flow at each mean distance from the pollen source. A binomial distribution was employed to estimate the frequency of gene flow from the pollen source in the eight blocks at each location-year. The data were fitted to the following exponential decay function (Hanson et al., 2005; McPherson et al., 2008),

$$p = a\, e^{-bd} \qquad \text{----------} \quad (2)$$

Where, p is predicted frequency of gene flow; a is intercept; b is curve parameter; and d is the mean distance from the source (m). Using this exponential decay function, the distance where frequency of outcrossing was reduced by 50 and 90% were estimated by the following equations,

$$O_{50} = \frac{\ln (0.5 * a) - \ln a}{-b} \qquad \text{----------} \quad (3)$$

$$O_{90} = \frac{\ln (0.1 * a) - \ln a}{-b} \qquad \text{----------} \quad (4)$$

Where, a is intercept and b is slope.

The same exponential decay function was also used to determine the combined O_{50} and O_{90} values of various sites and or years.

Results

Uncertainty resulting from testing random samples was reduced by combining binomial probability distribution and power analysis to define a sampling strategy (Table 4-1). Several thousand seeds were screened at each distance until the minimum power of 0.8 was obtained. For example, minimum 2,400 to as high as 476,000 seeds were screened for a single distance to maintain

high power values in test (Table 4-2). In many instances, power (0.95) was achieved, especially to detect low level presence of gene flow at longer distances (i.e. ≥ 15 m) from the pollen source (Table 4-3 to 4-6).

To evaluate differences in the gene flow frequency among directional blocks at each experimental site, the maximum likelihood ratio was calculated (Table 4-2). Results suggest that frequency of gene flow between various blocks was relatively similar; indicating that wind or wind direction has not played a consistent role in pollen dissemination from source to receptor plant population. Regular observations recorded during the growing season suggest that flowering of recipient and donor flax cultivars were synchronous and uniform at all the sites and years (data not shown). Therefore, it is unlikely that flowering time or flower density influenced gene flow frequency at different experimental sites.

Gene flow was tested at all distances (up to 50 m) and in all directional blocks (i.e. 8) at each location. At EdRS research site in 2006, 769,600 seeds were screened by TBA testing and 647 seeds found to be the result of gene flow from high ALA pollen source to low ALA flax cultivar with an average of 59,200 seeds screened per distance from the mean value of all eight blocks (Table 4-3). Maximum gene flow of 1.66% was observed at the minimum mean distance of 0.1 m from the pollen source after screening 14,400 seeds (Table 4-3). At ElRS site in 2006, 748,800 seeds were screened and gene flow was in the range of 1.45 to 0.011% at 0.1 to 35 m distance from the pollen source (Table 4-4).

The maximum percentage of out-crossing (2.42%) was recorded at the mean distance of 0.1 m from the pollen source at EdRS site in 2007 (Table 4-5;

Figure 4-2). Gene flow was quantified up to 25 m distance at EdRS site in 2007 with 13 seeds with high ALA out of 460,600 seeds screened to achieve the power value of 0.85 (Table 4-5). More than one million seeds in total were screened at this site with 475 high ALA seeds identified as result of gene flow. Some rare gene flow was observed (0.0028%) after screening 476,800 seeds at 35 m distance from the pollen source at ElRS site in 2007 (Table 4-6; Figure 4-2). Out of 113,0800 seeds screened, 372 seeds were found to be the result of gene flow at ElRS site in 2007 (Table 4-6). Value of power calculated for each distance and location suggested that except for a few sampling distances, the number of seeds screened was sufficient to achieve maximum power value of 0.95 (Tables 4-3 to 4-6).

Percent out-crossing was highest in the recipient plants closest to the source, 1.66% at EdRS, 2006 (Table 4-3); 1.44% at ElRS, 2006 (Table 4-4); 2.42% at EdRS, 2007 (Table 4-5) and 1.86% at ElRS, 2007 (Table 4-6). At all four locations, gene flow ranged from 0.01 to 0.1% at 7 m and from 0.0 to 0.01% 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) (Table 4-6). Several 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.

An exponential decay function was used to model gene flow at increasing

distance from the pollen source. Parameter estimates for intercept (a) and the rate of decline of gene flow (b) were significant, indicating that model was not over-parameterized (Table 4-7). The average distance at which gene flow was reduced by 50% (O_{50}) ranged from 0.85 to 2.64 m at all the locations-years. Minimum value of O_{50} (0.85 m) was recorded at ElRS site in 2007, whereas, the maximum distance (2.64 m) was recorded at ElRS site in 2006 (Table 4-7). Similarly, values for O_{90} ranged from 5.68 to 17.56 m, with the minimum distance of 5.68 m at ElRS in 2007, and maximum value of 17.56 m at ElRS in 2006 (Table 4-7).

Comparison was also made by combining data of different sites and years to estimate the distance from the source where 50 and 90% reduction in gene flow was occurred. The average distance where gene flow was reduced to 50 and 90% by combining the values of gene flow at all the sites and years was 1.62 and 5.37 m, respectively (Figure 4-3) and this distance was almost equal to the results of combined analysis of EdRS and ElRS sites at both the years (Table 4-8). This suggests that there was no difference in gene flow frequency for O_{50} and O_{90} distances at the same location but different year. In contrast to this, the combined results of O_{50} and O_{90} distance at EdRS and ElRS during the same year (2006 or 2007) indicate a site by year interaction. Therefore, gene flow at the same location but during different year (2006 and 2007) was relatively similar (Table 4-8).

Discussion

Gene flow studies have been conducted in many crops, however, the choice of sample size used to determine frequency of gene flow and the power of

test was rarely elucidated. A strategic approach to balance sample size with statistical power allowed for a maximization of the information generated for least sampling efforts.

Results indicate that maximum gene flow, ranging from 1.44 to 2.4%, was observed at the closest distance, a mean distance of 0.1 m. At 0.5 m, average gene flow was reduced to 0.77%; and at 1.0 m, 0.27%. Similar results were obtained in earlier studies on short distance out-crossing. Henry and Tu (1928) studied the extent of out-crossing in flax by growing blue and white flowered varieties in adjacent rows. Gene flow was reduced from 1.26% to 0.33%, when flax cultivars were grown 1.25 to 0.25 m apart, respectively. In a similar experiment, (Robinson, 1937) reported that out-crossing in flax varied from 0 to 3%, depending on the spacing between plants and climatic conditions. Kadam et al. (1938) reported gene flow was in the range of 0 to 6%, however, the average was < 3%.

Flax outcrossing did not differ significantly by direction (block), suggesting that wind may not have played an important role in flax gene flow. However, flax is an indeterminate species and flowers for 33 to 63 days (Chopde and Thakre, 1969) and wind speed and direction varies considerably over time. Flax flowers generally produce pollen in small quantities and it is relatively heavy and therefore, pollen dissemination by wind seems unlikely (Eyre and Smith, 1916). Insect-mediated pollination has been considered in several studies to determine the impact of insect on seed set. Dillman (1938) reported that sticky flax pollen was primarily disseminated by honey bees and thrips. Subsequent experiments suggest that honey bee was the major pollinator of flax followed by

bumble bees and thrips (Smirnov, 1956) While insects visit flax flowers, the important to gene flow has been disputed (Dillman, 1938; Gubin, 1945; Gill, 1987). Most recently Williams (1991) reported no increase in seed production in the presence of honeybees suggesting limited gene flow mediated by insects.

Gene flow declined exponentially with distance, the value of O_{90} was <18 meter at all site-year. No gene flow was detected beyond 35 m and from the power analysis we would accept the null hypothesis that the frequency of gene flow from these samples was equal to or less than 0.00005 at 95% confidence interval. Cultivar, environment and their interaction may have impact on the gene flow; results cannot be extrapolated for other flax cultivars or environmental conditions. Neither can these results be easily extrapolated to field scale gene flow as the size of the pollen source may affect distance of pollen movement (Matus-Cadiz et al., 2004; Matus-Cadiz et al., 2007).

Although pollen mediated gene flow has been studied in many crops including in canola (Beckie et al., 2003; Knispel et al., 2008), maize (Messeguer et al., 2006; Goggi et al., 2007; Weber et al., 2007; Mercer and Wainwright, 2008), wheat (Gaines et al., 2007; Gatford et al., 2007; Matus-Cadiz et al., 2007), soybean (Yoshimura et al., 2006), safflower (McPherson et al., 2008) and other crops reviewed by (Mallory-Smith and Zapiola, 2008)), it is not possible to directly compare the gene flow between crops. However, gene flow in flax appears lower than for maize, safflower and canola. When choosing a crop for the production of non-food products, one factor to consider may be the proclivity for long distance gene flow.

Strategy to reduce transgene movement

As recent as 2009, flax international trade was interrupted and market prices reduced following the detection by the EU of an unapproved GE flax in a shipment of Canadian flax. The variety CDC Triffid had not been registered to grow in Canada since 2001 and the source of contamination has not been established (Bedard, 2009). Unapproved GE crops have a zero threshold for adventitious presence. In Canada, approved GE crops are considered substantially equivalent to non-GE crops and segregation is not required between two cropping systems or their products (Smyth and McHughen, 2008). However, Europe has defined a 0.9% labeling threshold for the EU approved GE seeds in organic or conventional crop seeds (European Commission, 2003). Adventitious presence above this threshold triggers product labeling as originating from GE material (Devos et al., 2008). Prior to the release of GE flax, a strategy is required to mitigate transgene movement.

Given the length of the flower period in flax, a delay in seeding dates of organic flax to reduce the flowering overlap with GE flax may not be useful. This research does not specifically address the influence of the distance of gaps between crops, but increasing the isolation distances between GE and conventional flax grown for seed from the current 3 meters is suggested. Routine testing of flax seed for sale is also recommended to reduce the inadvertent planting of GE flax. We recommended the use of non-GE buffer zone around GE flax field and discarding boundary plants of organic flax fields after flowering (Jhala *et al.*, 2009). Our data suggests that >90% of the out-crossing could be

eliminated by removal of a 20 m strip between two flax crops. In combination with harvest blending and relatively large field sizes adventitious presence would be minimized. Recent experiments to determine efficiency of buffer zones and harvest discarding on gene flow containment in canola (*Brassica napus* L.) in France using the GENESYS model suggest that buffer zones were more effective in reducing harvest admixture because they increased the distance between GE and non-GE fields and also diminished the proportion of GE pollen in the total pollen cloud (Colbach et al., 2009).

Table 4-1 Power analysis assuming a binomial distribution to determine minimum number of seeds required to detect at least one GE seed for different frequency of gene flow; four α values (α= 5, 2.5, 1.0, 0.05%); and three power (1- β) values (0.80, 0.85, 0.95)

o^a Frequency of OC p(x)	Alpha value (α)											
	α = 5%	α = 2.5%	α = 1%	α = 0.05%	α = 5%	α = 2.5%	α = 1%	α = 0.05%	α = 5%	α = 2.5%	α = 1%	α = 0.05%
	Minimum sample size (n)[b] for Power[c] = 0.80				Minimum sample size (n)[b] for Power[c]= 0.85				Minimum sample size (n)[b] for Power[c] = 0.95			
0.01	1989	2588	3383	3985	2242	2875	3710	4340	3128	3869	4829	5544
0.005	3996	5199	6797	8007	4503	5776	7454	8719	6282	7771	9700	11136
0.0025	8010	10422	13625	16051	9026	11577	14941	17477	12590	15573	19441	22319
0.001	20052	26090	34109	40183	22595	28980	37402	43750	31513	38982	48664	55870
0.0005	40122	52204	68248	80402	45209	57985	74837	87540	63051	77995	97368	111788
0.00025	80262	104431	136528	160841	90437	115996	149707	175120	126127	156023	194777	223624
0.0001	200681	261114	341367	402159	226123	290028	374319	437860	315356	390106	487005	559132
0.00005	401379	522251	682765	804355	452266	580082	748671	875759	630737	780244	974051	1118312
0.000025	802776	1044526	1365561	1608746	904551	1160190	1497376	1751558	1261500	1560519	1948144	2236672
0.00001	2006968	2611350	3413949	4021921	2261406	2900514	3743490	4378955	3153787	3901347	4870422	5591751
0.000005	4013954	5222724	6827930	8043879	4522833	5801054	7487013	8757949	6307600	7802726	9740884	11183549
0.0000025	8027925	10445471	13655891	16087794	9045685	11602134	14974060	17515937	12615225	15605485	19481810	22367146

[a]Null hypothesis: theoretical value of frequency of gene flow

[b]Minimum sample size required to detect one or more GE seeds at a given theoretical frequency of gene flow and different values of α and power (1-β). Null hypothesis that the frequency $X \geq p$ is rejected at a given α value, when no GE seeds were found in a sample size of n or greater

[c]Value of power to ascertain how much power (1- β) would be available for a given sample size and confidence interval

Table 4-2 Gene flow directionality: log-likelihood ratio test among blocks for each location and year[f].

Location	Block	Direction[a]	Combined value[b]	Df[c]	Partitioned value[d]	Df[c]	X^2 value[e]	Df[c]
Edmonton, 2006	All	All	1.6	8	1.6	1	0	7
	1 *vs.* 5	N & S	0.1	2	0.7	1	0	1
	2 *vs.* 6	NE & SE	0.1	2	0.2	1	0	1
	3 *vs.* 7	E & W	0.7	2	0.6	1	0.1	1
	4 *vs.* 8	NW & SE	0.1	2	0.1	1	0	1
Ellerslie, 2006	All	All	1.4	8	1.4	1	0	7
	1 *vs.* 5	N & S	0.6	2	0.6	1	0	1
	2 *vs.* 6	NE & SE	0.2	2	0.2	1	0	1
	3 *vs.* 7	E & W	0.6	2	0.6	1	0	1
	4 *vs.* 8	NW & SE	0.0	2	0.0	1	0	1
Edmonton, 2007	All	All	1.5	8	1.3	1	1.2	7
	1 *vs.* 5	N & S	1.0	2	0.9	1	0	1
	2 *vs.* 6	NE & SE	0.0	2	0.0	1	0	1
	3 *vs.* 7	E & W	0.4	2	0.4	1	0	1
	4 *vs.* 8	NW & SE	0.0	2	0.0	1	0	1
Ellerslie, 2007	All	All	1.3	8	0.9	1	0.4	7
	1 *vs.* 5	N & S	0.6	2	0.6	1	0.0	1
	2 *vs.* 6	NE & SE	0.1	2	0.0	1	0.1	1
	3 *vs.* 7	E & W	0.6	2	0.3	1	0.3	1
	4 *vs.* 8	NW & SE	0.0	2	0.0	1	0.0	1

[a]Direction from the pollen source, where N is North, S is South, W is West, and E is East
[b]Overall -2 log-likelihood value calculated by SAS (2007) for the regression of the combined data set for blocks being compared
[c]Degree of freedom
[d]The sum of the individual value of -2 log-likelihood calculated by SAS (2007) for each individual regression of the partitioned data sets for the blocks being compared
[e]The chi-square value was calculated by subtracting the portioned value from the combined value
[f]the value was non-significant at $\alpha=0.05$ for all the blocks being compared for any site/year

Table 4-3 Harvest distances and laboratory screening results for the flax gene flow study at Edmonton Research Station (EdRS) in 2006

Mean Distance[a] (m)	Seeds screened[b]	Seeds with high ALA	Gene flow (%)	95% confidence interval[c]		Power[d] (1-β)
				Lower	Upper	
0.1	14400	239	1.6597	1.4199	1.8457	0.95
0.5	14400	130	0.9028	0.7275	1.0435	0.95
0.9	7200	33	0.4583	0.2901	0.6124	0.95
1.3	2400	13	0.5417	0.2459	0.8598	< 0.8
1.7	12000	37	0.3083	0.2007	0.4054	0.95
2.5	4800	2	0.0417	0.0021	0.1311	< 0.8
2.9	14400	29	0.2014	0.1230	0.2745	0.95
4.0	26400	37	0.1402	0.0912	0.1843	0.95
7.0	40800	46	0.1127	0.0771	0.1441	0.95
15	105600	41	0.0388	0.0259	0.0504	0.95
25	244800	40	0.0163	0.0108	0.0213	0.95
35	138400	0	0.0000	0.0000	0.0078	-
45	144000	0	0.0000	0.0000	0.0021	-
Total	769600	647	-	-	-	-
Mean	59200	49.77	-	-	-	-

[a] Mean distance from the pollen source was used in analysis for all observations

[b] Total number of seeds screened in all blocks

[c] The 95% confidence interval using a relationship between F and binomial distributions, with a correlation for the lower interval value when there is zero value of gene flow as described in Zar (1999), p.528.

[d] the value of power (1-β) was calculated for 95% confidence interval (α=5%) using equation 5 (see text) and Table 3

Table 4-4 Harvest distances and laboratory screening results for the flax gene flow study at Ellerslie Research Station (ElRS) in 2006

Mean Distance[a] (m)	Seeds screened[b]	Seeds with high ALA	Gene flow (%)	95% confidence interval[c]		Power[d] (1-β)
				Lower	Upper	
0.1	24000	347	1.4458	1.2713	1.5791	0.95
0.5	24000	201	0.8375	0.7055	0.9409	0.95
0.9	4800	4	0.0833	0.0148	0.1906	< 0.8
1.3	9600	21	0.2188	0.1208	0.3149	0.95
1.7	9600	47	0.4896	0.3364	0.624	0.95
2.5	9600	6	0.0625	0.0170	0.1233	< 0.8
2.9	14400	10	0.0694	0.0274	0.1178	< 0.8
4.0	48000	60	0.1250	0.0900	0.1550	0.95
7.0	52800	37	0.0701	0.0456	0.0922	0.85
15	148800	22	0.0148	0.0083	0.0211	0.95
25	168000	17	0.0101	0.0016	0.0086	0.95
35	100800	11	0.0109	0.0045	0.0181	0.95
45	134400	0	0.0000	0.0000	0.0022	-
Total	748800	783	-	-	-	-
Mean	57600	60.23	-	-	-	-

[a]Mean distance from the pollen source was used in analysis for all observations

[b]Total number of seeds screened in all blocks

[c]The 95% confidence interval using a relationship between F and binomial distributions, with a correlation for the lower interval value when there is zero value of out-crossing as described in Zar (1999)

[d]Value of power (1-β) was calculated for 95% confidence interval (α=5%) using equation 5 (see text) and Table

Table 4-5 Harvest distances and laboratory screening results for the flax gene flow study at Edmonton Research Station (EdRS) in 2007

Mean Distance[a] (m)	Seeds screened[b]	Seeds with high ALA	Gene flow (%)	95% confidence interval[c]		Power[d] (1-β)
				Lower	Upper	
0.1	9600	232	2.4167	2.0636	2.6906	0.95
0.5	9600	60	0.6250	0.4503	0.7743	0.95
1.3	9600	31	0.3229	0.2010	0.4356	0.95
2.5	14400	26	0.1806	0.1068	0.2504	0.95
4.0	91200	40	0.0439	0.0291	0.0571	0.95
7.0	115200	44	0.0382	0.0259	0.0491	0.95
15	96000	29	0.0302	0.0184	0.0412	0.85
25	460600	13	0.0063	0.0003	0.0073	0.85
35	176800	0	0.0000	0.0000	0.0039	-
45	176800	0	0.0000	0.0000	0.0039	-
Total	1159800	475	-	-	-	-
Mean	115980	47.5	-	-	-	-

[a]Mean distance from the pollen source was used in analysis for all observations

[b]Total number of seeds screened in all blocks

[c]The 95% confidence interval using a relationship between F and binomial distributions, with a correlation for the lower interval value when there is zero value of outcrossing as described in Zar (1999), p.528

[d] the value of power (1-β) was calculated for 95% confidence interval (α=5%) using equation 5 (see text) and Table 3

Table 4-6 Harvest distances and laboratory screening results for the flax gene flow study at Ellerslie Research Station (ElRS) in 2007

Mean Distance (m)	Seeds screened	Seeds with high ALA	Gene flow (%)	95% confidence interval[c]		Power[d] (1-β)
				Lower	Upper	
0.1	9600	179	1.8646	1.5551	2.1080	0.95
0.5	9600	70	0.7292	0.5395	0.8889	0.95
1.3	9600	33	0.3438	0.2175	0.4594	0.95
2.5	9600	22	0.2292	0.1286	0.3271	0.85
4.0	100800	27	0.0268	0.0160	0.0369	0.95
7.0	76800	11	0.0143	0.0060	0.0237	0.95
15	91200	13	0.0143	0.0065	0.0227	0.95
25	176800	0	0.0000	0.0000	0.0039	-
35	476800	17	0.0028	0.0000	0.0062	0.85
45	170000	0	0.0000	0.0000	0.0039	-
Total	1130800	372	-	-	-	-
Mean	113080	37.2	-	-	-	-

[a]Mean distance from the pollen source was used in analysis for all observations

[b]Total number of seeds screened in all blocks at a particular distance from the pollen source

[c]The 95% confidence interval using a relationship between F and binomial distributions, with a correlation for the lower interval value when there is zero value of outcrossing as described in Zar (1999), p.528.

[d] the value of power (1-β) was calculated for 95% confidence interval (α=5%) using equation 5 (see text) and Table 3

Table 4-7 Parameter estimates and the distance where 50% (O_{50}) and 90% (O_{90}) reduction in gene flow occurred with their respective standard errors and confidence intervals from the regression analysis at various sites in 2006 and 2007.

Experiment	Parameter[a]	Estimate[b]	Standard error	Df[c]	95% confidence interval	
					Lower	Upper
Edmonton, 2006	*a*	0.009570	0.03162	60	-0.05368	0.07282
	b	0.3024	1.1767	60	-2.0514	2.6563
	O_{50}	2.29191	8.91769	60	-15.5461	20.1299
	O_{90}	15.2271	59.2478	60	-103.286	133.74
Ellerslie, 2006	*a*	0.007363	0.02665	63	-0.04589	0.06062
	b	0.2622	1.1557	63	-2.0473	2.5717
	O_{50}	2.64364	11.6528	63	-20.6427	25.9300
	O_{90}	17.5640	77.4198	63	-137.147	172.275
Edmonton, 2007	*a*	0.01566	0.05022	64	-0.08466	0.1160
	b	0.6588	2.1599	64	-3.6561	4.9737
	O_{50}	1.05209	3.44916	64	-5.83840	7.94258
	O_{90}	6.98994	22.9157	64	-38.7895	52.7694
Ellerslie, 2007	*a*	0.01518	0.05221	64	-0.08911	0.1195
	b	0.8106	2.8400	64	-4.8630	6.4842
	O_{50}	0.85513	2.99613	64	-5.13032	6.84058
	O_{90}	5.68136	19.9058	64	-34.0851	45.4478

[a]Parameters a and b were estimated using equation 1. The distance (O_{50} and O_{90}) where outcrossing was reduced by 50 and 90% were estimated using equations 2 and 3 respectively.

[b]Estimates of the parameters for intercept (a), slope (b) and the estimates of the distance where outcrossing was reduced by 50 and 90%.

[c]degree of freedom

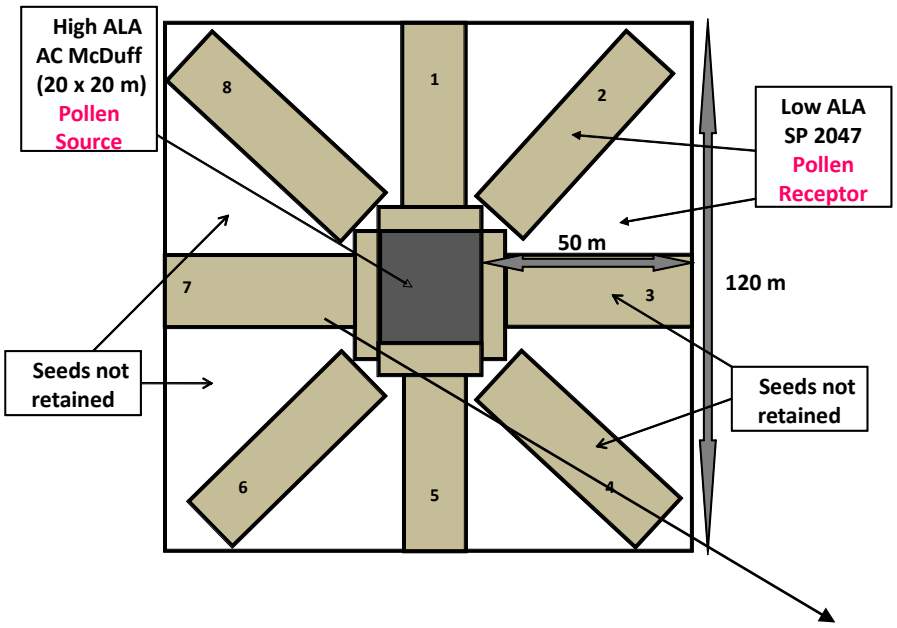
Table 4-8 Comparison of parameter estimates and the distance where 50% (O_{50}) and 90% (O_{90}) reduction in gene flow occurred with their respective standard errors and confidence intervals from the regression analysis of combined values of various sites and or years

Experiments combined	Parameter[a]	Estimate[b]	Standard error	Df[c]	95% confidence interval	
					Lower	Upper
Edmonton, 2006 & 2007	*a*	0.0119	0.0276	124	-0.0427	0.0665
	b	0.4291	1.0773	124	-1.7032	2.5614
	O_{50}	1.6153	4.0552	124	-6.4111	9.6416
	O_{90}	5.3658	13.4710	124	-21.2971	32.0287
Ellerslie, 2006 & 2007	*a*	0.0100	0.0254	127	-0.0402	0.0603
	b	0.4273	1.1723	127	-1.8925	2.7471
	O_{50}	1.6221	4.4501	127	-7.1839	10.4281
	O_{90}	5.3885	14.7829	127	-23.8643	34.6412
Edmonton & Ellerslie, 2006	*a*	0.0084	0.0206	123	-0.0324	0.0493
	b	0.2831	0.8274	123	-1.3548	1.9210
	O_{50}	2.4485	7.1566	123	-11.7176	16.6146
	O_{90}	8.1337	23.7738	123	-38.9251	55.1925
Edmonton & Ellerslie, 2007	*a*	0.0153	0.0360	128	-0.0558	0.0865
	b	0.7251	1.7233	128	-2.6882	4.1313
	O_{50}	0.9606	2.2943	128	-3.5791	5.5004
	O_{90}	3.1912	7.6217	128	-11.8896	18.2720
All years and locations	*a*	0.0197	0.0188	251	-0.0260	0.0480
	b	0.4283	0.7934	251	-1.1343	1.9909
	O_{50}	1.6182	2.9975	251	-4.2852	7.5216
	O_{90}	5.3756	9.9574	251	-14.2351	24.9864

[a]Parameters *a* and *b* were estimated using equation 1. The distance (O_{50} and O_{90}) where gene flow was reduced by 50 and 90% were estimated using equations 2 and 3 respectively.

[b]Estimates of the parameters for intercept (a), slope (b) and the estimates of the distance where gene flow was reduced by 50 and 90%.

[c]degree of freedom


High ALA
AC McDuff
(20 x 20 m)
Pollen
Source
Low ALA
SP 2047
Pollen
Receptor
50 m
120 m
Seeds not
retained
Seeds not
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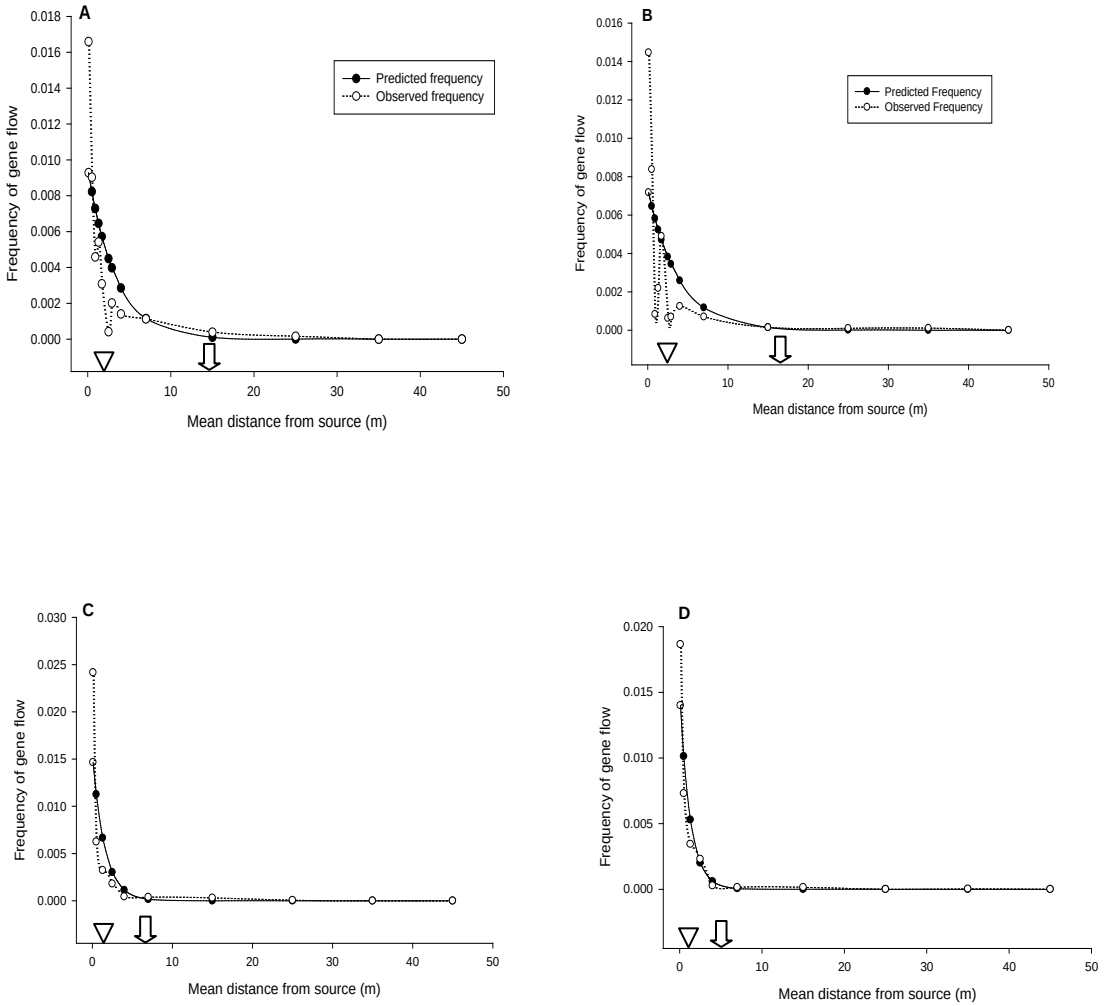

Figure 4-2 Pollen mediated gene flow in flax at various locations and years (A) Edmonton Research Station (EdRS), 2006; (B) Ellerslie Research Station (ElRS), 2006; (C) Edmonton Research Station (EdRS), 2007; and (D) Ellerslie Research Station (ElRS), 2007. The triangle indicates the distance, where 50% (O_{50}) reduction in the frequency of gene flow and the arrow indicates the distance where 90% (O_{90}) reduction in gene flow from the pollen source (m).

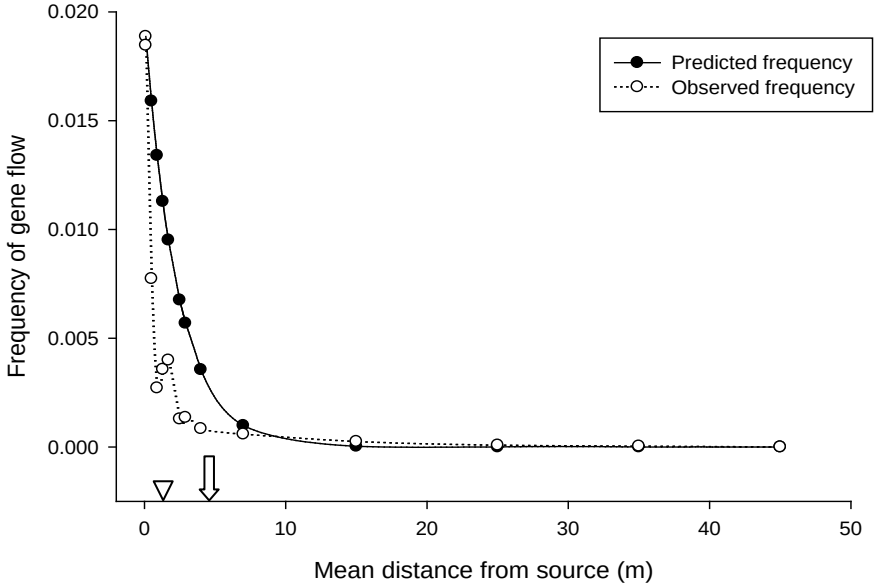

Figure 4-3 Pollen-mediated gene flow in flax (average of all locations and years). The triangle shape indicates 50% reduction in gene flow at particular distance (m) and the arrow indicates 90% reduction in gene flow at particular distance from the source (m).

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Chapter 5

Adventitious presence: Volunteer flax (*Linum usitatissimum* L.) in herbicide resistant canola (*Brassica napus* L.)[5]

Introduction

Since the commercialization of genetically engineered (GE) crops in 1996, the total area and number of countries growing GE crops has increased rapidly (Brookes and Barfoot 2008). In 2008, 25 countries cultivated GE crops on approximately 125 million hectares (James 2008). Many GE crops have been developed and commercialized, but the four most extensively grown GE crops are canola (*Brassica napus* L.), cotton (*Gossypium hirsutum* L.), maize (*Zea mays* L.), and soybean (*Glycine max* L.). Several other crops, including flax (*Linum usitatissimum* L.) are being considered for the development of novel bio-industrial products using genetic transformation technologies (Kymalainen and Sjoberg 2008; Moryganov et al. 2008)

Flax, also known as linseed, is an annual, eudicot oilseed crop. In temperate and subtropical countries, flax has been grown either for oil extracted from the seed, or for fiber extracted from stems. Flax is a well adapted crop in western Canada. In 2008, Canadian growers produced ~861 thousand tonnes of flaxseed from approximately 631 thousand hectares (Statistics Canada 2009) and exported about 675 thousand tonnes (AAFC 2009). Current research on medicinal applications, especially reducing the risk factors contributing to cardiovascular

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diseases (Bloedon and Szapary 2004) and cancer (Thompson et al. 2005) has opened an opportunity to use this oilseed crop for functional food applications (Fitzpatrick, 2007). Now flax can be found in cereals, dairy products, bakery products, prepared foods and snacks (Morris, 2007). The use of flaxseed in poultry diets for increasing the level of α-linolenic acid (ALA, 20:3$^{cis\Delta 9,12,15}$), an ω-3 fatty acid in eggs, has increased demand of flaxseed in North America (Jhala and Hall, 2009). It has been estimated that in 2005, nearly 200 new functional foods and personal care products based on flax or flax ingredients were introduced into the USA market (Morris 2007). Using its unique oil biosynthesis pathways, GE cultivars of flax are under development to supply the market demand for novel bioproducts (Sorensen et al. 2005; Jhala et al. 2009; Wrobel-Kwiatkowska et al. 2007).

While herbicide-resistant flax was one of the first GE crops to receive regulatory approval in Canada (McHughen et al. 1997), currently no GE flax cultivars are available on a commercial scale in Canada or elsewhere (Jhala et al. 2008). Consumer and political concerns about GE crops for food and animal feed continue to be pervasive in Europe and these concerns could effectively block future GE crop development (Demeke et al. 2006; Devos et al. 2005). Although currently released GE crops and their products are considered substantially equivalent to conventional crops in the USA and Canada, if GE flax is to be cultivated in western Canada, it must be segregated from conventional and organic flax to preserve these valuable markets in the European Union (EU).

Trust in GE crops has eroded in part because transgenes from GE crops

have been detected in non-GE feed and food products and have been widely reported in the media and in the scientific literature (Demeke et al. 2006; USDA/APHIS 2008). Unapproved transgenes are illegal in food or feed and detection has lead to serious economic consequences (Holst-Jensen 2008; Krueger and Le Buanec 2008; Ramessar et al. 2008). For example, GE maize intended to produce a specialty pharmaceutical protein was found as crop volunteers in a soybean field grown in rotation in Nebraska (USDA/APHIS 2008). These GE maize volunteers were subsequently harvested with soybean and were transported to storage and mixed with a half-million bushels of stored soybeans (Ellstrand 2003). Since Prodigene Inc. failed to confine the GE maize, the company received a fine of $250,000 but the United States Department of Agriculture (USDA) was required to buy and destroy the contaminated soybean at an approximate cost of $ 3.5 million (USDA/APHIS 2008). This has led to changes in government regulatory policies and resulted in more stringent confinement procedures for field experiments and commercialization of GE crops.

The presence of GE seed can occur through pollen-mediated gene flow between conventional and GE crops and seed handling in the commodity system (Ellstrand et al. 1999; Hall et al., 2000). For crops that have low rates of out-crossing and thus limited pollen-mediated gene flow, volunteer seed production may be the most likely source of AP (Devos et al., 2009). Information about gene dissemination by seeds and volunteers has been compiled for some important GE crops (Gressel, 2005). A study of post harvest gene movement for GE canola, maize, sugar beet (*Beta vulgaris* L.) and wheat (*Triticum aestivum* L.)

suggest that all species may disseminate transgenes via crop volunteers (Gruber et al., 2008).

Genetically engineered crop volunteers that emerge in subsequent crops can also be a significant agronomic concern (Beckie, 2001; Beckie and Owen, 2007). They are considered weeds because they compete with crops for nutrients, moisture, space and light (Blackshaw et al., 2005; O'Donovan et al., 2007); and thereby reduce crop yield, quality and may also interfere in harvest operations (O'Donovan et al., 2005; O'Donovan et al., 2007; Williams and Boydston, 2006). Herbicide-resistant volunteers may become more problematic and difficult to control if a crop with the same trait is planted in rotation. For example, soybean is grown in rotation with cotton in the southeast states of the USA. Volunteer soybean in cotton became a problem with the commercialization of glyphosate resistant soybean and cotton, because glyphosate was ineffective for the control of glyphosate-resistant soybean volunteers (York et al., 2005). Similar problems have been observed in Ontario, Canada for controlling glyphosate resistant volunteer maize in maize-soybean cropping systems after the commercialization of glyphosate resistant traits in both crops (Deen et al., 2006).

Where flax is grown, it volunteers in subsequent crops (Leeson et al., 2005). A survey conducted in Manitoba suggested that relative abundance of volunteer flax has increased from 2.0 to 15.3 in last two decades (Thomas et al., 1997). An understanding of the biology of volunteer flax and the agronomic practices which mitigate its occurrence in agro-ecosystems is essential to the reduction of seed-mediated gene flow and persistence of transgenes in the

environment.

Flax is usually grown in rotation with cereals to interrupt cereal disease cycles (Wall and Smith, 1999), however, farmers may also grow canola after flax in western Canada (Dexter et al., 2006). Few pre-emergence (PRE) and post-emergence (POST) herbicides have been registered for control of volunteer flax in cereals in Canada (Brook 2008). Three herbicide-resistant canola systems: glyphosate, glufosinate and imidazolinone have been rapidly adopted by Canadian growers and now comprise > 90% of canola acres (Buth, 2007). Volunteer flax may be a significant weed problem in canola, if not controlled.

Best management practices have been introduced to reduce vertical movement of transgenes in the environment (Devos et al., 2004). Uncontrolled GE crop volunteers are major routes of seed-mediated gene flow. An integrated weed management strategy is required to effectively control volunteer flax and reduce AP in the crops grown in rotation with flax. In addition to other cultural practices and tillage, chemical control of volunteer flax may be an effective method of reducing the risk of seed-mediated gene flow. Currently there is no information available on control of volunteer flax in canola, volunteer seed production, or potential AP. Therefore, the objectives of this study were:

- To determine the potential of PRE or POST herbicides used alone or in combination for mitigating AP of volunteer flax in glufosinate-resistant (GR) and imidazolinone-resistant (IR) canola
- To evaluate the viability of uncontrolled volunteer flaxseed affected by crop competition or weed management practices

- To quantify the amount of AP (w/w) of volunteer flax in GR and IR canola under herbicides treated and untreated conditions

Materials and Methods

Two separate field experiments were conducted for GR (cv. Invigor 5030) and IR (cv. 45H73-CL) canola systems in 2007 at one location in Edmonton, Alberta, Canada; and in 2008 at three locations in Edmonton, Ellerslie, and St Albert, Alberta, Canada. Soil texture at Edmonton was a clay loam consisting of 34% sand, 37% silt, and 28% clay with a pH of 5.6 and 13% organic matter. Soil texture at Ellerslie was a clay loam with 28% sand, 41% silt and 31% clay with a pH of 6.5 and 11% organic matter. The soil texture at St Albert was a clay loam and consisted of 23% sand, 40% silt, and 36% clay with a pH of 7.6 and 13% organic matter. The experiments at all the sites and years were established in areas that had not been seeded to flax for at least five years.

To simulate volunteer flax infestations, flax cv. CDC Bethune was seeded at a rate of 12.2 kg/ha with the target population of 150 plants/m^2 (based on survey of volunteer flax emergence in western Canada, Dexter, 2009) in row spacing of 20 cm using a low disturbance airseeder[1] at a depth of 2 to 3 cm (Table 5-1). A light tillage operation followed immediately after to incorporate the seeds. Flax plants were allowed to emerge and Pre-plant herbicide was applied when flax plants were at least at the 3-leaf stage (Table 5-1). After herbicide application, either canola cv. Invigor 5030 (glufosinate[2] resistant canola) or 45H73-CL (imidazolinone-resistant canola) were seeded perpendicular to the flax seeding direction using a low disturbance airseeder at a target population of 160 plants/m^2

and with a row spacing of 20 cm. Canola seeding dates were delayed at all the locations compared to recommended timings for this region because of the need to establish volunteer flax populations and PRE herbicide applications (Table 1). Fertilizer rates for canola were based on soil test recommendations for each site-year (data not shown).

The plots were 2.0 m wide by 8.5 m long and were arranged in a completely randomized block design (CRBD) with 9 treatments (9 for GR and IR canola each) and four replications at all the locations and years (Table 1). In GR canola, treatments consisted of PRE application of glyphosate[3] at the recommended rate (1.25 kg ae/ha); the sole application of glufosinate applied POST at three application rates (150, 300, and 600 g ai/ha); and a combination of glyphosate (Pre-plant) at recommended rate (1.25 ae kg/ha) followed by glufosinate (POST) at three application rates (150, 300, and 600 g ai/ha). Weed-free plots were maintained by removing all weeds (including volunteer flax) by hand weeding at each location and untreated control plots were left uncontrolled. Similarly, nine treatments were applied in IR canola, except POST treatments were imazamox+imazethapyr[4] was applied POST at three application rates (10.5, 21, and 42 g ai/ha) alone; and as POST at three application rates (10.5, 21, and 42 g ai/ha) in combination after PRE application of glyphosate (1.25 kg ae/ha). The adjuvant Merge[5] was mixed with imazamox+imazethapyr at a final concentration of 0.5 L/100 L of spray solution (0.5% v/v). Glyphosate was applied when the flax plants were 6-8 cm in height and the third pair of leaves was unfolded in GR and IR canola. Glufosinate (in GR canola) and imazamox+imazethapyr (in IR

canola) were applied POST when canola plants were 3-5 leaf stage and volunteer flax plants were about 20 to 30 cm in height with > 20 leaves. Herbicides were applied with a self-propelled, high clearance Spider sprayer[6] equipped with Teejet XR 110015 nozzles[7] delivering 100 L/ha at 214 kPa.

Volunteer flax densities were assessed during the growing season within pre-established 0.25 m^2 quadrats (3 quadrats/plot), after PRE herbicide treatments and at harvest (Table 1). Volunteer flax that survived herbicide treatments in pre-established quadrats were cut at the stem base close to the soil surface, placed in paper bags, dried at room temperature for a week and dry weight (g) of volunteer flax was recorded. Flax seed bolls were threshed by hand and weight of seed (g/m^2) was recorded and seeds tested for viability (see below) after counting individual seed. Canola biomass was also determined in established 0.25 m^2 quadrats by cutting the plants near the soil surface and by drying for 72 h at 60 C and biomass weight was recorded. Plots were harvested at maturity (Table 1) and seeds were dried to uniform moisture content for 72 h at 62 C. The seeds of volunteer flax and canola were separated from the harvested admixture from the each plot and site and the recovered seed was weighed (g) and used to determine the seed yields (kg/ha) of volunteer flax and canola. AP of volunteer flax seed in harvested canola seed was determined by using the following formula and expressed as the percentage (w/w) of volunteer flax AP in harvested canola,

$$\text{Volunteer flax AP (\%)} = Wf / Wc \times 100$$

Where, AP is the adventitious presence of volunteer flax in canola expressed in percent, *Wf* is the weight of flaxseed (g), *Wc* is the weight of canola

seed (g).

Volunteer flax seed viability test.

To determine flax seed viability, seeds from volunteer flax were collected from plants that survived herbicide treatments. These plants were hand harvested from the fixed quadrats (three 0.25 m^2 quadrats/plot) and the seed capsules were threshed by hand. A sub-sample of 300 seeds from each quadrat of harvested flax volunteers (if available) were randomly selected and further divided into three replications of 100 seeds each, to be replicated in time over three consecutive days. Seeds were placed in acrylic germination boxes[8] (24 x 16 x 3.8 cm) and lined with 15x23 cm absorbent blue filter paper[9] to prevent the seeds from drying out. To reduce the fungal growth, the fungicide Helix Xtra[10] was added to each germination box at a concentration of 0.2% (40 ml/box). The germination trays were stored in the dark at ambient temperatures for 72 h to induce germination. Seeds were considered to have germinated when the radicle emerged through the seed coat. The weathered and moldy seeds were considered dead and they were counted and removed from the germination boxes. Non-germinated seeds were transferred to petri dishes[11] lined with white filter paper[12] equivalent to Whatman No. 1 and moistened with 5.0 ml of 0.005 M gibberellic acid[13] (GA_3) solution. After 72 h on the GA_3 solution, the number of seeds that did and did not germinate were recorded. Germinated seeds were considered to be viable and non-germinated seeds were considered to be non-viable.

Statistical analysis

Data were analyzed separately for GR and IR canola systems. All data were

subjected to analysis of variance (ANOVA) using the general linear models procedure of statistical analysis software (SAS[14] Institute 2007). Normality, homogeneity of variance; and interactions of treatment, year, and locations were tested. In this experiment, year by treatment and treatment by location interactions were non-significant, therefore, the data of all four locations were pooled and combined data were presented. Volunteer flax density at various stages, flax and canola biomass, flax seed production, canola and volunteer flax yield and AP (%) of volunteer flax were analyzed using ANOVA within RCBD in SAS (SAS Inc. 2007). To meet assumptions of variance analysis; the data of volunteer flax density and dry weight were log transformed prior to analysis. Because of zero values in data, one was added to each observation value prior to transformation to avoid the problem of log of zero as explained in Little and Hills (1978). However, non-transformed data were presented with statistical interpretation based on transformed data. Where the ANOVA indicated that treatment effects were significant, means were separated at $P \leq 0.05$ lsmeans and adjusted with Fisher's Protected Least Significant Difference (LSD) test

Results and Discussion

Volunteer Flax Control

In GR canola, glyphosate applied PRE at the recommended rate (1.25 kg ae/ha) reduced volunteer flax densities from 55 to 3 plants/m^2, 12 days following application (Table 2). Densities of volunteer flax at harvest were reduced by glufosinate applied POST from 11 to 2.5 plants m^{-2} for low (300 g ai/ha) and recommended rates (600 g ai/ha), respectively, compared to the untreated control (44 plants/m^2) (Table 2). The combined application of pre-plant glyphosate at 1.25 kg ae/ha followed by POST application of glufosinate at reduced (150 or 300 g ai/ha) or recommended rates (600 g ai/ha) reduced volunteer flax densities to < 2 plants/m^2 in GR canola (Table 2). For reducing seed-mediated gene flow by crop volunteers from canola, maize, sugar beet and wheat, Gruber et al. (2008) reported that PRE herbicides followed by POST applications were most effective for reducing densities of GE crop volunteers. Tillage operations may also aid in control herbicide-resistant crop volunteers.

Volunteer flax dry weight was also influenced by application of PRE and POST herbicides and their combinations in comparison to the untreated control in GR canola (Table 2). Highest dry weight of volunteer flax was recorded in the untreated control (150 g/m^2) followed by a POST application of glufosinate at 150 g ai/ha (57.3 g/m^2), suggesting that glufosinate alone at the reduced rate was not effective for reducing volunteer flax density and dry weight (Table 2). Dry weight of volunteer flax was reduced by a PRE application of glyphosate alone (3.3 g/m^2) and the POST application of glufosinate alone at 600 g ai/ha (4 g/m^2). PRE glyphosate (1.25 kg ae/ha)

followed by POST glufosinate at all the rates were effective for reducing volunteer flax dry weight to ~2 g/m^2 in GR canola.

Volunteer flax was not controlled well within the IR canola system. Imazamox+imazethapyr at any dosage (10.5, 21 or 42 g ai/ha) did not reduce the density and dry weight of volunteer flax in IR canola, suggesting that flax has natural tolerance to POST imazamox+imazethapyr applied at reduced or recommended rates. Flax has also been reported to tolerate foliar applications of some POST sulfonylurea herbicides (Wall and Kenaschuk 1996), although soil residues may injure juvenile flax plants (Friesen and Wall 1991; McHughen and Holm 1995). When glyphosate was applied at 1.25 kg ae/ha, the density of volunteer flax at harvest was reduced to 2 plants/m^2 compared to untreated plots (48 plants/m^2) regardless of imazamox+imazethapyr treatment. PRE application of glyphosate at recommended rate (1.25 kg ae/ha) was required to reduce volunteer flax density.

In summary, PRE glyphosate (1.25 kg ae/ha) followed by POST glufosinate at 600 g ai/ha were most effective for reducing volunteer flax population densities and dry weight in GR canola.

Crop response

All herbicide treatments increased GR canola biomass compared to untreated plots (Table 3). Interestingly, weed free plot did not have the highest canola biomass (784 g/m^2). This might be because of excellent control of volunteer flax by glyphosate applied PRE, remaining or later emerged volunteer flax were controlled by a POST application of the recommended rates of glufosinate (data not shown), and/or minor damage to canola plants during hand weeding in weed free plots. Canola biomass was similar for all

herbicide treatments, except when only glufosinate was applied POST at 150 g ai/ha (712 g/m^2).

The highest GR canola yields were recorded with pre-plant glyphosate alone or when followed by POST glufosinate at all the rates (2,787 to 2,929 kg/ha) and in fact also when glyphosate was applied alone (2,774 kg/ha) in compare to untreated plots (1,431 kg/ha) (Table 3). Field experiments conducted in western Canada have previously shown that to achieve high yields of canola, early weed removal is critical (Blackshaw et al. 2008; Harker et al. 2008).

Highest IR canola biomass (941 g/m^2) was recorded in the weed free treatment, followed by glyphosate applied PRE at 1.25 kg ae/ha (887 g/m^2), PRE glyphosate followed by POST imazamox+imazethapyr at 10.5 g ai/ha (865 g/m^2) and PRE glyphosate followed by POST imazamox+imazethapyr at 42 g ai/ha (825 g/m^2) (Table 3). IR canola yield increased with application of herbicides in compared to untreated plots (1,326 kg/ha), except for POST imazamox+imazethapyr alone applied at 42 g ai/ha (Table 3). Uncontrolled volunteer flax may reduce IR canola yields by 51%. In a similar experiment in wheat, Wall and Smith (1999) reported that uncontrolled volunteer flax may reduce wheat yields up to 27% (Table 3).

Volunteer flax seed production, viability, yield and AP

Volunteer flax seed production in GR canola measured within established quadrats of untreated controls was 32 g/m^2 or 5,963 seeds/m^2 (Table 4). Volunteer seed production may have been influenced by the experimental conditions in which the flax was allowed to emerge prior to seeding the canola crop. Volunteer flax seed yield was dramatically reduced by

all herbicides (Table 4). Unlike volunteer flax population density and dry weight, volunteer flax seed production and yield were not affected by increasing glufosinate rates in GR canola. In IR canola, flax volunteers produced 30 g/m^2 or 5,571 seeds/m^2 in untreated plots (Table 4). Pre-plant glyphosate alone effectively in reduced volunteer flax seed production to 472 seeds/m^2 in IR canola.

Herbicides can cause mortality, reduce plant growth and delay maturity of weeds and thus influence seed viability as well as fecundity (McPherson et al., 2009). In untreated plots, viability of volunteer flaxseed averaged 69.6% and reflects the later maturity of flax compared to GR canola, even when planted earlier. Viability was reduced to about 11% by pre-plant glyphosate alone (Table 4). Increasing POST glufosinate doses decreased seed viability from 26 to 7%. The use of both PRE followed by POST herbicides reduced viability to < 3% (Table 4). Volunteer flax plants were stunted by herbicide treatments and thus they produced smaller, malformed seeds.

Seed viability in untreated controls was 75% in IR canola and when glyphosate was applied pre-plant alone, this was reduced to 20%. Imazamox+imazethapyr applied POST also reduced volunteer flax viability (Table 4). The combination of both treatments (pre-plant followed by POST) reduced viability in the range of 17 to 25% in IR canola. While the reduction in seed viability has implications on propagule number, the ability of volunteer populations to perpetuate and the potential initiation of inadvertent populations following seed loss, viability may not influence the AP, depending on the methods of quantifying AP (Gaines et al., 2007; Jorgensen et al., 2007).

Uncontrolled or escaped volunteer GE flax may be harvested with subsequent crops and may contribute to AP. Uncontrolled volunteer flax that emerged prior to seeding canola produced 321 kg/ha flax seeds which may contribute 26.2% AP in harvested GR canola seeds (Table 4). Flax seed harvested with GR canola was variable. All the herbicides reduced admixture and AP in the range of 0 to 32 kg/ha in GR canola. However, when only pre-plant glyphosate was applied at 1.25 kg ae/ha, AP decreased to as low as 0.5% suggesting that pre-plant glyphosate was very effective in reducing AP in GR canola. When pre-plant glyphosate was followed by POST glufosinate, AP was 0.1 to 0.0% (Table 4).

Volunteer flax yield and AP were also reduced when herbicides were applied in IR canola. Glyphosate applied pre-plant alone reduced volunteer flax seed yield to 27 kg/ha and resulted in AP of 1.1% in compared to 301 kg/ha and 25.5% AP in untreated plots (Table 4). Because the POST application of imazamox+imazethapyr alone was not effective for reducing the density and dry weight of volunteer flax (Table 2), the results were also reflected in volunteer flax seed yields which was in the range of 179 to 195 kg/ha and AP 13 to 15%, when imazamox+imazethapyr was applied POST alone at different rates (Table 4). However, the pre-plant treatment of ghyphosate followed by POST imazamox+imazethapyr at 21 g ai/ha reduced flax yield and AP to 13 kg/ha and < 1%, respectively in IR canola. Single pre-plant application of glyphosate reduced volunteer flax AP in both the canola systems (0.5 and 1.1% in GR and IR canola, respectively).

Canada is the largest producer and exporter of flaxseed in the world. Canada exports > 80% of domestically produced flaxseed mainly to the

European Union (EU), Japan, Korea and the USA every year (Flax Council of Canada 2007). Concerns regarding volunteerism, ferality and AP have increased with expanding area and production of GE crops (Gressel 2005). Crops are grown in an open system where complete isolation is not possible. To facilitate co-existence of GE, conventional and organic crop production systems, threshold levels are required (Weber et al. 2007). Compliance with international standards is complicated because various countries have different threshold levels for AP of GE seeds in non-GE crops/seeds. The EU has established a labeling threshold level of 0.9% (Devos et al. 2009). Japan and South Korea have a 5 and 3% tolerance limit, respectively (Demeke et al. 2006). In addition, international standard testing methods for AP of GE seeds have yet to be resolved. Non-viable seeds, while unable to propagate, may register as GE. To avoid market risk of flaxseed export, especially to the EU, Canadian growers will be required to adopt best management practices to allow GE flax production to co-exist with commodity or organic flax production and in other crops grown in rotation with flax.

These experiments represent a worst-case scenario for volunteer flax AP in herbicide-resistant canola. Flax populations were seeded prior to canola at a target population of 150 plants/m^2. Seeded flax emerged early and uniformly, enhancing seed production potential. Volunteer populations under the natural field conditions may emerge over a long period of time, frequently after the crop has emerged (personal observation). Canola is usually seeded early and a delay in flax emergence may reduce relative competitive ability. Neverthless, when assessing risk, worst-case scenarios provide valuable information to decision makers.

This is the first report quantifying and mitigating volunteer flax AP in two types of herbicide-resistant canola. With effective control of volunteer flax in GR canola, AP was reduced. However, the IR canola system did not reliably reduce AP and therefore, growers in western Canada are advised not to grow IR canola in the year following GE flax production. Other imidazolinone-resistant crops [for example IR lentil (*Lens culinaris* L.), pea (*Pisum sativum* L.), wheat (*Triticum aestivum* L.)], in rotation with flax may have similar concerns. PRE application of glyphosate at the recommended rate provided better canola yields in both canola systems by reducing volunteer flax density and therefore reducing cop-weed competition. Effective pre-seeding control of volunteer populations is suggested to increase control and reduce potential AP.

This study demonstrated that when proper mitigation strategies were adopted, AP of volunteer flax in canola can be reduced to a great extent. Effective herbicides applied the year following GE flax production would control flax volunteers, and reduce pollen and seed-mediated gene flow from GE volunteer flax. Herbicides are one component of the best management system approach. Other practices include: reducing harvest loss by properly adjusting combine settings, adopting isolation distances between GE and organic flax fields, diversification of crop rotations, cleaning of equipment and separate supply chains for GE and organic flax. This information will be useful to the flax industry, growers and regulators for policy development and risk assessment for potential commercial release of GE flax. Integrated management of currently available (herbicide and insect-resistant GE crops) and future GE crop volunteers (abiotic stress-tolerant and crops for

biopharmaceuticals) will be required in order to reduce AP in subsequent crops and minimize impacts on market and international trade.

Sources of Materials

[1]Fabro Enterprises Ltd., 2545, North Service, Rd (W), Swift Current, Saskatchewan, S9H 5L3, Canada.

[2]Glufosinate, Liberty Link®, herbicide, Bayer Canada, 77 Belfield Road, Toronto, Ontario, M9W 1G6, Canada.

[3]Weathermax®, herbicide, Monsanto Canada, 900 - One Research Road, Winnipeg, Manitoba R3T 6E3, Canada.

[4`]Imazamox+imazethapyr®, herbicide Odyssey, 100 Milverton Drive, 5th Floor, Mississauga, Ontario, L5R 4H1, Canada.

[5]Merge®, surfactant blend + solvent (petroleum hydrocarbons), BASF Canada, 100 Milverton Drive, Mississauga, Ontario, L5R 4H1, Canada.

[6]Fabro Enterprises Ltd., 2545, North Service, Rd (W), Swift Current, Saskatchewan, S9H 5L3, Canada.

[7]Max-Quip, 11423-163 Street, Edmonton, Alberta T5M 3Y3, Canada.

[8,9,11,12]Hoffman Manufacturing, Inc, 16541 Green Bridge Road, Jefferson, OR 97352-9201, USA

[10]Helix XTra™, Insecticide with fungicides (thiamethoxam, difenoconazole, mefenoxam, fludioxonil) Syngenta Crop Protection Canada, Inc. Suite 300, 6700 Macleod Trail South, Calgary, Alberta, T2H 0L3, Canada.

[13]Gibberellic acid, Sigma-Aldrich Corp., P.O. Box 14508, St. Louis, MO 63178, USA.

[14]Statistical Analysis Systems, The SAS systems for windows, SAS Institute Inc., P.O. Box 8000, Cary, NC 27512, USA.

Table 5-1 Dates of pre-plant and post-emergence herbicides sprayed and agronomic operations conducted at various locations in 2007 and 2008.

Operations	2007	2008		
	Edmonton	Ellerslie	Edmonton	St Albert
Flax cv. CDC Bethune seeded	April 29	May 5	May 16	May 8
Pre-plant herbicide applied	May 22	May 29	June 2	June 5
GR and IR canola seeded	May 24	May 29	June 2	June 5
POST herbicides applied	June 26	June 23	June 24	June 27
Volunteer flax counts after PRE herbicide application	June 2	June 10	June 13	June 17
Volunteer flax counts at harvest	Aug. 28	Sep. 25	Sep. 27	Sep. 28
Volunteer flax and canola biomass cut	Aug. 28	Sep. 25	Sep. 27	Sep. 28
Canola harvest	Sep. 27	Oct. 9	Oct. 9	Oct. 15

Table 5-2 Volunteer flax density and dry weight in glufosinate- and imidazolinone-resistant canola as influenced by herbicide treatments.[b,c]

Treatment	Application timing	Flax density after Pre-plant[d]	Flax density at harvest[d]	Flax dry weight[d]
Glufosinate resistant Canola		plants/m^2	plants/m^2	g/m^2
Weed free	-	1.0 *d*	1.0 *d*	1.0 *e*
Untreated	-	54.6 *a*	44.3 *a*	150 *a*
Glyphosate (1.25 kg ae/ha)	PRE	3.2 *b*	1.8 *cd*	3.3 *d*
Glufosinate (150 g ai/ha)	POST	61.1 *a*	31.7 *a*	57.3 *b*
Glufosinate (300 g ai/ha)	POST	65.5 *a*	11.0 *b*	15.7 *c*
Glufosinate (600 g ai/ha)	POST	45.5 *a*	2.5 *c*	4.0 *d*
Glyphosate (1.25 kg ae/ha) *fb*[a] Glufosinate (150 g ai/ha)	PRE/POST	1.6 *cd*	1.5 *cd*	2.1 *de*
Glyphosate (1.25 kg ae/ha) *fb* Glufosinate (300 g ai/ha)	PRE/POST	1.9 *c*	1.0 *d*	1.0 *e*
Glyphosate (1.25 kg ae/ha) *fb* Glufosinate (600 g ai/ha)	PRE/POST	2.1 *bc*	1.2 *d*	1.0 *e*
Imidazolinone-resistant Canola				
Weed free	-	1.0 *c*	1.0 *b*	1.0 *c*
Untreated	-	55.7 *a*	48.3 *a*	175 *a*
Glyphosate (1.25 kg ae/ha)	PRE	2.1 *b*	2.0 *b*	2.7 *b*
Imazamox+ imazethapyr (10.5 g/ha)	POST	67.1 *a*	61.7 *a*	205 *a*
Imazamox+ imazethapyr (21 g/ha)	POST	56.8 *a*	51.4 *a*	169 *a*
Imazamox+ imazethapyr (42 g/ha)	POST	66.2 *a*	43.9 *a*	149 *a*
Glyphosate (1.25 kg ae/ha) *fb* Imazamox+ imazethapyr (10.5 g/ha)	PRE/POST	1.9 *b*	2.0 *b*	2.9 *b*
Glyphosate (1.25 kg ae/ha) *fb* Imazamox + imazethapyr (21 g/ha)	PRE/POST	1.6 *b*	1.7 *b*	2.2 *bc*
Glyphosate (1.25 kg ae/ha) *fb* Imazamox+ imazethapyr (42 g/ha)	PRE/POST	2.1 *b*	1.4 *b*	1.9 *bc*

[a] Abbreviation: *fb*, followed by; NA, not applicable

[b] each data represents a pooled value over locations and years

[c] the data was log transformed for homogenous variance prior to analysis; non-transformed data are presented in this table with statistical interpretation based on transformed data. One was added to each data prior to transformation which is reflected in the values of weed free treatment

[d] Least square means within columns with no common letters are significantly different according to Fisher's Protected LSD test where $P \leq 0.05$

Table 5-3 Canola biomass and yield as influenced by herbicide treatments[b].

Treatment	Application timing	Canola biomass[c]	Canola seed yield[c]
Glufosinate resistant Canola		**g/m^2**	**kg/ha**
Weed free	-	784 *bc*	2,826 *ab*
Untreated	-	517 *d*	1,431 *e*
Glyphosate (1.25 kg ae/ha)	PRE	871 *ab*	2,774 *ab*
Glufosinate (150 g ai/ha)	POST	712 *c*	2,365 *d*
Glufosinate (300 g ai/ha)	POST	827 *abc*	2,501 *cd*
Glufosinate (600 g ai/ha)	POST	842 *abc*	2,648 *bc*
Glyphosate (1.25 kg ae/ha) *fb*[a] Glufosinate (150 g ai/ha)	PRE/POST	934 *a*	2,787 *ab*
Glyphosate (1.25 kg ae/ha) *fb* Glufosinate (300 g ai/ha)	PRE/POST	896 *ab*	2,864 *ab*
Glyphosate (1.25 kg ae/ha) *fb* Glufosinate (600 g ai/ha)	PRE/POST	960 *a*	2,929 *a*
Imidazolinone-resistant Canola			
Weed free	-	941 *a*	2,654 *a*
Untreated	-	416 *d*	1,326 *d*
Glyphosate (1.25 kg ae/ha)	PRE	887 *a*	2,349 *b*
Imazamox+imazethapyr (10.5 g ai/ha)	POST	602 *c*	1,677 *c*
Imazamox+imazethapyr (21 g ai/ha)	POST	656 *c*	1,569 *cd*
Imazamox+imazethapyr (42 g ai/ha)	POST	546 *cd*	1,777 *c*
Glyphosate (1.25 kg ae/ha) *fb* Imazamox+imazethapyr (10.5 g ai/ha)	PRE/POST	865 *ab*	2,341 *b*
Glyphosate (1.25 kg ae/ha) *fb* Imazamox+imazethapyr (21 g ai/ha)	PRE/POST	702 *bc*	2,381 *ab*
Glyphosate (1.25 kg ae/ha) *fb* Imazamox+imazethapyr (42 g ai/ha)	PRE/POST	825 *ab*	2,282 *b*

[a] Abbreviation: *fb*, followed by

[b] each data represent a pooled value over locations and years

[c] Least square means within columns with no common letters are significantly different according to Fisher's Protected LSD test where $P \leq 0.05$

Table 5-4 Volunteer flax seed production, seed viability and adventitious presence in glufosinate- and imidazolinone-resistant canola as influenced by herbicide treatments.[b]

Treatment	Application timing	Volunteer flax[c]		Seed viability[c]	Yield[c]	AP[a,c,d]
		g/m^2	seeds/m^2	%	kg/ha	%
Glufosinate resistant Canola						
Weed free	-	0.0 *b*	0 *b*	0.0 *e*	0 *b*	0.0 *b*
Untreated	-	31.9 *a*	5,963 *a*	69.6 *a*	321 *a*	26.2 *a*
Glyphosate (1.25 kg ae/ha)	PRE	1.2 *b*	233 *b*	10.9 *cd*	17 *b*	0.5 *b*
Glufosinate (150 g ai/ha)	POST	3.2 *b*	590 *b*	25.8 *b*	30 *b*	1.5 *b*
Glufosinate (300 g ai/ha)	POST	1.4 *b*	261 *b*	14.6 *c*	18 *b*	0.5 *b*
Glufosinate (600 g ai/ha)	POST	0.5 *b*	92 *b*	7.4 *cde*	7 *b*	0.2 *b*
Glyphosate (1.25 kg ae/ha) *fb*[a] Glufosinate (150 g ai/ha)	PRE/ POST	0.2 *b*	37 *b*	2.1 *de*	4 *b*	0.1 *b*
Glyphosate (1.25 kg ae/ha) *fb* Glufosinate (300 g ai/ha)	PRE/ POST	0.1 *b*	8 *b*	0.5 *e*	1 *b*	0.1 *b*
Glyphosate (1.25 kg ae/ha) *fb* Glufosinate (600 g ai/ha)	PRE/ POST	0.0 *b*	0 *b*	NA[a]	0 *b*	0.0 *b*
Imidazolinone-resistant Canola						
Weed free	-	0.0 *c*	0 *c*	0.0 *d*	0 *c*	0.0 *c*
Untreated	-	29.8 *a*	5,571 *a*	74.8 *a*	301 *a*	25.5 *a*
Glyphosate (1.25 kg ae/ha)	PRE	2.5 *c*	472 *c*	20.2 *c*	27 *c*	1.1 *c*
Imazamox+imazethapyr (10.5g/ha)	POST	19.2 *b*	3,594 *b*	58.0 *b*	194 *b*	13.0 *b*
Imazamox+imazethapyr (21 g/ha)	POST	18.1 *b*	3,393 *b*	64.2 *ab*	179 *b*	14.8 *b*
Imazamox+imazethapyr (42 g/ha)	POST	19.3 *b*	3,613 *b*	56.7 *b*	195 *b*	14.7 *b*
Glyphosate (1.25 kg ae/ha) *fb* Imazamox+imazethapyr (10.5g/ha)	PRE/POST	2.3 *c*	431 *c*	18.7 *c*	26 *c*	1.0 *c*
Glyphosate (2.32 L ha^{-1}) *fb* Imazamox+imazethapyr (21 g/ha)	PRE/POST	1.5 *c*	283 *c*	17.0 *c*	13 *c*	0.7 *c*
Glyphosate (1.25 kg ae/ha) *fb* Imazamox+imazethapyr (42 g/ha)	PRE/POST	2.5 *c*	457 *c*	25.4 *c*	27 *c*	1.2 *c*

[a] Abbreviation: AP, adventitious presence; *fb*, followed by; NA, not applicable

[b] each data represent a pooled value over locations and years

[c] Least square means within columns with no common letters are significantly different according to Fisher's Protected LSD test where $P \leq 0.05$

[d] AP of volunteer flax was calculated from the formula given in text

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Chapter 6

Genetically engineered flax (*Linum usitatissimum* L.): Potential benefits, risks, regulations and mitigation of transgene movement[6]

Introduction

Flax, *Linum usitatissimum* L., also known as linseed is the third most important oilseed crop after canola (*Brassica napus* L.) and soybean (*Glycine max*) in Canada. Although the genus *Linum* is composed of approximately 230 species, cultivated flax is the only species of economic importance (Rowland et al., 1995). It is one of the oldest plants cultivated for fiber and oil (De Candolle, 1904). Archaeological evidence of flaxseed and flax based products dates back to 5500-5000 BC at Tepe Sabz, Iran; 5800-5600 BC at Telles Sawwan, Iraq; and 8050-7542 BC at Tell Mureybat (Gill, 1987). The use of flax for food, fiber and medicine is well established (Lee, 2003).

Flax is an annual, inbreeding plant species grown on almost all continents. Flax varieties are distinguished as fiber flax (for bast fiber) and linseed (for seed oil). Flax fiber is used in the textile industry for linen cloth and also in the paper and pulp industry to make paper products including cigarette paper (Belonogova and Raldugina, 2006). Traditionally, linseed oil was obtained by extraction of seed with an organic solvent (Dillman, 1953). Currently, flax oil is used for manufacturing varnishes, paints, printing ink,

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linoleum, oilcloths and plastics (Rowland et al., 1995). The fiber from these varieties may also be useful for bio-product applications such as geotextiles and insulation (Anonymous, 2006; Kymäläinen and Sjöberg, 2008).

The five major flax producing countries are Canada, China, India, the USA and the European Union (EU) (Figure 6-1) (FAOSTAT data, 2007). World linseed production has ranged from two to three million tonnes per year for the last several years (AAFC, 2005). Canada is the world leader in the production and export of linseed (Flax Council of Canada, 2007). Canada produced ~39% of the world's total flax output in 1997 (AAFC, 1997). Production of flax in the last five years in Canada fluctuated annually depending on the markets (Figure 6-2). In 2007, world linseed production was ~2.3 million tonnes, with Canada accounting for ~29% (0.67 million tonnes) of this production (FAOSTAT data, 2007).

It is a challenge for flax breeders to develop flax cultivars to meet the requirements and demands of the changing markets and environment (Diederichsen, 2007). The introduction and development of recombinant deoxyribonucleic acid (rDNA) technology has provided an additional gene pool not previously attainable through conventional plant breeding methods such as hybridization, mutagenesis and somaclonal variation to modify genomes and create genetic variability between and within species (McLaren, 2005). Many field crops containing single gene modifications conferring resistance to insects and herbicides have been commercialized (Miller and

Conko, 2005). The next generation of GE crops will likely involve complex, multigene traits for industrial and pharmaceutical markets (Stewart and Knight, 2005; CFIA, 2005).

Flax was among the first commodity crop species to be genetically engineered by recombinant DNA technologies (McHughen, 2002). It was also among the first plant species to be genetically engineered for imparting agronomic traits such as herbicide resistance (Jordan and McHughen, 1988; McHughen, 1989) and salt tolerance (McHughen, 1987). Flax has been transformed with resistance to several herbicides including glyphosate, glufosinate and sulfonylurea (McHughen, 2002). The only GE flax cultivar in the world, "CDC Triffid", resistant to sulfonylurea herbicide was considered for commercial release in Canada in 1998 (McHughen, 2002), but after commercialization for six years, it was deregistered at the request of the flax industry because of the EU's concern with importing GE flaxseeds. Canada exports most of its flaxseed (> 80%) to the EU, Japan, Korea and the USA (Flax Council of Canada, 2007). This traditional linseed export market has remained GE free, essentially excluding the use of genetic engineering to further improve the crop for fiber and other non-food applications.

As a minor crop, flax has not had benefited from intense breeding efforts and genetic engineering approaches to improve yield, increase competition with weeds, and decrease maturation time. In contrast, China, India and the Ukraine have recently adopted large-scale flax production strategies

(FAOSTAT data, 2007) and may become global competitors to the export market currently dominated by Canada. Recently, the fiber-flax growing region in the EU has been faced with the problem of low quality fiber which has led to a rapid decrease in the demand of this product and the area committed to growing the crop (Wrobel-Kwiatkowska et al., 2007a). Governments and the flax industry in Canada and abroad have been slow to invest in genomics, molecular biology, and genetic engineering to enhance the performance of this multipurpose crop.

Prior to and during the introduction of GE crops, many scientists expressed grave concerns about ecological risks associated with GE crops (Rissler and Mellon, 1996; Snow and Palma, 1997; Ellstrand et al., 1999; Stewart et al., 2003). For conventional crop cultivars developed by conventional plant breeding methods, gene flow and the potential for non-target effects were not a concern (Stewart and McLean, 2004) or was limited to maintain the genetic purity of breeder seeds (Hucl, 1996). In contrast, GE technology, especially after its commercialization in 1996, began to bring about concern with the general public and government regulatory agencies regarding possible environmental effects of these crops (Poppy, 2000; Wilkinson et al., 2003).

The issues surrounding GE crops can be divided into environmental; food and feed safety concerns; and issues of market access. While the former two issues can be addressed using science-based risk assessment tools, the

latter issue of market access can be intractable. Information on the potential benefits, risks, and regulations of GE flax may be useful to flax industry for the development and commercialization of GE flax for bio-industrial and nutraceutical markets. The objectives of this paper are to:

- discuss the history and current status of genetic engineering of flax,
- discuss the potential benefits and risks of growing GE flax commercially,
- provide information on the registration and regulation of GE flax in Canada, and
- describe the best management practices to mitigate transgene movement from GE flax to conventional, organic flax and wild or weedy species and their potential impact on environment.

History and current status of genetic engineering of flax

An *Agrobacterium*-mediated transfer system has been successfully used as a vector to transfer desired genes to flax (Hepburn et al., 1983; Mlynarova et al., 1994). Hepburn et al. (1983) reported that flax was susceptible to *Agrobacterium tumefaciens* induced crown gall infection. Herbicide resistance was the first GE agronomic trait in flax (Jordan and McHughen, 1988). A glyphosate resistant plant was derived by delivering the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene to flax hypocotyl tissue (Jordan and McHughen, 1988). In another study,

Agrobacterium-mediated gene transfer successfully incorporated chlorsulfuron resistance in flax (McHughen, 1989). When the GE flax progeny were grown in soil containing chlorsulfuron, in the greenhouse, the plants survived (McHughen, 1989). There was no significant difference in the overall agronomic performance of GE lines when grown in sulfonylurea-treated versus untreated soils in the field (Mchughen and Holm, 1991; McSheffrey et al., 1992).

The first GE flax cultivar, "CDC Triffid", received regulatory clearance in 1996 in Canada (McHughen, 2002). It was expected that CDC Triffid would provide a broadleaf cropping option to summer fallowing or continuous cropping to cereals in soils previously treated with sulfonylurea herbicides (McHughen et al., 1997). CDC Triffid was deregistered, however, in 2001 upon the request of the Flax Council of Canada and the Saskatchewan Flax Development Commission. A lot of the discussion surrounding this cultivar probably conjured up recollections of the science fiction novel *"The Day of the Triffids"* by Wyndham (1951) where man-eating plants wreak havoc and attempt to take over the world!

The transformation of flax with a phosphonothricin acetyltransferase *(PAT*) gene conferring tolerance to the non-selective herbicide glufosinate was attempted and field tested (McHughen and Holm, 1995). The particle gun bombardment system was also used for genetic engineering of flax (Wijayanto, 1998; Wijayanto and McHughen, 1999). The *GUS* (β-glucuronidase) reporter

gene was used to test different seed specific promoters in flax (Drexler et al., 2003). The results suggested that the β-ketoacyl-CoA synthase (*KCS*) gene and the gene encoding napin (a major storage protein in *Brassica napus* L.) would not be expressed at high enough levels to be useful promoters, but *USP* (encoding an unknown seed protein from *Vicia faba*) and *LeB4* (encoding a legumin protein from *Vicia faba*) promoters could be successfully used for heterologous gene expression in flax (Drexler et al., 2003).

In order to find an alternate source of antibiotic resistance genes, Lamblin et al. (2007) introduced the phosphomannose isomerase gene, *PMI*, as an alternative selectable marker for *Agrobacterium*-mediated transformation in flax. The results indicated that the PMI/mannose selection system could be successfully used for isolation of GE flax plants. Finally, expression of a cDNA encoding potato β-1,3-glucanase in flax improved the resistance of GE lines to *Fusarium oxysporum* and *Fusarium culmorum* (three fold higher) compared to non-transformed plants (Wrobel-Kwiatkowska et al., 2004).

Tissue culture-derived systems have played a critical role in the incorporation of genetic engineering approaches into germplasm improvement programs. Flax has a long history of being transformed by tissue culture techniques including regeneration from protoplasts, and hypocotyl-, cotyledon- and leaf-derived callus (Barakat and Cockling, 1983; 1985). Haploid plants of flax have been derived through microspore-derived culture (McHughen, 2002) and anther culture (Obert et al., 2004). These approaches

and their applications in flax have been discussed in detail (McHughen, 2002; Millam et al., 2005).

Quality traits in flax were improved utilizing new information on gene identification and molecular expression. Several methods have been compared for genetic transformation in fiber flax (Polyakov et al., 1998). An alternate antibiotic selection method involving the use of hygromycin B has been developed (Rakousky et al., 1999). In addition, non-GE methods including somaclonal variation (O'Conner et al., 1991) and mutagenesis to develop Solin flax cultivars have been developed and successfully implemented in flax breeding programs (Dribnenki et al., 1999; Green and Marshall, 1984; Green, 1986).

The market value of flax fibers strongly depends on their mechanical properties. To reduce lignin content, gene silencing, ribonucleic acid interference (RNAi) technologies were successfully employed to produce plants with modified elastic properties. A significant increase in the lignin precursor content and a reduction in the pectin and hemicellulose were obtained in GE lines (Wrobel-Kwiatkowska et al., 2007b), which may increase the extractability of fibers (Wrobel-Kwiatkowska et al., 2007b).

The biotechnological production of bio-plastics, including polyhydroxybutyrate (PHB) and polyhydroxyalkanoate (PHA), has been explored in both micro-organisms and plants (Suriyamonglok et al. 2007). To produce biodegradable composites, flax was transformed with bacterial genes

that encoded enzymes catalyzing the formation of PHB (Wrobel-Kwiatkowska et al., 2007b). The protocol resulted in a modification of the mechanical properties of the stem wherein PHB accumulated in growing fiber cells. This study has paved the way for the large-scale production of biodegradable composites in the future. Recent developments in plant genomics, the availability of microarray technology and development of metabolomics technology will soon make it possible to understand the complex relationship between genes and flax fiber quality (Kymäläinen and Sjöberg, 2008).

Although several genetic modifications have been attempted in agronomic and other value-added traits in flax, no GE cultivars of flax are currently available for commercial production.

Potential benefits of GE flax

With unique oil and fiber properties, flax is considered as a model plant species for multipurpose use with whole plant utilization. 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 GE flax for quality traits has been developed (Ebskamp, 2002; Kymäläinen and Sjöberg, 2008). Agronomic limitations of flax production may also be minimized by developing GE flax cultivars. The potential benefits of flax are discussed in detail below.

Increased agronomic performance

Flax is a poor competitor with weeds (McHughen, 2002) and flax yield

can be reduced in the presence of weeds (Anonymous, 2006), in part because flax has a comparatively small photosynthetic surface (Mani and Bhardwaj, 1965). Weeds can also adversely affect the quality of flaxseed oil, and decrease the flaxseed oil content and iodine value (Bell and Nalewaja, 1968). Although herbicides are registered for controlling weeds in flax, they are limited in their utility. The development of herbicide resistant flax (especially glyphosate or glufosinate resistant cultivars) would result in enhanced weed control.

Flax is a relatively long season crop, taking about 100-120 days to mature depending on cultivar (Anonymous, 2006). To avoid damage by early spring frosts, early maturing cultivars are preferred by Canadian farmers. Genetic engineering may be useful in developing flax cultivars with early maturity, which would reduce the risk of crop damage by spring or fall frosts or lodging from snow fall. Flax is also sensitive to salt, which is the major limitation in some flax growing areas of North America and Egypt (McHughen, 2002). An attempt was made to develop a flax cultivar tolerant to salinity by somaclonal variation with cellular selection (Rowland et al., 1995). Other abiotic stress tolerant traits being developed in crops such as cold tolerance and improved nitrogen use efficiency could benefit flax (Warwick et al., 2009). Recently, Monsanto developed the first GE drought tolerant maize (*Zea mays*) and has applied to the United States Department of Agriculture (USDA) and Food and Drug Administration (FDA) for regulatory approvals

(Anonymous, 2009). The development of drought-tolerant flax also has enormous potential to increase yield where moisture is a limiting factor.

Linseed oil for industrial applications

Flaxseed oil has several industrial applications because of higher levels of ALA compared to other oilseed species including canola (*Brassica napus* and *B. rapa*), safflower (*Carthamus tinctorius* L.) or sunflower (*Helianthus annuus* L.). Currently available flaxseed oil for industrial applications has a maximum 70% of ALA which was achieved by a traditional plant breeding germplasm development program (Ntiamoah and Rowland, 1997). The drying quality of flaxseed oil is useful for industrial applications in manufacturing paints, varnishes, linoleum, printer's ink and other coatings (Rowland et al., 1995). Although the use of traditional plant breeding methods like natural or induced mutations for increasing the level of ALA within the genus *Linum* remains a valid option, the GE approach may play an important role. The manipulation of the fatty acid composition of oil to produce oils with > 70% ALA by genetic engineering may be possible. This would increase the drying quality of linseed oil and thus extend its industrial applications (Rowland et al., 1995).

Novel oil products

Flax can be modified to produce oil for human consumption and manufacturing margarines. The first strategy was to replace ALA in flax with palmitic acid (Rowland et al. 1995). The level of ALA (< 3.0% ALA) in

linseed oil by traditional mutation breeding methods has been reduced to produce “Solin” (the name given by the Flax Council of Canada to describe the flax cultivars with less than 5.0% ALA for use in the food industry) varieties (Rowland, 1991; Ntiamoah and Rowland, 1997). Zero percent ALA cultivars of flax could not be obtained by traditional plant breeding methods, but may be achieved through genetic engineering methods by reducing the activity of delta-15 desaturase (Jain et al., 1999). Linseed oil modification can be achieved by adopting GE approaches including: elucidation of the basic biochemical pathways of oil synthesis, availability of promoter elements that restrict expression of introduced genes, and silencing or cloning of the genes encoding enzymes involved in flaxseed oil synthesis.

Flax for the nutraceutical market

Modern North American diet is high in total and saturated fats, ω-6 fatty acids and *trans*-fatty acids; and low in ω-3 fatty acids (Burdge and Calder, 2005). This nutritional imbalance has led nutrition experts to recommend increasing ω-3 fatty acid intake (Fitzpatrick, 2007). Flax is valuable for the nutraceutical market because it is rich in fat, protein and dietary fiber (Jenkins et al., 1999). Flax is considered as the richest plant source of ALA, an ω-3 fatty acid. It has been estimated that the oil of common flax cultivars in Canada contains about 55% ALA (Rowland et al., 1995). ALA can, however, serve as the precursor of ω-3 very long chain polyunsaturated fatty acids (VLCPUFAs): eicosapentaenoic acid (EPA or $20{:}5^{cis\Delta 5,8,11,14,17}$) and

docosahexaenoic acid (DHA or 22:6$^{cis\Delta 4,7,10,13,16,19}$). VLCPUFAs are the subject of interest because of their roles in human health and nutrition; in particular DHA is an important constituent of the brain and retina (Hoffman et al., 2009; Neuringer and Connor, 1986). Special GE flax can also be engineered for enhanced use in animal feed, especially for poultry, to increase the level of ω-3 fatty acids in eggs.

Triacylglycerol (TAG) biosynthesis has been studied extensively in a number of oilseed crops (Weselake, 2005). Research in this area in flax, however, is limited (Abbadi et al., 2004; Stymne et al., 1992). Based on experiments on storage lipid accumulation and acyltransferase action in developing flax seed, Sorensen et al. (2005) have suggested that if the appropriate acyl-CoA-dependent desaturation/elongation pathways are introduced and expressed in flax, ALA-CoA may be converted into EPA-CoA through a pathway which uses enzymes that only work on acyl-CoA. This has now been demonstrated by Hoffman et al. (2009) in the seeds of *Arabidopsis thaliana.* Modifying the biosynthesis of VLCPUFAs in oilseed crops by genetic engineering has been a major objective among plant biotechnologists hoping to provide novel ω-3 oils for the nutraceutical market (Abbadi et al., 2004). For example, GE soybean with elevated levels of ω-3 fatty acids is under field testing in the USA (Anonymous, 2008). The accumulation of ω-3 VLCPUFAs in GE flax seed could someday represent a breakthrough in the search for alternative vegetarian source of fish oil.

Flax for functional foods

Functional food can be defined as food claimed to have health-promoting or disease-prevention properties in addition to basic nutritional properties. Many health claims have been made for whole flax seed, flax meal and milled flax. Studies have shown that consumption of flax seed in the daily diet can modestly reduce serum total and low-density lipoprotein cholesterol, decrease inflammation, and raise the level of ω-3 fatty acids, especially ALA and EPA (Jenkins et al., 1999; Thompson et al., 2005). Flaxseed has recently gained attention in the area of atherosclerotic cardiovascular disease (ASCVD) prevention because it contains ALA, lignan, phytoestrogen and soluble fiber. Daily consumption of 15-50 grams of flaxseed can improve cardiovascular risk factors, primarily by modestly improving blood lipid profiles (Bloedon and Szapary, 2004). In addition to ALA, flax is also one of the richest plant sources of lignans, which play a role in plant growth and act as antioxidants in human metabolism (Morris, 2007). Lignan metabolism is a complex process and can be studied by adopting GE approaches, which may increase the use of flax based lignans (Muir and Westcott, 2003). Thus, because flax has functional food properties, it may fit well as a plant source for the development of drugs and therapeutics in the future, especially to reduce the risk of cardiovascular diseases (Paschos et al., 2007).

Recent research also indicates that flax seed is useful in controlling

inflammation and reducing the risk of diabetes and cancer (Thompson et al., 2005). It is beyond the scope of this manuscript, however, to discuss the medical applications of flax; however, readers are directed to (Tarpila et al., 2002; Vaisey-Genser and Morris, 2003; Bloedon and Szapary, 2004; Morris, 2007).

Value added products

Genetic engineering of flax may lead to new opportunities for fiber production, with several applications in the textile industry. Bast fibers of flax are used as insulation due to their thermal properties and eco-friendly features (Smeder and Liljedahl, 1996). Recent developments in plant genomics, proteomics, molecular biology and microarray technology have made it possible to understand the relationship between genes and fiber quality (Wrobel-Kwiatkowska, 2007). High yielding fiber flax cultivars can be produced by genetic mapping and recombinant DNA technologies (Ebskamp, 2002). Biocomposites made up from flax fiber based on polyhydroxybutyrate (PHB) polymers may be an eco-friendly and biodegradable alternative to conventional plastics (Wrobel-Kwiatkowska et al., 2007). For more information on the uses of flax fibers see Foulk et al. (2002); Rennebaum et al. (2002) and Moryganov et al. (2008). The secondary cell wall of flax stem contains high levels of cellulose, hemicellulose and smaller amounts of lignins and pectins. Research on flax fiber cultivar development by genetic engineering is necessary to promote the use of flax for fibers (Rennebaum et

al., 2002).

The fine fraction obtained as a byproduct of dehulling (a process of preparing flaxseed for value added industrial products) could be a potential ingredient in pet food, whereas the medium and mix fractions can be blended into poultry feed formulations (Oomah and Mazza, 1998).

Potential risks of GE flax

Potential risks of GE crops to the food, feed and environmental systems have been delineated by the Government of Canada. Before the commercial cultivation, plant with novel traits (PNTs) are subject to environmental biosafety regulations administered by the Plant Biosafety Office of the Canadian Food Inspection Agency (CFIA) in Canada (CFIA, 2007b), and by USDA / Animal and Plant Health Inspection Service (APHIS) in the USA (ERS-USDA, 2008). The risks of GE crops depend on both the biology of the species, and construction, insertion and function of the GE trait. Transformation of unknown genes into crops may have potential to create new food allergens in humans and animals (Andow and Zwahlen, 2006). Conventional flax has no inherent food and feed safety concerns and recently a panel of experts from the United States Food and Drug Administration (FDA) has given Generally Recognized As Safe (GRAS) status to whole and milled flax seeds (Flax Council of Canada, 2009). Health risks of GE flax (like other GE crops) will be compared to conventional flax to determine if they are substantially equivalent through testing in animal and human systems, prior to

release (Chassy, 2007; Kok et al., 2008).

Environmental risks include both, those associated with the crop (the trait) and effects of changes in conventional agronomic practices. Environmental concerns of GE crops raised by stakeholders include the potential of GE crops to become weeds (Ellstrand and Schierenbeck, 2006), which may become invasive in nature and require a costly management strategy (Gaines et al., 2007; Knispel et al., 2008). Flax is a highly domesticated species and volunteer flax populations are ephemeral under agronomic conditions, diminishing over three years. There is no evidence that volunteer flax is invasive of ruderal or natural areas. Unless a GE trait confers a significant fitness advantage, flax is unlikely to be invasive. GE flax may also need to be examined for its potential to become a plant pest and for impacts on non target organisms and biodiversity. GE crops may cross pollinate with wild and weedy species (Beckie et al., 2003; Hall et al., 2003; Ellstrand, 2005; Warwick and Stewart, 2005). If transgenes introgress with the genomes of wild or weedy relatives, they may cause changes to those populations. Flax has the ability to hybridize with at least nine species of *Linum* occurring in Asia and Europe with the same chromosome number as cultivated flax (n=15) (Jhala et al., 2008). While there are eight *Linum* species identified in Canada, only *L. rigidum* Pursh var. rigidum and *L. sulcatum* Riddell have the same chromosome number, indicating a potential for GE introgression (Jhala et al., 2008). Their inter-specific hybridization with flax

needs to be quantified.

In Canada and the USA, approved GE crops are assumed to be as safe as conventional and are commingled (Brookes and Barfoot, 2004). Segregation and product labeling are not required in North America, but they are required in Europe and other countries (Devos et al., 2009). Because of non-uniform GE acceptance and legislation regulating GE-labeling in food and feed, market disruptions continue to be a major risk for developers, producers and commodity traders (Ramessar et al., 2008). For example, the first GE flax resistant to sulfonylurea (Millam et al., 2005), and more recently, glyphosate resistant wheat, were not commercialized because of market issues (Stokstad, 2004). Coexistence between GE and conventional flax production systems will depend on the limiting seed- and pollen- mediated gene flow through good management and identity preservation practices. Flax is an inbreeding species and the rate of outcrossing has been described in the range of 1-4% (Robinson, 1937; Dillman, 1938). Recent studies conducted in western Canada, however, suggest that intra-specific pollen-mediated gene flow was less than 2.0%, when two different cultivars of flax were grown 0.1 m apart. Some rare outcrossing events were recorded up to 35 m, but at a very low frequency (Jhala *et al.*, in prep). Gene flow beyond 1.0 m was less than 0.9%. Thus, pollen-mediated gene flow from GE flax to non-GE or organic flax may be mitigated through best management practices to reduce market risk.

Seed-mediated gene flow, the movement of GE flax seeds during harvest, handling, grading and transportation may increase the volunteerism and ferality and may also lead to AP in conventional crops. A recent study on post-harvest gene escape in four important GE crops:, canola (*Brassica napus*), maize (*Zea mays*), sugar beet (*Beta vulgaris*) and wheat (*Triticum aestivum*) suggest that seed-mediated gene flow cause seed dissemination and volunteerism (Gruber et al., 2008). The pathways of seed-mediated gene flow are numerous and stochastic. One of the keys to reduce seed-mediated gene flow is to control volunteer flax populations in subsequent crops.

Regulation of GE flax in Canada

Canada has a unique, science-based regulatory system administered by the Plant Biosafety Office (PBO) of the Canadian Food Inspection Agency (CFIA) for the import and environmental release of plants with novel traits (PNTs). CFIA has defined *PNTs as those plants containing a trait that is not present in plants of the same species already existing in Canada, or is present at a level outside the range of that trait in stable, cultivated populations of that plant species in Canada* (CFIA, 2007a). PNTs can be produced by many techniques including conventional breeding, mutagenesis or by modern genetic engineering. The CFIA regulates the PNTs, regardless of method of breeding, requiring data, especially to document environmental, food, and feed safety. The Plant Biosafety Office of the CFIA is responsible for regulating the agronomic and horticultural PNTs, including post-commercialization

monitoring and inspection (CFIA, 2007b). Health Canada is responsible for food derived from GE crops (Health Canada, 1994). Environment Canada is also the authority for regulating other products derived from PNTs. The CFIA has successfully approved >70 PNTs irrespective of their production method in Canada.

In Canada, GE flax requires registration before seed can be sold commercially in the market under the statutory authority of the Seeds Act. Based on a positive recommendation by an independent body, the federal Minister of Agriculture issues a certificate of registration for a new flax cultivar. In addition to this, there are five assessment criteria for determining environmental biosafety of GE flax as described in CFIA Directive 94-08 (CFIA, 2007b). The applicant is responsible for providing the appropriate data and relevant scientific information to the CFIA including the environmental risk of the new GE flax cultivar to its counterpart(s) already present in Canada. The PBO compares this information with the available biology document of flax, which contains information on reproductive biology, origin, occurrence and distribution of closely related species of flax, breeding history, interaction of flax with different species and other relevant information (CFIA, 2001). , For several plant species including flax, however, the biology documents available on CFIA website were prepared prior to experience with GE cultivars. Thus, there is need to update CFIA's crop biology documents including the biology of flax, based on current scientific data.

Confined field trials of any new GE flax cultivar are required to minimize the potential environmental impact of the new trait while conducting environmental biosafety experiments at various locations in Canada. CFIA officers are responsible for inspection of the applicant's confined field trials to ensure they meet the conditions of the confinement. For unconfined release of GE flax, the CFIA relies on the report of the CFIA officer responsible for the inspection of the confined trials, confidential research reports and also on the peer-reviewed published research (if any) developed from the testing of new PNTs (Corbet et al., 2007).

The Canadian regulatory framework is flexible to address the regulation of the PNTs through the ongoing development of clear and transparent criteria before commercial production of GE crops. For more details on the Canadian regulatory framework for PNTs see (Demeke et al., 2006; Corbet et al., 2007; Smyth and McHughen, 2008) and the USA regulatory framework see (McHughen and Smyth, 2008; USDA/APHIS, 2008).

Best management practices to mitigate transgene movement

Canadian flax seed is being exported mainly to the Europe, Japan, USA and South Korea (Flax Council of Canada, 2007). The EU has established a labeling threshold level of 0.9% of AP defined as " the percentage of GE-DNA copy numbers in relations to target taxon specific DNA copy numbers calculated in terms of haploid genomes" (Council of the European Parliament, 2003). The EU threshold and wide applicability of analysis methods have been

challenged. Weighardt (2006) has described the EU labeling thresholds as impractical and unscientific and suggested that caution should be exercised when analyzing GE content in processed food and feed (Weighardt, 2007). Japan and South Korea have 5 and 3% tolerance limits, respectively (Demeke et al., 2006). To preserve the export market for conventional flax seed, it must be segregated from GE material. Gene flow, either by pollen and/or seed during the production and transport process are major routes of transgene movement. For effective coexistence of GE, conventional and organic flax, Canadian flax growers will be required to mitigate the pollen and seed-mediated gene flow.

Mitigating seed-mediated gene flow

In the case of an autogamous species like flax, seed-mediated gene flow will be more significant and stochastic than pollen-mediated gene flow. Although the growing area of flax has not changed significantly over the past few years, the relative abundance of volunteer flax has increased from 2.0 to 15.3 in western Canada (Thomas et al., 1997). Volunteer flax was ranked as the 32nd most abundant weed in the Canadian Prairies in the 1970s, but in the 1990s and 2000s it was replaced as the 26th most abundant weed (Leeson et al., 2005), indicating that seed-mediated gene flow may become a problem if GE volunteer flax will not be effectively controlled. Left uncontrolled, volunteer flax seeds can be harvested with subsequent crops, usually cereals. In the year following flax production, volunteers are numerous.

There are some herbicides registered for the control of volunteer flax. Quinclorac has been registered for control of volunteer flax in spring wheat providing consistently good control at 100 or 200 g a.i. ha^{-1} (Wall and Smith, 1999). Pre-emergence application of glyphosate or glyphosate plus tribenuron reduced volunteer flax densities from 39 plants to < 4 plants m^{-2} (Dexter et al *in prep.*). Post-emergence application of either fluroxypyr plus MCPA or 2,4-D ester reduced the volunteer flax density up to 2 plants m^{-2}. Experiments to mitigate volunteer flax in glufosinate and imidazolinone resistant canola indicate that combination of glyphosate applied pre-plant and glufosinate applied post-emergence at recommended rates can control volunteer flax in glufosinate resistant canola (personal observation). Pre-plant application of glyphosate was effective to reduce AP of volunteer flax in both the canola traits. Without recharge, flax volunteer populations are ephemeral, and do not persist more than three years. Sound agronomic practices for controlling volunteer populations, including cultivation of competitive crops in rotation, timely and effective weed control practices, and pre and post-harvest monitoring and mitigation will reduce populations of volunteer GE flax and the risk of seed-mediated gene flow.

Mitigating pollen-mediated gene flow

The frequency of gene flow in flax declines rapidly with increasing distance from the donor field. Since maximum out-crossing has been observed in the area of 3.0 m near the pollen donor, an isolation distance of 3.0 m

between fields would likely reduce pollen-mediated gene flow significantly (<0.14%). Alternatively, if the area of 3.0 m around the GE flax is removed after flowering, but before seed set, it can reduce the out-crossing significantly between GE and conventional flax cultivars. If the buffer zone around the GE flax field is strictly followed, the EU standard (0.9%) for adventitious presence of GE flax in non-GE or organic flax production systems can be achieved. Border or trap rows (non-GE crop borders grown around a GE *crop)* of non-GE crop and barren zone both have been tested for efficacy in transgene containment. The experiments conducted by Morris et al. (1994) in canola suggest that the trap rows were effective in reducing gene flow to distant populations, but their effectiveness was highly dependent on the width of the trap. In comparison to canola, flax has a lower outcrossing frequency, and thus may need less area (1-3 m) for trap rows.

In the event that GE flax is grown beside organic flax, with no established thresholds for presence of AP, an increased level of pollen-mediate gene flow reduction may be required, including the following practices.

- Flowering synchrony can be reduced by sowing organic flax at different dates than GE flax to reduce the pollen-mediated gene flow between GE and organic flax cultivars.
- In addition to isolation distance, a trap crop may be required for organic growers. The removal of 1.0 m of the crop adjacent to

other flax crops after flowering may reduce the risk of transgene dissemination.

- Harvest loss should be reduced to reduce the volunteer flax population in subsequent years.
- For organic growers, without the application of pre-seeding herbicides, Delay the seeding of subsequent crops should be delayed to allow pre-seeding tillage may reduce the presence of volunteers.
- Diversification of crops in rotation should be adopted.

A better understanding of crop-to-wild gene flow and the best management practices to mitigate the transgene movement from GE flax to its wild and weedy species is required as a part of the ecological risk assessment to access potential negative consequences to biodiversity. The majority of flax wild relatives which are likely to hybridize with GE flax are distributed in the Mediterranean and Southeast Asia, the probable centers of the origin of flax (Jhala et al., 2008). The Canadian Prairies is the largest flax growing region in the world, and only three wild relatives of flax (*Linum lewisii*, *Linum rigidum* and *Linum sulcatum*) are found in this region (Scoggan, 1993). It is important to note, however, that the presence of wild relatives of flax does not imply that successful hybridization will occur. There is no evidence to date for inter-specific hybridization of these three species with cultivated flax. None of these species has been reported as weeds on the Canadian Prairies in

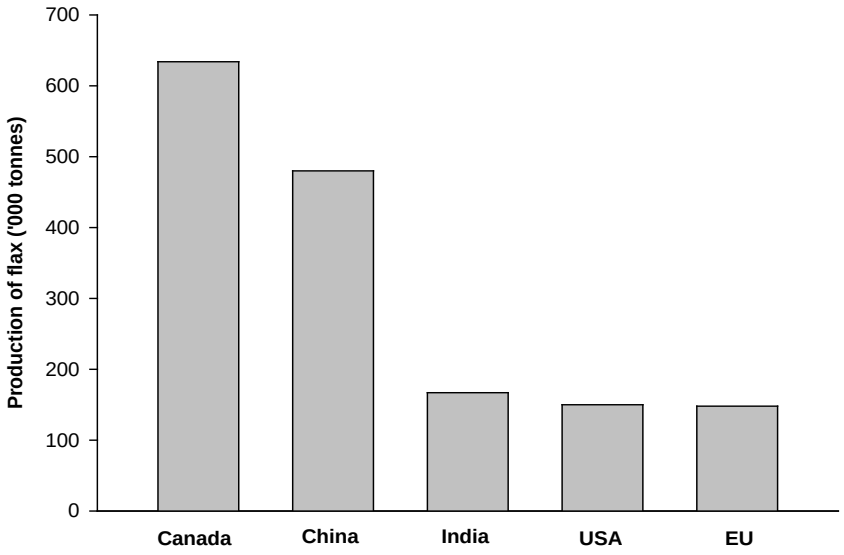
agronomic weed surveys conducted during the 1970s to the 2000s (Leeson et al., 2005). The limited number of flax wild relatives and their limited occurrence and distribution in Canada make it less likely for inter-specific transgene movement. Several integrated weed management practices during crop season including herbicide applications may also reduce the populations of closely related species, and thus the chances of gene flow from GE flax to wild relatives.

Transgene movement cannot be prevented, but can be reduced by adopting best management practices to below threshold levels in conventional flax. If GE flax were cultivated in western Canada, volunteer flax could be effectively controlled by herbicides in cereals and canola. If herbicide-resistant flax is introduced into market, extension agronomists should play a vital role in advising growers to adopt integrated weed management systems, crop rotation, spot application of herbicides and use of herbicide mixtures for reducing herbicide-resistant weeds and for controlling herbicide resistant flax volunteers (Beckie, 2007; Blackshaw et al., 2008; Devos et al., 2004).

Conclusion

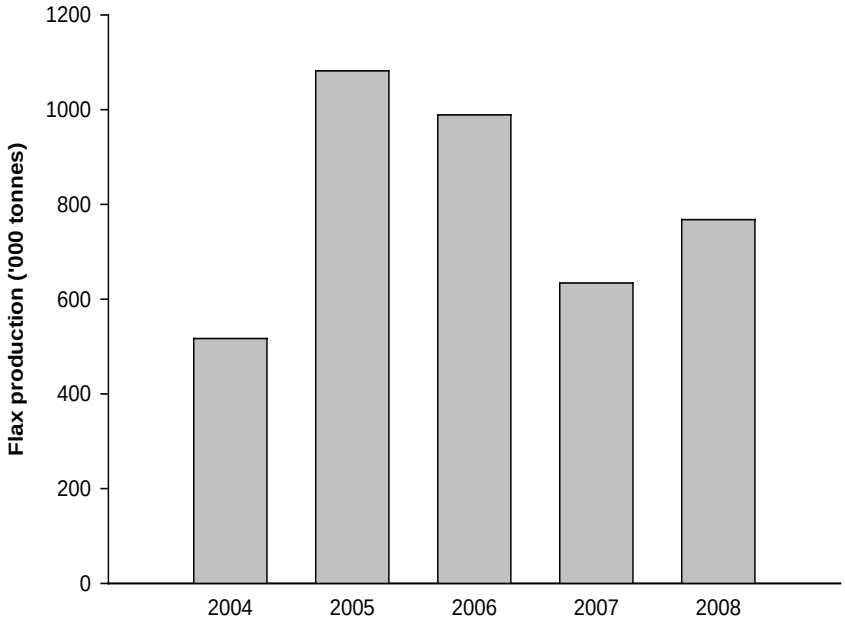
The utility of modifications for flax have been apparent to flax breeders for many years. The resources available for development of flax have been relatively small compared to resources applied to the development of Canada's other oilseed crops including canola and soybean. Advances in cell and molecular biology now allow plant breeders to respond more quickly to agronomic limitations of flax and fiber production, and increasing consumer demands for functional food. GE technologies could increase the utility and value of oils and fiber products, and reduce production risk to growers through improvements in agronomic performance discussed in this paper. As one of the first genetically engineered crops, the tools for rapid improvement of flax germplasm are available.

Without the consent of society at large, GE crops will fail in the market place. While questions remain to be answered about the risk of each proposed trait on a case by case basis, there is little evidence that domestic flax itself poses an inherent risk to food, feed or the environment. While market harm remains the primary concern for developers and growers, coexistence between conventional and GE flax can be achieved with reasonable AP thresholds and with appropriate mitigation and testing measures in place.



***Source: FAOSTATE data (2007)**

Figure 6-1 Production of flax in five major flax producing countries in 2007*



*Source: Flax Council of Canada (2008)

Figure 6-2 Production of flax in the last five years in Canada*

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Chapter 7

General discussion and Conclusions

Flax is the second most important oilseed crop in western Canada. To support this industry, three major breeding programs develop flax and solin cultivars in Canada; Agriculture and Agri-Food Canada program located at the Morden Research Centre in Manitoba, the Crop Development Centre at the University of Saskatchewan in Saskatoon and the Vittera flax breeding program at Vegreville, Alberta.

Because flax has unique oil and fiber attributes, it is being evaluated as a model plant species for bio-industrial and nutraceutical products. Several of these products are predicated on the deployment of recombinant DNA techniques. This thesis research is a part of a large project to develop flax as a platform crop for bio-products. The flax project includes several research groups and objectives including accelerate flax transformation for the development of modified long chain fatty acids; development of genetic use restriction technologies (GURT) and visible traits for GE flax cultivars; to study the processes of vascular development, especially the development of phloem fibers in flax by high-throughput technologies such as DNA microarrays and environmental biosafety assessment of GE flax as a part of pre-commercialization risk assessment of GE flax.

Biosafety research in flax to determine risk of seed-mediated gene flow including emergence and persistence of flax, seed dormancy and seed bank

dynamics, controlling volunteer flax in western Canadian cropping systems, suggested that risk of transgene movement by seed can be reduced by adopting management practices (Dexter et al., 2009). I conducted a meta-analysis and field experiments to quantify pollen-mediated gene flow and mitigate adventitious presence of transgenes from GE flax to predict the co-existence of GE flax with conventional flax prior to commercial production.

Intra-specific pollen-mediated gene flow (flax-to-flax) was quantified through the transfer of the dominant, xenia effect of high α-linolenic acid (ALA, $18{:}3^{cis\Delta 9,12,15}$) trait to the low ALA flax cultivars. Field experiments were conducted at four locations during 2006 and 2007 in western Canada in a concentric donor (20x20 m) receptor (120x120 m) design. Binomial distribution and power analysis were used to develop a decision tool to quantify the minimum number of seeds required statistically to detect the frequency of gene flow in relation to various α (confidence interval) and power (1-β) values. A total of ~4 million seeds were screened for ALA content and the high ALA seeds were considered a product of pollen-mediated gene flow. Results from the average value of all location-year suggest that the frequency of gene flow at 0.1 m distance was 0.0185 and declined rapidly with distance from the pollen source. The results of an exponential decay function to measure the distance at which the gene flow was reduced by 50% (O_{50}) and 90% (O_{90}) suggest that it was in the range from 0.85 to 2.64 m, and 5.68 to 17.56 m, respectively. However, the average combined value of O_{50} and O_{90}

for all the locations and years was 1.62 and 5.37 m, respectively. Thus, pollen-mediated gene-flow from GE flax to commodity flax may occur at short distances but would be rare beyond 35 m.

The genus *Linum* contains approximately 300 species which are distributed in many parts of the world and may grow in sympatry with cultivated flax. The potential for gene introgression from GE flax to wild relatives, the occurrence, the phylogeny of flax wild relatives and reported interspecific hybridization was reviewed to initiate the evaluation of environmental risk of inter-specific transgene movement. 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*. Hybridization of flax with many other wild relatives has either not been studied or reported. However, based on the evidence of reported work, gene flow from flax to wild or weedy relatives is possible; however, in species native to Asia and Europe. Information on species distribution, sympatry, concurrent flowering, ploidy level and sexual compatibility, indicates that inter-specific gene flow will be negligible in North America.

To quantify and mitigate adventitious presence of flax volunteers in canola, multi year site experiments were conducted. Results revealed that pre-plant treatment of glyphosate in glufosinate- and imidazolinone-resistant canola was effective to reduce volunteer flax density and dry weight. A

combination of pre-plant followed by post-emergence (POST) herbicide treatment was found to be superior in reducing adventitious presence of volunteer flax in glufosinate resistant canola. Imazethapyr/imazamox applied POST was not effective for controlling volunteer flax in imidazolinone-resistant canola.

Best management practices were proposed to mitigate transgene movement from GE flax. If an isolation distance of 1 and 3 m would be followed between GE and conventional flax, pollen-mediated gene flow would be reduced to 0.27% and 0.14%, respectively. Furthermore, no hybridization between flax and its wild and weedy species has been reported to occur in Canada and the limited number of flax wild relatives and their distribution and occurrence in western Canada (where majority of flax is grown) make it unlikely for inter-specific transgene movement. Other approaches including the use of a buffer zone around GE flax, cleaning of equipment, delay seeding dates of organic flax to reduce flowering synchronization and genetic use restriction technologies (GURTs) may facilitate co-existance of GE, conventional and organic flax. Mitigation of volunteer flax in herbicide-resistant canola illustrates that management practices including the choice of crop grown in rotation and herbicides are essential to reduce adventitious presence of volunteer flax in subsequent crops.

Environmental biosafety and risk assessment studies for commercialization of GE flax suggest that with the adoption of best

management practices, pollen- and seed-mediated gene flow from GE flax can be reduced below threshold level. By comparing the risk-benefit ratio, GE flax may provide more benefits and very few potential risks. Therefore, considering the need for rapid cultivar development for various purposes, flax should be a prime target for cultivar improvement using genetic engineering techniques.

This type of environmental biosafety evaluation is extremely important prior to commercialization of GE crops to avoid any negative consequences. The government in respective countries, industry, producers and stakeholders should be involved in risk analysis frame work to make the regulatory streamline approval.

Future research objectives

As discussed in chapter 3, flax has only three wild and weedy relatives which are reported to occur in western Canada. While, two wild relatives, *L. sulcatum* and *L. perenne* have diploid chromosome number 30, and thus they are likely to hybridize with GE flax based on the reported work in other *Linum* species. Using tiered risk assessment, following research should be conducted,

- Occurrence, population dynamic and biology of the three western Canadian *Linum* species should be studied
- Interspecific hybridization work should be done by crossing cultivated flax with *L. sulcatum* and *L. perenne* under worst case scenario conditions in greenhouse

- If iterspecific hybridization would be successful in greenhouse, fecundity of F1 hybrid shoyld be studied and should be back cross should be attempted with cultivated flax to understand the degree to which crop-specific genes are integrated into hybrid populations
- If iterspecific hybridization would be successful artificially, field experiments should be conducted to learn hybridization potential of cultivated flax with *L. sulcatum* and *L. perenne*
- If hybridization is confirmed in nature, the potential impact on fitness or weediness and the impact of transgenes on biodiversity should be addressed
- When GE flax is developed and available for commercialization, larger scale gene flow work should be conducted to predict the landscape scale gene flow in flax
- Co-existence between GE and conventional crops is only possible if a threshold value of adventitious presence above zero is accepted, so more research and network is required within industry, government and regulators to identify threshold levels for GE flax market acceptance and trade between countries

Canadian growers have rapidly adopted GE crops in canola, maize, soybean and some other crops at a commercial scale. However, the major export market for conventional Canadian flaxseed is the EU which is sensitive to GE crops and their products. If the Canadian government would encourage

industry to process flaxseeds in Canada, for example by manufacturing linoleum flooring or several other industrial applications, a large amount of flaxseed can be consumed domestically. Revenue could be generated through exporting high value processing products derived from flax oil without market harm. However, more work is required to get more alternatives, so economic benefits of flax or flax based products can be derived without environmental or market harm.

Alternately, segregation of GE flax from conventional flax will be required, similar to segregation of high oleic acid canola cultivars in Canada or GE and conventional canola in Australia. However, in this case, there are more chances of seed-mediated gene flow from GE flax and following research should be addressed,

- Loss of the GE flaxseeds during transportation or trade should be studied
- More research is also required to mitigate adventitious presence of GE flax in the crops grown in rotation especially pulse and legume crops
- Flax has natural resistivity to imazamox+imazethapyr, so herbicide or tank mix of herbicides should be identified to control GE volunteer flax in imidazolinone-resistant crops grown in western Canada
- Best management practices should be identified including separate equipments for GE and conventional flax and separate transportation, testing and marketing channels to reduce level of adventitious presence

Curriculum Vitae

Family Name : JHALA

First Name : AMIT

Date of Birth : 28th April, 1979

Office Address : Dept. of Agricultural, Food and Nutritional Science
University of Alberta, Edmonton, T6G 2P5, AB, Canada

Tel No. : 780-492-7994 (O); 780-716-6877 (Cell)

Fax No. : 780-492-4265

E-mail ID : jhala@ualberta.ca OR amit_jhala@yahoo.co.in

Personal webpage : http://lindahall.pbwiki.com/Amit-Jhala

Educational qualification

University	Degree	Start Date	End Date	Status
University of Alberta, Canada	Ph. D. (Plant Sci.)	March, 2006	Dec, 2009	Completed
Ghent University, Belgium	Ambassadorial Scholarship	Jan, 2005	Jan, 2006	Completed
Gujarat Agricultural University, India	M.Sc. (Agronomy)	Nov, 2000	May, 2003	Completed
Gujarat Agricultural University, India	B.Sc. (Agri)	July, 1996	Aug, 2000	Completed

HONORS AND AWARDS

1. Ph.D. student travel award by the Canadian Weed Science Society (CWSS) to attend 63rd CWSS Annual Conference at Charlottetown, Prince Edward Island, Canada, Nov. 24-26, 2009 ($1000)

2. Best oral presentation award (1st place) by the Canadian Society of Agronomy at the joint annual conference of the Canadian Society of Agronomy, Canadian Society of Soil Science and Canadian Society of Agricultural and Forest Meteorology held during 5-7 Aug. at the University of Guelph, Canada (Aug. 2009; $ 300)

3. Post Doctoral fellowship, University of California, Davis

4. Natural Science and Engineering Research Council (NSERC) of Canada Industrial Post Doctoral R & D Fellowship (May, 2009; $ 60,000)

5. Pest Management Award by the Canadian Society of Agronomy (Apr., 2009; $ 1500)

6. The Government of Alberta Graduate Citizenship Award for Student Leadership (March, 2009; $ 2000)

7. Pansy and George Strange Graduate Scholarship, University of Alberta (Nov. 2008; $ 1400)

8. Ph.D. student travel award by the International Weed Science Society, sponsored by Bayer Crop Protection, Canada to attend 5th International Weed Science Congress, Vancouver, Canada, 23-27th June, 2008 ($ 1200)

9. Professional Development Grant (PDG) Award by Graduate Students' Association (GSA), University of Alberta (June, 2008; $ 200)

10. Alberta Ingenuity Ph.D. student scholarship sponsored by Alberta Ingenuity Fund, Alberta, Canada (May, 2008 to April, 2010; $ 26,000/annum)

11. Canadian Weed Science Society (CWSS) Ph.D. student travel award to attend 61st CWSS Annual Conference at Mont Tremblent, Quebec, Nov. 25-27, 2007. ($1000)

12. Graduate student scholarship sponsored by Alberta Innovation and Science, Alberta Government, Canada ($22,000/annum; from Sep. 2006 to March, 2008)

13. Awarded as an Entrance Award for Tuition to new graduate students by Dept. of Agricultural Food and Nutritional Science, University of Alberta to pay the fall term 2006 tuition fees (Award amount $3942.00)

14. Traveling Award sponsored by European Weed Research Society (EWRS) for attending 13th International EWRS Symposium held at Bari, Italy during 19– 23rd June, 2005 (€500; Declined)

15. Awarded as an Ambassadorial Scholarship Sponsored by Ministry of Flemish Community, Brussels, Belgium to conduct one year research project at the University of Ghent, Belgium (From Jan., 05 to Jan., 06)

16. Best Poster Presentation Award for the paper "Management of root-knot nematode *Meloidogyne incognita* through soil solarization and intercropping systems" at the National Nematology Symposium on "Paradigms in Nematological Research for biodynamic farming", GKVK, Bangalore, India, November 17-19, 2004

17. Awarded as a senior research scholarship sponsored by Government of India, Department of Science and Technology, New-Delhi (from Feb.04 to Dec.04)

PUBLICATIONS

Research Papers in Peer Reviewed Journals,

1. **Jhala A.J**., Weselake R.J. and Hall L.M. (2009). Genetically engineered flax (*Linum usitatissimum* L.): Potential benefits, risks, regulations and mitigation of transgene movement. *Crop Science* 49:1943-1954

2. **Jhala A.J.,** Lisa Raatz, Jody E. Dexter and Linda M. Hall. (2010). Adventitious presence: Volunteer flax (*Linum usitatissimum* L.) in herbicide resistant canola (*Brassica napus* L.). *Weed Technology* (Accepted, Manuscript # WT-D -09 – 00003R).

3. **Jhala A.J**. and L.M. Hall (2009). Flax (*Linum usitatissimum* L.): Current uses and future applications. *Australian Journal of Basic and Applied Sciences* (In Press).

4. Rathod P.H., Patel J.C., Shah M.R. and **Jhala A.J**. (2009). Recycling gamma irradiated sewage sludge as fertilizer: A case study using onion (*Alium cepa*). *Applied Soil Ecology* 41:223-233.

5. Dexter J.E., **Jhala A. J.**, Hills M.J., Yang R.C., Topinka K.C, Weselake R.J. and Hall L.M. (2010). Quantification and mitigation of adventitious presence of volunteer flax (*Linum usitatissimum* L.) in wheat (*Triticum aestivum* L.). *Weed Science* (In Press, Manuscript # WS-09-104R).

6. Rathod P.H., Patel R.B. and **Jhala A.J.** (2010). Persistence and management of dinitroaniline herbicides residues in sandy loam soil. *International Journal of Environment and Sustainable Development* 9:58-73.

7. Rathod P.H., Patel J.C. and **Jhala A.J**. (2009). Potential of gamma irradiated sewage sludge as fertilizer in radish (*Raphanus sativus* L.): Evaluating heavy metal accumulation in sandy loam soil. *Communications in Soil Science and Plant Analysis* (Under Review, Manuscript # LCSS-2009-0225).

8. Hall L.M., Dexter J. E., **Jhala A.J.** and McPherson M. (2009). Biology matters: Seed and pollen-mediated gene flow in three oilseed species. Peer reviewed Proceedings of the Fourth international conference on coexistence between genetically engineered (GM) and non-GM based agricultural supply chains (GMCC-09) to be held during 12-15 Nov., 2009 in Melbourne, Australia.

9. ***Jhala A.J.,** Hall L.M. and Hall J. C. (2008). Potential hybridization of flax with wild and weedy species: An avenue for movement of engineered genes? *Crop Science*. 48 (2): 825-840 (**Selected as a quality paper by the Crop Science Editorial Board*)

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12. **Jhala A. J.,** Rathod P. H., Patel K. C. and P. VanDamme (2005). Growth and yield of groundnut (*Arachis hypogeae*) influenced by weed management practices and *Rhizobium* inoculation. *Communications in Agricultural and Applied Biological Sciences*, 70 (3): 493-500.

13. Parmar R. S., B. Akula, Shekh A. M. and **Jhala A. J.** (2004). Inter seasonal climatic variability of Gujarat State. *Journal of Agrometeorology*, 7 (2): 214-219.

Research Papers in International Conference / Symposium Proceedings

14. Jhala A.J., Bhatt H., Topinka K. and Hall L.M. (2009). Gene flow and co-existence of genetically engineered and conventional flax in western Canada. 63rd Canadian Society of Weed Science Annual Conference held at Charlottetown, Prince Edward Island, Canada, Nov. 24-26, 2009.

15. **Jhala A.J.**, Dexter J.E. and Hall L.M. (2009). Best management practices to mitigate transgene movement from transgenic flax (*Linum usitatissimum* L.). Joint Annual Conference of the Canadian Society of Agronomy, Canadian Society of Soil Science, and Canadian Society of Agricultural and Forest Meteorology during 4-7 Aug., 2009, University of Guelph, Ontario, Canada (Presentation).

16. **Jhala A. J**., Hall L. M. and Hall J.C. (2008). Potential introgression of transgenes from genetically engineered flax to wild and weedy species in Canada. 62nd Canadian Weed Science Society Annual Conference held at Banff, Canada during 25-27 Nov. 2008. (Presentation).

17. **Jhala A. J**., Linda M. Hall and Jocelyn C. Hall (2008). Potential (trans) gene movement from flax to wild and weedy species in North America. 5th International Weed Science Congress going to be held at Vancouver, British Columbia, Canada during 22-27 June, 2008 (Poster).

18. **Jhala A. J**., A. Keith Topinka, Marc McPherson and Linda M. Hall (2007). Intra specific pollen-mediated gene flow in flax (*Linum usitatissimum* L.). In Proceedings of 61st Canadian Weed Science Society Annual Meeting at Mont Tremblant, QC, Canada, 27-29th Nov. 2007 (*Abstract*), pp. 65 (Presentation).

19. **Jhala A.J.,** Topinka A.K., McPherson M., and Hall L.M. (2006). Intra-specific gene flow in flax (*Linum usitatissimum* L.): Experimental plan. Annual poster presentation at the Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Nov. 2006 (Poster).

20. **Jhala A.J.,** Topinka A.K., McPherson M., and Hall L.M. (2006). Inter-specific gene flow in flax (*Linum usitatissimum* L.): Experimental plan. Annual poster presentation at the Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Nov. 2006 (Poster).

21. **Jhala A. J.**, Rathod P. H., Patel K. C. and P. Van Damme. (2005). Growth and yield of groundnut (*Arachis hypogaea* L.) as influenced by weed management practices and *Rhizobium* inoculation. Proceedings of the 57th International Symposium on Crop Protection held at Ghent University, Belgium on 10th May, 2005 (Poster).

22. **Jhala A. J.**, Rathod P. H., Shah S. C. and P. Van Damme. (2005). Effect of seed rates and weed management practises on weeds and yield of wheat (*Triticum aestivum* L.). Proceedings of the 57th International Symposium on Crop Protection held at Ghent University, Belgium on 10th May, 2005 (Poster).

23. **Jhala A. J.**, Rathod P. H. and Trivedi G. C. (2005). Herbicidal weed control in peas (*Pisum sativum* L.) today. Proceedings of the 92nd Indian Science Congress, January 3-7, 2005 at Nirma University, Ahmedabad, India (Presentation).

24. Rathod P.H., **Jhala A. J.** and Patel R.B. (2005). Minimization of herbicidal residues in mustard crop. Proceedings of the 92nd Indian Science Congress, January 3-7, 2005 at Nirma University, Ahmedabad, India (Poster).

25. **Jhala A. J.** and Patel R.H. (2004). Management of root-knot nematode *Meloidogyne incognita* through soil solarization and intercropping. Proceeding of the National Nematology Symposium on "Paradigms in nematological research for biodynamic farming", GKVK, Bangalore, India November 17-19, 2004 (Poster).

Non Refereed Contribution (i.e. reports, popular articles etc.)

26. **Jhala A. J**., Hall L.M. and Hall J.C. (2008). Investigating the potential for gene flow of transgenic flax with its wild relatives. *Crop Science Society of America Newsletter*. 53, (7): 2-3.

Manuscripts Under Preparation for Peer Reviewed Journals,

27. **Jhala A.J**., A. Keith Topinka and Linda M. Hall (2009). Pollen-mediated gene flow in flax (*Linum usitatissimum* L.) and strategy to confine transgene movement: Can biotech and organic flax co-exist? *Nature Heredity*

28. **Jhala A.J.** and Hall L. M. (2009). Biology of Canadian Weeds: Volunteer flax (*Linum usitatissimum* L.). *Canadian Journal of Plant Science*

29. Dexter J.E., **Jhala A.J.,** M.J. Hills, R.K.Yang, R.J. Weselake and L.M. Hall (2009). Persistence and occurrence of volunteer flax (*Linum usitatissimum* L.) in western Canadian cropping systems. *Agronomy Journal.*

30. Ryan N.L., **Jhala A.J.**, K.N. Harker, and Hall L.M. (2009). Quantifying dose response and fecundity of volunteer wheat in herbicide resistant canola seeded at reduced and recommended rates. *Weed Technology*

Book Chapters (Under preparation)

1. **Jhala A.J**., Weselake R.J., Duguid S. and Hall L.M. (2009). Flax (*Linum usitatissimum* L.): Domestication, agronomy, breeding, genetic engineering and industrial applications. *In* Oilseeds for Industrial Applications (ISBN 978-1-893997-98-1). American Oil Chemists' Society (AOCS) Oilseed Monograph Series, AOCS Press, Urbana, IL, USA.

2. **Jhala A.J**. and Hall L.M. (2009). Environmental biosafety of transgenic crops: Flax (*Linum usitatissimum* L.) as a model system *In* "*Plant Biotechnology and Transgenic Research*" edited by

Thangadurai D., Othman R.Y. and Biradar D.P., Bentham Science Publisher, Oak Park, IL, USA.

EXTENSION ACTIVITIES

- A television talk was given on the topic "Intercropping of pigeonpea *(Cajanus cajan* L.) with green gram and pearlmillet and their effect on weed seed bank. The program was telecasted on August 8, 2004 at 6:30 to 7:00 am on E-TV, India
- A television talk was given on the topic "The advantages of green manuring". The program was telecasted on January 3, 2005 at 6:30 to 7:00 am on E-TV, India

LEADERSHIP / VOLUNTEER ACTIVITIES

- Graduate Student Representative of Graduate Program Committee, Dept. of Agricultural, Food and Nutritional Science, University of Alberta (Sep. 2006 to Aug. 2007).
- Graduate Student Representative of General Faculty Council, University of Alberta (March, 2007 to Feb. 2009).
- Volunteered as a Peer Leader with International Center, University of Alberta, Canada (Sep. 2006 to Sep. 2007)

MEMBERSHIP IN PROFESSIONAL ORGANIZATIONS

- Weed Science Society of America
- Canadian Weed Science Society
- Weed Science Society of America
- Indian Weed Science Society
- Crop Science Society of America
- American Society of Agronomy
- Soil Science Society of America
- International Weed Science Society