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Environmental biosafety of genetically engineered camelina [*Camelina sativa* (L.) Crantz.] for use as a bio-product crop

by

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Abstract

Camelina is currently being evaluated as bioindustrial platform crop on the Canadian Prairies. Prior to unconfined release of genetically engineered (GE) camelina, an environmental risk assessment must be conducted.

Camelina pollen-mediated gene flow (PMGF) was assessed using a dominant DsRed seed-expressed transgene in small and large plot experiments. Intraspecific small plot PMGF examined approximately 8 M seeds. Outcrossing was low, ranging from 0.09 to 0.28% at up to 0.6 m distance. Large plot assessment screened over 19 M seeds and detected a maximum PMGF of 0.78% immediately adjacent to the pollen source. However, PMGF rapidly declined by 99% at 9.99 m (± 0.18 m) from the pollen source with rare events (≤ 0.001%) at 20 m. Interspecific PMGF to weedy relative shepherd's purse was examined under greenhouse and small plot conditions. Zero hybrids were detected in 103,000 and 30,000 seeds respectively which corresponds to PMGF at or below 0.1 and 0.025%. Camelina is self-fertile with a low propensity for interspecific gene flow that should not constrain novel cultivar development.

A study of camelina seed-mediated gene flow quantified seed bank inputs, longevity, and emergence in growers fields. Seed losses incurred at harvest were high and variable (1,202 to 43,430 viable seeds m^{-2}). Seed banks became 99% depleted within 15 months. In the year following production, camelina volunteer populations were initially high (1,208 plants m^{-2}) but declined to nearly extinct (0.6 plants m^{-2}) by two years post-production. While seed bank

inputs can be high, camelina's brief persistence limits weediness in agricultural areas.

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Contributions of Authors

The contributions of the candidate and the co-authors to the completion of this work are described here. Chapter 3 of this thesis was co-authored by the candidate and by Keith Topinka, Dr. Rong-Cai Yang, Debbie Puttick, Dr. Melissa Hills, and Dr. Linda Hall. In Chapter 3, the candidate was responsible for processing and screening samples, statistical analysis, and writing the manuscript. The co-author Keith Topinka was responsible field trial design and execution. Dr. Rong-Cai Yang provided statistical analysis guidance and Debbie Puttick performed PCR to confirm putative hybrids. The co-author Dr. Melissa hills provided critical review of the manuscript. Chapter 4 of this thesis was co-authored by the candidate and by Keith Topinka and Dr. Linda Hall. The candidate was responsible for sample processing and screening, statistical analysis, and construction of the manuscript. The co-author Keith Topinka provided guidance for trial design and execution. Chapter 5 was co-authored by the candidate and Dr. Linda Hall. Chapter 6 of this thesis was co-authored by the candidate and by Keith Topinka, Lisa Raatz, and Dr. Linda Hall. The candidate was responsible for data collection, sample processing, statistical analysis and writing of the manuscript. Keith Topinka was responsible for experimental design while Lisa Raatz gave guidance for statistical analysis and provided valuable manuscript improvements. Chapter 7 of this thesis was co-authored by the candidate and by Dr. Linda Hall, Dana Sanderson, and Dr. Melissa Hills. In Chapter 7, the candidate was responsible for data collection and statistical analysis as well as writing of the manuscript. The co-author Dana Sanderson was

responsible for trial conduction, and data collection. For Chapter 7, Dr. Linda Hall provided critical review of the manuscript. The co-author Dr. Melissa Hills acted as an advisor for Chapter 7, involved in the experimental design and manuscript reviews. The co-author Dr. Linda Hall of Chapters 3 to 6 served as the PhD advisor involved in experimental design, manuscript reviews, and advising of the candidate.

List of Abbreviations

ANOVA	Analysis of variance
AP	Adventitious presence
bar	bialaphos resistance
CFIA	Canadian Food Inspection Agency
˚C	Celsius
DsRed	*Discosoma* ssp. red fluorescent protein
EC	Commission of the European Communities
EPA	Environmental Protection Agency
EU	European Union
EX_{50}	Extinction of 50%
EX_{99}	Extinction of 99%
GE	Genetically engineered
HR	Herbicide resistant
LLP	Low level presence
OC	Outcrossing
PBO	Plant Biosafety Office
PMGF	Pollen-mediated gene flow
PNT	Plant with novel trait(s)

POSTHARV	After harvest
POSTHERB	After application of an in-crop herbicide
PREHARV	Prior to harvest
PREHERB	Prior to the application of an in-crop herbicide
PRESEED	Prior to seeding
SMGF	Seed-mediated gene flow
TKW	Thousand kernel weight
USDA	United States Department of Agriculture

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Chapter 1. Introduction

1.1. Background

Camelina sativa (L.) Crantz is an old world European oilseed crop and a member of the Brassicaceae family. Camelina has applications in cosmetics, human nutrition, and bio-industrial oils, however, North American production is largely devoted to biodiesel feedstocks (Blackshaw et al., 2011; Gugel and Falk, 2006; Schillinger et al., 2012). Camelina grows well in arid, cool climates and is well suited to the more northerly regions of Europe, North America, and Asia. Traits including early maturity (80 to 100 days), high yield potential, low fertility requirements, and compatibility with existing farm equipment (Gugel and Falk, 2006; Urbaniak et al., 2008) have increased interest in this novel crop. Research and development of genetically engineered (GE) camelina lines is underway (Linnaeus Plant Sciences Inc., 2011) but prior to commercialization, a detailed risk assessment must be conducted.

In Canada, introduction of GE crops is governed using a framework of science-based risk assessment and risk management measures designed to protect Canada's plant resource base. The Canadian Food Inspection Agency (CFIA) regulates all plants with novel trait(s) (PNT). A PNT is defined as, "A plant that contains a trait(s) which is both new to the Canadian environment and has the potential to affect the specific use and safety of the plant with respect to the environment and human health. These traits can be introduced using biotechnology, mutagenesis, or conventional breeding techniques," (CFIA,

2011b). CFIA regulates PNTs according to substantial equivalence to its conventional counterpart to determine if the novel plant poses additional risks. Environmental assessments are conducted on a case by case basis per PNT applicant and if deemed safe, unconfined environmental release will be authorized. Approved PNTs may be grown commercially and are generally not subjected to terms and conditions although stewardship may be required (CFIA, 2011a). Examinations are conducted on three broad categories of possible impacts (CFIA, 2011b):

1. The potential of the plant to become a weed
2. The potential of the plant to create a weed bu cross-pollinating with another plant
3. The potential impact on biodiversity

Assessment data is intended to contribute to a growing knowledge base about the unconfined release of GE crops into the environment. This enables CFIA scientists to determine how PNTs are likely to behave, interact, and grow in Canadian environments.

Pollen-mediated gene flow (PMGF) occurs between two genetically distinct plants of the same species (intraspecific gene flow) or to a sexually compatible related species (interspecific gene flow). In Canada, PMGF makes it necessary to develop isolation distance for crop breeders. In Europe PMGF is an important consideration for coexistence of conventional and GE crops. Intraspecific gene movement from GE plants may contribute to undesirable,

unintended contaminants in conventional crops termed adventitious presence (AP) (Kershen and McHughen, 2005). Intraspecific gene flow is a naturally occurring process. However, transgene escape into wild and weedy relatives whose hybrid offspring is more weedy may displace native vegetation, evade control measures, and be disadvantageous to biodiversity (Ellstrand et al., 1999).

Seed-mediated gene flow (SMGF) may occur naturally (animals, wind, and water) or as the result of human activities (Mallory-Smith and Zapiola, 2008). Processes include farming and seed shipment permit movement and retention of transgenes in seed. Unlike pollen, seeds are a repository of germplasm capable of enduring substantial time periods. Seed-mediated gene flow generally initiates as seed loss incurred at the time of harvest, or as contaminants in sown seed (Nielson et al., 2009; Warwick et al., 2009). The formation of a 'seed bank' at and below the soil surface enables a species to buffer unfavorable change and persist over time (Thompson, 1987). Weedy traits including proclivity for shatter and the presence of seed dormancy will impact seed bank formation and persistence (Warwick and Stewart, 2005). Species specific seed bank characterization is therefore a key component in developing best management practices and recommendations that mitigate SMGF.

Coexistence of GE, conventional and organic agriculture requires that the respective types are sufficiently segregated to ensure crop purity (Kershen and McHughen, 2005). However, agriculture is an open process in which complete segregation is impossible. Mitigating PMGF and SMGF through stewardship and the adoption of reasonable practices will support coexistence and minimize AP.

1.2. Research objectives

1.2.1. Determine the intraspecific gene flow in camelina

Pollen-mediated gene flow informs decisions about the suitability of the crop for GE applications as well as precautions including isolation distances that may be required during varietal development. Although camelina is reported to be a primarily self-fertilizing species (Francis and Warwick, 2009), outcrossing (OC) rates have not been quantified at a field scale. As part of a detailed risk assessment, the following questions need to be addressed:

- What are camelina OC rates?
- What factors will influence PMGF?

Therefore, the following hypotheses were made and addressed in Chapters 3 and 4:

1. Camelina OC is hypothesized to be low relative to highly outcrossed crops like corn (*Zea mays* L.). It is hypothesized that OC rates will be less than 5% immediately adjacent to the pollen source as seen in other self-fertile crops including wheat (*Triticum aestivum* L.) (Hanson et al., 2005) and safflower (*Carthamus tinctorius* L.)(McPherson et al., 2009). We predict that camelina OC will follow a standard diffusion process that can be characterized as leptokurtic, highest immediately adjacent to the pollen source and diminish rapidly with distance (Warwick et al., 2009) (**Chapters 3 & 4**).

2. There are many factors that influence gene glow including temperature, humidity, presence of winds and other vectors, and size of the pollen source (Mallory-Smith and Zapiola, 2008). The collection of meteorological and observational data was included in the experimental design to account for any anomalies. We anticipate that prevailing winds may influence PMGF and that increasing pollen donor size will increase maximal OC values (**Chapters 3 & 4**).

1.2.2. Determine the frequency of introgression between transgenic camelina and closely related species (interspecific gene flow)

Gene flow to wild and weedy relatives from GE camelina is a significant risk that will need to be addressed prior to unconfined release. Interfertility within the camelina genus has been reported (Séguin-Swartz et al., 2011), but compatibility between members of the Camelineae tribe including shepherd's purse (*Capsella bursa-pastoris* L. Medikus) and ball mustard (*Neslia paniculata* L.) has yet to be established. Therefore, a tiered approach was designed to address the propensity for gene flow within the Camelineae tribe. Tiered assessments, based on scenarios of decreasing severity, limit the amount of data required to address a specific research question without compromising the validity of the results (Raybould and Cooper, 2005; Raybould, 2006). The experimental tiers were as follows:

Tier I: Assess the sexual compatibility of camelina and shepherd's purse by performing pollination on stigmas of emasculated flowers.

Tier II: Assess the propensity for interspecific introgression with insect pollinators present under confined greenhouse conditions.

Tier III: Assess the propensity for interspecific introgression under small-scale field conditions.

Due to the considerable scope of this investigation, our research was only intended to conduct experimental tiers II and III and make preliminary conclusions. Therefore, the following question needs to be addressed:

- What is the likelihood of transgene escape from GE camelina to the weedy relative shepherd's purse?

The following hypothesis is posed in **Chapter 5**:

1. If no events of introgression are detected under worst case conditions, we hypothesize that the probability for harm will be much smaller in a natural setting. We predict interspecific GE introgression to be very unlikely, but due to the high abundance of shepherd's purse, further investigation may be warranted.

1.2.3. Characterize seed-mediated gene flow in camelina

Seed-mediated gene flow may contribute more significantly than pollen to the spread of transgenes for small seeded, self-pollinated species (Beckie and Hall, 2008). Moreover, population dynamics of a weedy species is determined by seed inputs, longevity, and emergence from the seed bank (Buhler et al., 1997).

Seed bank characterization is intended to identify avenues of gene flow that may impact coexistence. In an agricultural setting, seed bank inputs are typically seed loss incurred at the time of harvest that subsequently emerge as volunteer weeds in subsequent crops. Characterization of seed bank additions, longevity, and successful emergence therefore is a key component to developing mitigation measures. There is limited data regarding camelina seed banks, therefore this research was intended to answer the following questions:

- What are typical harvest losses (seed bank additions) in Western Canadian commercial camelina fields and what factors influence loss?
- Do camelina seeds have dormancy?
- How long do camelina seed banks persist?
- How long do populations of volunteer camelina persist in an agricultural environment?

The corresponding hypotheses were posed and addressed in **Chapter 6**:

1. We hypothesize that seed losses at the time of harvest will be numerous due to small seed size.
2. There is a paucity of information on camelina seed dormancy. However, reports indicate that camelina seeds are vigorous and rapid to emerge from the soil (Crowley, 1999; Urbaniak et al., 2008), therefore, we expect that camelina seeds will lack dormancy.

3. We hypothesize that if camelina lacks seed dormancy, it is unlikely to form persistent seed banks. However, if additions are numerous and frequent camelina seed banks may prevail longer.
4. Recently, a study in Montana evaluated invasion potential of camelina in disturbed and undisturbed plots in a rangeland ecosystem (Davis et al., 2011). They found that population growth rate (λ) was low, never exceeding 0.03, and that only plants in disturbed plots survived to maturity. We therefore hypothesize that camelina volunteer populations will be limited in duration. Farm weed control operations including herbicide application and cultivation are anticipated to further reduce longevity.

1.2.4. Determine any inherent allelopathic activity in camelina

Allelopathy describes the inhibitory or stimulatory effect one plant has on another through chemical interactions (Rice, 1974). Camelina has been previously reported to be allelopathic (Grümmer and Beyer, 1960; Lovett and Sagar, 1978). Canola (*Brassica napus* L.; family *Brassicaceae*) has also been reported to have allelopathic activity (Ackroyd and Ngouajio, 2011; Jafariehyazdi and Javidfar, 2011; Uremis et al., 2009), and was therefore used as a comparator. Our research objective was to assess the allelopathic potential of camelina and answer the following questions:

- Does camelina possess allelopathic activity?
- Is camelina allelopathy specific to certain species recipients?

These questions are addressed in **Chapter 7**:

1. We hypothesize that camelina will be allelopathic, but due to discordant findings (Grümmer and Beyer, 1960; Lovett and Sagar, 1978; Lovett and Jackson, 1980; Lovett and Duffield, 1981), we are unsure if the effect will be stimulatory or inhibitory.
2. Camelina, commonly known as 'false flax', was once considered a weedy species in association with flax crops (Francis and Warwick, 2009; Zohary and Hopf, 2000). We hypothesize that allelopathic responses will vary, but ecological relationships may influence responses.

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Chapter 2. Literature review

2.1. Environmental risk assessments

Genetically engineered (GE) crops are assessed for food, feed, and environmental safety prior to approval for unconfined release in Canada (CFIA, 2011a). The responsibility for regulating GE crops in Canada lies with two government agencies, the Canadian Food Inspection Agency (CFIA) and Health Canada. Environmental safety is the responsibility of the Plant Biosafety Office (PBO) of the CFIA, feed safety is determined by the Animal Feed Division of the CFIA, and food safety by Health Canada. The PBO is also responsible for post-commercialization and monitoring activities of GE crops (CFIA, 2011a). When regulatory decisions are made the CFIA publishes publicly available "decision documents" that summarize the information used in decision making.

In Canada, GE crops are classified as plants with novel traits (PNTs). A PNT is defined as "a plant that is novel to stable cultivated species of that particular plant species with the potential to have an environmental effect" (CFIA, 2011b), and includes plants derived from mutagenesis and conventional breeding. Environmental safety assessments examine three broad categories (CFIA, 2011a):

- The potential of the plant to become a weed
- The potential of the plant to create a weed bu cross-pollinating with another plant
- The potential impact on biodiversity

Assessments cannot prove the complete absence of risk. The principle of substantial equivalence is therefore employed so that a PNT may be evaluated relative to its conventional counterpart (CFIA, 2011a). Regulatory decisions are made following examination of outlined risks in association with environmental and government policy thresholds that determine if risks are "acceptable" or "unacceptable" (Raybould and Cooper, 2005). Regulatory decisions are based on scientific data and each assessment is conducted on a case-by-case basis.

Assessments must consider not only the potential risks of a PNT, but the likelihood that harm will result. Risk is therefore defined as a function (f) of hazard (harm) and its probability of exposure [risk = f(hazard, exposure)] (Wilkinson et al., 2003). For example, transgenic *Bacillus thuringiensis* corn (*Zea mays* L.) pollen can be harmful to monarch butterfly (*Danaus plexippus* L.) larvae (Losey et al., 1999). However, lethal concentrations are unlikely to be present in an agricultural setting, therefore the risk is considered to be acceptable (Shelton and Sears, 2001; Dively et al., 2004). If properly formulated, a risk assessment can provide high confidence of minimal risk (Raybould, 2006).

2.1.1. Tiered risk assessments

Tiered risk assessments begin with artificial 'worst case' scenarios and then move to more environmentally realistic ones (Wilkinson et al., 2003; Raybould and Cooper, 2005). It follows a non-linear approach, so that the most relevant lines of evidence are brought forward (Wolt, 2009). Tiered testing is

designed to be time and resource efficient and limit the collection of superfluous data. This system is routinely used to test the toxicity of pesticides, assess food safety, and to quantify environmental risks associated with GE crop cultivation.

The first tier (Tier 0), is a preparatory phase where the experiment is planned (Garcia-Alonso et al., 2006). Tier 0, also known as the 'problem formulation step', focuses the assessment and ensures that conclusions drawn will be appropriate to the decision making process (Garcia-Alonso et al., 2006). This includes research on the plant species, the GE trait, the environment in which it is to be introduced, and the potential hazards of introduction. All information gathered at this stage will be retained and utilised in the synthesis and interpretation of subsequent tiers.

Tier I, the first experimental tier, is conducted under 'worst case' conditions (Raybould and Cooper, 2005). The analytical phase of any risk assessment is initiated with tests that conservatively address broad questions using simple experimental designs (Wolt, 2009). This assessment is not intended to be realistic, rather to maximize the chance for detection of hazard occurrence and minimize the chance of committing Type II errors. In tier I, laboratory or greenhouse conditions are generally preferred over field studies, as they remove environmental factors that may complicate observations and results (Garcia-Alonso et al., 2006). For example, if one is assessing the potential for interspecific hybridization between two species, tier I may be the emasculation of the pollen receptor followed by manual pollination, and perhaps even embryo rescue of the hybrid (Raybould, 2006). If no hybrids are detected (no occurrence of hazard)

testing can be terminated based on the assumption that hybrid formation (hazard) will be less likely under more natural (less artificial) conditions and risk can be deemed low. If hazards are detected in tier I, higher tier tests (tier II) should be performed to better assess risk (Raybould and Cooper, 2005; Wolt, 2009).

Tier II experiments are intended to be more realistic. They may include additional laboratory or greenhouse experiments, or progress to small-plot field experiments (Raybould and Cooper, 2005). For instance, tier II may involve small-plot experiments that encourage hybridization events by minimizing the distance between pollen donor and acceptor populations. If tier II results indicate that risk is acceptable or negligible, testing may terminate (Garcia-Alonso et al., 2006). However, if results from tier II indicate a potential risk, higher tiered tests will be employed.

Tier III tests are designed to mimic more natural conditions and may be conducted as medium to large-scale field experiments (Raybould and Cooper, 2005). These studies are often laborious and the results provided can be difficult to interpret without the aid of the previous tiers' data (Garcia-Alonso et al., 2006). If the results from Tier III indicate the risk level to be acceptable, no further experiments will be required. If the results from Tier III confirm sufficient risk, further refinement may be required, or a decision of unacceptable risk may be made.

This systematic scientific tiered approach is iterative, where knowledge obtained in lower tiers directs data collection at higher tiers (Wilkinson et al.,

2003; Garcia-Alonso et al., 2006). Risk assessments analyze the probability that harm will occur, the likely magnitude of harm and the uncertainty associated with those predictions (Raybould and Cooper, 2005). A poorly formulated risk assessment will increase uncertainty and negatively affect proper decision making. A tiered approach to risk assessment provides a foundation of knowledge which informs subsequent regulatory decision making.

2.2. Gene flow

Gene flow is the change in gene frequency in a population due to the movement of gametes, individuals, or groups of individuals from one location to another (Slatkin, 1987). Within an agronomic system avenues of gene flow are not mutually exclusive and are often linked (Warwick et al., 2009). As such, the potential for gene flow from GE to non-GE crops exists. The unintentional presence of GE materials, also known as adventitious presence (AP), is a technical inevitability as agriculture is an open process (Kershen and McHughen, 2005). Adventitious presence may result from extraneous pollen movement, sown seed contaminants, crop volunteers, and commingling of seed during planting, harvesting, transportation, and storage (Mallory-Smith and Zapiola, 2008). The concern is that the mere presence of transgenes will reduce the value of its receptor population or commodity, notably in export markets. Due to the scale of GE agriculture, it is unrealistic to maintain a zero level of AP.

The ability for conventional, organic, and GE agriculture to co-exist requires establishment of verifiable, attainable threshold levels for AP. In Canada, any GE plant currently granted unconfined environmental release is considered substantially equivalent and does not require segregation (CFIA, 2011a). However, in exports, AP must remain at or below country-specific thresholds. For example, the European Union (EU) requires that any materials containing 0.9% or more approved GE events be labeled as genetically modified (European Commission, 2003).

Globally, there is a 0% threshold for unapproved GE events. The inadvertent presence of small amounts of GE events that have received regulatory approval in one or more countries, but remain unauthorized in others is known as low level presence (LLP) (Kalaitzandonakes, 2011). Reasons for LLP include asynchronicity of approval between countries, expiration of time limited approval, or transgene escape from confined research trials (Kalaitzandonakes, 2011).

Trade disruption can result from LLP. In 1998, sulfonylurea-resistant flax (*Linum usitatissimum* L.)(CDC Triffid; Event PF967) was approved for safe release in the US and Canada (CFIA, 2001). The application was withdrawn prior to commercialization at the request of the Flax Council of Canada to avoid trade concerns with the EU, the primary export market. Approval of event P967 was not completed in the EU, thus a zero tolerance was established. In September of 2009, GE material was discovered in Canadian flax shipments to Europe (Flax Council of Canada, 2009). The original source was not conclusively determined. This incident led to a disruption in the trade of Canadian flax to many countries. The

United States encountered similar problems in the late 1990's with the release of GE events LL601rice and Starlink corn (Schmitz et al., 2005; Quirasco et al., 2008). Although both events were approved, LL601 rice and Starlink corn were not approved for commercial and food applications, respectively. In 2000 and 2001 Starlink corn was found in food products and LL601 in commercial shipments in 2006. The discovery of unapproved events lead to considerable litigation and market disruption. Given the consequences, gene flow quantification is an essential element of risk assessment.

2.3. Pollen-mediated gene flow

Pollen-mediated gene flow (PMGF) is the transfer and introgression of genes or genetic information via pollen. This may occur between two genetically distinct plants of the same species (intraspecific gene flow) or between sexually compatible related species (interspecific gene flow). Successful PMGF requires sympatry between donor and receptor populations, synchronous flowering, the presence of pollen vectors, and minimal physical barriers (Lamkey, 2002). Moreover, the event must produce a viable seed and genetic information from the donor must stably introgress into the genome of the hybrid (Warwick et al., 2009).

In agriculture, the potential for PMGF is influenced by a crop's reproductive biology. Pollen-mediated gene flow is high for obligate outcrossers, like corn (Aylor et al., 2003), when compared to self-pollinated crops including flax (Jhala et al., 2011) and wheat (*Triticum aestivum* L.) (Beckie et al., 2011).

Corn produces large amounts of wind-dispersed pollen; PMGF has been reported to range from 9 to 47% 1 m from the pollen source, but drop to less than 0.5% at 35 m and beyond (Goggi et al., 2006). However, PMGF in flax was 1.85% at 1 m and less than 0.005% at 35 m (Jhala et al., 2011).

For both highly outcrossed and highly selfed species, PMGF is highest immediately adjacent to the pollen source and rapidly declines as distance from the source increases (Beckie and Hall, 2008). This distribution, often described by a leptokurtic curve, is well documented and has been reported in species including canola (*Brassica napus* L.) (Salisbury, 2002), safflower (*Carthamus tinctorius* L.) (McPherson et al., 2009b), soybean (*Glycine max* L.) (Abud et al., 2007), triticale (x *Triticosecale* Whitm. ex A. camus) (Kavanagh et al., 2012), and rice (*Oryza sativa* L.) (Rong et al., 2010). Pollen-mediated gene flow is asymptotic and theoretically never reaches zero. For instance, in Oregon, PMGF in creeping bentgrass (*Agrostis stolonifera* L.), a wind-pollinated species, was detected at distances greater than 20 km (Watrud et al., 2004). Even for the self-fertile wheat, PMGF has been detected as far as 2.75 km (Matus-Cadiz et al., 2007). Therefore some level of AP should be assumed, even at long distances from the pollen donor population.

2.3.1. Wild and weedy interspecific hybridization

Transgene escape into wild and weedy relatives has been identified as a key consideration in environmental risk assessment (Ellstrand and Hoffman,

1990; Raybould and Gray, 1993; Raybould and Cooper, 2005). Over the past two decades, considerable growth in the area and number of GE crops cultivated (James, 2011) has increased awareness of transgene movement into non-agricultural ecoregions. Crops and weeds have exchanged genes for centuries (Ellstrand et al., 1999), however, introgression of novel genes into wild populations may negatively impact Canadian biodiversity.

Typically, interspecific hybridization occurs with great difficulty. In addition to the challenges associated with PMGF discussed previously, the potential for interspecific hybridization is further reduced by factors including genomic compatibility and impaired F_1 hybrid fertility and fitness (Warwick et al., 2009). First generation hybrids are often unstable and require the formation of backcross generations for fit progeny (Ellstrand, 2003). While the risks may be low, a dominant advantageous transgene may theoretically only arise once in a population and become established.

Crop genes generally confer limited fitness benefits to wild plants (Warwick and Stewart, 2005). However, the impacts are dependent upon the introduced trait and ecology of the recipient population (Snow et al., 2005). Hybrids have been detected between canola and field mustard (*Brassica rapa* L.) (Simard et al., 2006). Hybridization rates ranged from 1.1 to 17.5% and were dependent on density, sympatry, and the spatial arrangement of related species. Similarly, gene flow between herbicide resistant wheat and jointed goatgrass (*Aegilops cylindrical* L.) was observed with hybrids found at up to 40 m (Hanson et al., 2005). Low rates of PMGF were reported between herbicide-resistant rice

and relative red rice ranging from 0.003 to 0.008% (Shivrain et al., 2007). To date, the few instances of transgene movement into wild or weedy species have been more of "an academic interest than of any sort of environmental problem", (Ellstrand, 2012). However, the potential for interspecific hybrids to form and persist remains a necessary risk assessment component.

2.4. Seed-mediated gene flow

Seed-mediated gene flow (SMGF) enables transgenes to quickly move long distances and persist over time (Warwick et al., 2009). Volunteer and feral populations, high seed production, high shatter propensity, and long distance seed transport favor SMGF, while the cleaning of machinery, using certified seed, and covering shipments are limiting (Mallory-Smith and Zapiola, 2008). Long distance gene flow is beyond the scope of this review.

Short distance SMGF typically initiates as loss incurred during harvest, or as seed contaminants when planting a conventional crop (Figure 2-1) (Nielson et al., 2009; Warwick et al., 2009). While management of volunteer weeds can help mitigate gene flow incurred from harvest losses, SMGF from a contaminated seed source may not be immediately evident. Prediction of SMGF is difficult as it is influenced by cropping practices, equipment, and complex human decisions, and therefore can be estimated but not predicted solely on the biology of the plant (Lu, 2008).

2.4.1. The soil seed bank

The seed bank is comprised of all viable seeds at and below the soil surface at a specific location and time (Thompson and Grime, 1979), where 'seed' includes both fruit and seeds (Cavers, 1995). Seed bank formation enables plant populations to buffer against unfavorable environmental events and persist over time. Continuity over time requires that a portion of seeds are physiologically capable of germination when favorable environmental conditions occur (Buhler et al., 1997). The population's regenerative ability is therefore dependent on seed output as well as the resultant offspring's ability to exploit opportunities for regeneration (Thompson and Grime, 1979). Nielson et al. (2009) illustrate the general flow of seeds through the seed bank highlighting the most prominent factors affecting entry and exit (Figure 2-1).

Seed bank entry occurs via seed rain when seeds disperse from the parent plant. Seeds that disperse from the parent plant may land on the soil surface or become buried. The quantity dispersed is dependent on a complex interplay of biological and abiotic factors including the fecundity and fitness of the parent plant, environmental conditions, and the competitive ability of surrounding plants. Following seed rain, secondary dispersal by animals, wind, water, and humans makes seed susceptible to long distance transport (Bakker et al., 1996). In agricultural regions, crop seed dispersal incurred at the time of harvest generally constitutes the most fundamental crop seed bank addition (Figure 2-1) (Gulden et al., 2003; Nielson et al., 2009).

Numerous domesticated crops have been investigated to quantify seed losses incurred at the time of harvest and results have varied by crop, possibly related to seed size, and were spatially variable. Losses in wheat averaged ~300 seeds m^{-2} (Clarke, 1985); 231 to 1,069 seeds m^{-2} in safflower fields (*Carthamus tinctorius* L.) (McPherson et al., 2009a); losses in flax ranged from 22 to 1,986 seeds m^{-2} (Dexter et al., 2011); and loss of up to ~3,000 seeds m^{-2} was observed in canola fields (Gulden et al., 2003). In all studies seed loss exceeding the recommended seeding rate was documented, with the highest quantified loss being in canola with a loss 56- fold greater than seeding rate (Gulden et al., 2003).

Seed bank additions are linked to environmental conditions and grower practices. Seed bank deposition can occur naturally by shatter which may be aided by hail, disease, lodging, or insect herbivory (Willenborg and Van Acker, 2008). However, cultivar selection may be used as a tool to reduce loss incurred from shatter (Wang et al., 2007). Grower choices including harvest timing, type of combine, and equipment settings may influence the density and distribution of seed dispersal (Gulden et al., 2003). Settings including travel speed and harvesting method (direct vs. windrow harvested crops) greatly influence seed loss. Dexter et al. (2011) reported losses in flax ranging from 53 to 117 seeds m^{-2} in inter-windrow spaces and 696 to 1,896 seeds m^{-2} under windrows. Large harvest losses are attributed in part to grower practices. Therefore, it is possible that seed loss may be reduced by optimizing management practices.

Seeds may exit the seed bank though predation, decay, disease or germination (Figure 2-1) (Nielson et al., 2009). Seeds on the soil surface are

vulnerable to predation and adverse environmental conditions, while those that are below the soil surface may exit by means of exhaustion and decay. Currently, the role of seed pathogens in seed persistence is poorly understood (Dalling et al., 2011). Emergence from the seed bank occurs via seed germination, but, failure to reach the soil surface or seedling death will prevent seed bank replenishment. Only a portion of seed bank entrants can contribute genes to future generations. Therefore factors that influence propagule depletion are an important determinant of the seed bank's genetic makeup.

Seed predation, also termed granivory, is a type of predation where a mobile predator consumes a sessile prey (Menalled et al., 2006). Seeds may be predated upon before (pre-dispersal) and after (post-dispersal) release from the mother plant. Seed predators include vertebrates (birds, mammals) and invertebrates (primarily insects). Most pre-dispersal predators are sedentary, small, specialist feeders belonging to insect orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Crawley, 2000). Pre-dispersal predation prevents seeds from entering the seed bank. Conversely, most post-dispersal predators tend to be larger in size, more mobile, generalist herbivores including granivorous birds and rodents (Crawley, 2000). The influence of post-dispersal predators on seed banks in western Canada has not been evaluated.

Germination does not guarantee plant survival. Seed depth influences exit from the soil and can negatively impact seedling emergence. Schwinghamer and Van Acker (2008) reported that kochia seeds [*Kochia scoparia* (L.) Schrad.] were unable to emerge from a depth of 80 mm. Limited seed reserves, particularly with

small seeds and in instances of great soil depth, may become exhausted resulting in seedling death (Forcella et al., 2000). Seedlings that successfully emerge are then susceptible to plant competition, adverse weather conditions, control measures including mowing or herbicide application, and any other barriers that prevent seed set and dispersal.

Choice of tillage system influences seed bank composition. Tillage operations disrupt the soil distribution, destroy seedlings, trigger new germination, and stratify the seed bank (Mohler et al., 2006; Schwinghamer and Van Acker, 2008; Légère et al., 2011). Consequently, reduced tillage systems will have a greater proportion of seeds on the soil surface. High density weed populations have been observed in reduced tillage systems as surface seeds may be better suited for germination (Légère et al., 2011). However, a positive correlation between tillage and secondary dormancy exists in canola (Gulden et al., 2004). Surface seeds are exposed to adverse conditions including exposure to environmental conditions, vulnerability to predation, and contact with crop residues (Marino et al., 2005; Menalled et al., 2007; Blackshaw and Molnar, 2008). Temperature, moisture, and soil environment immediately surrounding the seed, known as the microsite, are responsible for differential seedling emergence (Bullied et al., 2012). Due to the relative complexity of seed banks, species specific characterization is required to understand the contribution that seed banks may make to gene flow.

2.4.2. Seed dormancy

Mechanisms of dormancy and seed germination will impact seed bank composition (Thompson and Grime, 1979). Dormancy enables seeds to persist and buffer genetic change over time. Dormancy is defined as the "temporary failure of a viable seed to germinate, after a specified length of time, in a particular set of environmental conditions that later evoke germination when the restrictive state(s) has been terminated either by natural or artificial means" (Simpson, 1990). Alternatively, non-dormant seeds have the capacity to germinate over the widest range of normal physical environmental factors possible for that genotype (Baskin and Baskin, 2004). Seed dormancy is not to be confused with quiescence, when seeds in a fully viable state fail to germinate due to limiting external conditions including moisture, light, oxygen, and temperature (Foley, 2001).

There are two distinct phases of seed dormancy: primary and secondary (Baskin and Baskin, 2004). Primary, also known as innate, dormancy is inherited and develops during seed maturation (Bewley, 1997). It is present on, or soon after, release from the mother plant (Foley, 2001). After-ripening occurs as seeds dry and are released from primary dormancy and is influenced by air temperature, seed moisture content, time, and plant species (Murdoch and Ellis, 2000; Foley, 2001). As after-ripening develops, seeds become able to germinate over a widening range of conditions (Baskin and Baskin, 2004). Secondary, also known as induced, dormancy occurs when previously non-dormant seeds become dormant upon introduction to unfavorable conditions (Baskin and Baskin, 2004).

Seeds may undergo dormancy cycling when conditions are unfavorable, gradually moving in and out of dormancy until seed germination or death is realized (Finkelstein et al., 2008).

Seed banks are classified according to longevity which is linked to seed dormancy. Plants whose seed banks are 'transient' are those in which seeds primarily germinate or expire within one year of plant maturation, whilst those that have viable seed beyond one year are considered 'persistent' (Thompson and Grime, 1979; Cavers, 1983). Transient seed banks are typical of plants with short term primary dormancy and little to no secondary dormancy. Those with persistent seed banks may have extended primary dormancy, or a high degree of secondary dormancy, which is often triggered in response to unfavorable environmental conditions (Foley, 2001). Domesticated crop cultivars are most often selected for reduced dormancy to ensure expedient and uniform germination, followed by prompt seedling establishment to attain maximum yields (Warwick and Stewart, 2005). However some crops, including canola, may possess levels of secondary dormancy that have been linked to weedy populations (Gulden et al., 2004). Seed bank dynamics of a particular crop species will influence the potential for seed and pollen-mediated gene flow in years following cultivation.

2.4.3. Artificial Seed Bank Experiments

Artificial seed banks are an accepted research method that estimates longevity and capability for seed survival under a relatively constant environment (Conn et al., 2006). They are practical tools for gaining information, but have limitations. These include: differential responses to biotic and abiotic factors that would otherwise influence germination, emergence, and loss from the seed bank (Leon and Owen, 2004). Artificial seed banks may aggregate seeds for experimentation and data collection. Densities higher than naturally observed may underestimate persistence through increased vulnerability to soil borne pathogens and fungi and predators (Van Mourik et al., 2005). Artificial seed banks may also ignore the stimulatory effects of tillage on germination (Conn et al., 2006). Any containment measure employed may prevent vertebrate and invertebrate predation which would otherwise occur in natural conditions. Findings from artificial seed bank experiments are to be viewed with caution.

2.4.4. Agricultural volunteer populations

Crop volunteers are abundant agricultural weeds, both species without secondary dormancy (wheat, barley) and those with (canola). In weed surveys of the Canadian Prairies conducted post in-crop herbicide for the 1970's through 2000's, volunteer wheat was ranked between 12 and 31 of the most abundant weeds (Leeson et al., 2005). Wheat forms transient seed banks that are depleted to near extinction within two years of dispersal due to germination and degradation (De Corby et al., 2007; Harker et al., 2005; Nielson et al., 2009) (Figure 2-1).

However, canola seeds possess secondary dormancy, influenced by genotype, burial depth, and temperature (Gulden et al., 2004), and have been observed to persist for at least 4 years following cultivation (Simard et al., 2002). Best management practices including the choice of a suitable crop rotation can aid in volunteer management. For instance, wheat volunteers may be difficult to control in barley (*Hordeum vulgare* L.), however, planting a broad leaf crop like flax may increase control options.

In addition to competing for resources and affecting crop quality, volunteers may contribute to gene movement via PMGF and SMGF (Warwick et al., 2009). If volunteer plants are not controlled, mature seed may replenish the seed bank or constitute a source of AP if harvested with another crop. The propensity for PMGF and SMGF of volunteers is dependent, in part, on seed bank persistence and the efficacy of control measures.

2.5. Camelina sativa

2.5.1 History of camelina

Camelina sativa (L.) Crantz. is a European old world oilseed crop (Francis and Warwick, 2009) considered to have originated in the steppes of Southeastern Europe and Southwestern Asia (Knörzer, 1978). Historical evidence suggests camelina was present in Neolithic western France, nearly 3000 years before cultivation throughout France (Bouby, 1998). It is believed that camelina was cultivated sporadically in Europe in the Medieval times and became widespread in

Russia and some European countries before being abandoned in the 1940's (Knorzer, 1978; Zubr, 1997). The decline in cultivation was attributed to a low yield relative to other oilseeds, the complexity of its oils, and a general lack of interest and knowledge (Crowley, 1999; Johnson, 2007).

Camelina's entrance to Canada was most likely facilitated by a weed infestation in flax and cereals fields (Zohary and Hopf, 2000; Francis and Warwick, 2009). As its common name, 'false flax', implies camelina was often seen as a contaminant in early European flax fields. *Camelina sativa* was the first of the *Camelina* ssp. to be mentioned in North American botanical literature, occurring in fields and cultivated grounds (Torrey and Gray, 1838-1840). It was initially identified at the Red River settlement, Manitoba in 1863 (Scoggan, 1957). In the 1960's it was first grown as an oil seed crop in Canada (Plessers et al., 1962). There has a been a recent resurgence of interest in camelina due in part to its adaptation to various climatic conditions, low nutrient requirement, resistance to disease and pests of the brassicas, and the potential for use as a biodiesel feedstock (Urbaniak et al., 2008; Francis and Warwick, 2009; Johnson et al., 2010). In February of 2013, the United States Environment Protection Agency qualified camelina oil as a biomass-based diesel and advanced biofuel for utilization in biodiesel and renewable diesel (including jet fuel and heating oil) (United States EPA, 2013).

2.5.2. Biology of camelina

Camelina is a member of the Brassicaceae family, in the Capparales order within the Dilleniidae subclass (USDA, 2013). Camelina is a hexaploid (Hutcheon et al., 2010) with 2n=40 being the most common chromosomal count (Francis and Warwick, 2009). It is an annual, herbaceous, dicotyledonous, tap rooted species. Stems are single, branched above, erect, hairy, and become woody at maturity. Plants form a rosette prior to stem elongation which can reach heights of 30 to 90 cm (Meakin, 2007). Leaves are sessile, oblanceate to lanceolate, sparsely pubescent, toothed to entire in an alternate arrangement (Francis and Warwick, 2009). Inflorescences are ebracteate, borne on racemes in terminal clusters. Each flower has four spatulate yellow petals, 4 to 5 mm in length, four green sepals, and 6 stamens in 3 pairs of unequal length (Francis and Warwick, 2009). Camelina seed pods are siliques; globular and rounded, divided by a septum and typically contain 10 to 25 seeds (Schuster and Friedt, 1998). Camelina seeds are brown to light brown with a thousand kernel weight (TKW) of 1 to 2 g (Meakin, 2007) and 33 to 44% oil content (Francis and Warwick, 2009).

Flowers exhibit perfect sexual reproduction, previously described as self-fertile (Tedin, 1922; Schultze-Motel, 1986). Camelina reproduces by seed and does not propagate vegetatively (Francis and Warwick, 2009). The flowers are protogynous (stigma is ripe before anthers) and are open for several hours throughout the day, primarily by mid-morning. One to two hours prior to flower opening, the four tall anthers become ripe, but may not burst until the flower opens. Upon flower opening, the four long stamens reach stigmal height and

begin to release their pollen followed by the short stamens several hours later. Flowers close by evening, thus directing stamens towards the stigma, facilitating major pollen deposition from the tall anthers. By day two, the tall anthers are empty while the short pair still contains a small amount of pollen. By day three, anthers are empty and petals and sepals begin to wilt and fall off. Flowering is initiated at the base of the raceme and the process is repeated upwards as the stem elongates. Currently there are no reports of pollen vectors, however, Tedin (1922) suggested that pollination by bees may account for a small percentage (less than 3%) of outcrossing. At present, camelina outcrossing rates have yet to be documented in a field setting.

2.5.3. Agronomy of camelina

Camelina is suited to a wide variety of environments as demonstrated by a history of cultivation throughout Europe followed by more recent introductions into North American environments. Traits including early maturity (80 to 100 days), high yield potential, and tolerance to drought and frost make it suitable for cultivation in Canada (Gugel and Falk, 2006). Compatibility to existing farming methods permits relatively facile cultivation (Gugel and Falk, 2006; Johnson et al., 2010). Currently, a limited number of camelina cultivars are grown in Canada, but breeding efforts are ongoing to develop new lines that may increase yields and production (Urbaniak et al., 2008).

As diversified crop rotations becoming increasingly important, adoption of this minor use crop is expected to rise. Camelina fits well into most crop rotations, suitable at the end due to minimal fertility requirements or a good diversification crop, seeded before or after a cereal crop (Meakin, 2007). Moreover, early maturity and seeding date flexibility may alleviate harvest time constraints (Plessers et al., 1962).

Although camelina is touted as a low input crop, it responds positively to nitrogen additions (Crowley, 1999). Trials conducted on the Canadian prairies found that yield plateaued around 100 kg of nitrogen ha^{-1} similar to other Brassicaceae crops (Johnson et al., 2008). Another study conducted out of the north west United States reported that camelina requires 12 kg N ha^{-1} per 100 kg of expected seed (Wysocki et al., 2013). Despite the benefits, risk of lodging and propensity for weed infestations increased with the amount of nitrogen applied (Zubr, 1997; Crowley, 1999).

Camelina is typically spring sown, but fall seeding, primarily in the US, has also been explored (Zubr, 1997). Schillinger et al. (2012) investigated planting dates in the fall through spring and found that late winter to early spring planting dates achieved the highest yields. This small seeded crop can be challenging to establish as seeding depth must be approximately 1 cm with good soil contact (Putnam et al., 1993; Meakin, 2007). Urbaniak et al. (2008) reported that utilization of a seed drill and a forage seeder were both effective methods of crop establishment, but the forage seeder proved more effective at creating a uniform crop stand. Recommended seeding rates range from 5 to 8 kg ha^{-1},

approximately corresponding to 400 to 900 seeds m^{-2}, intended to establish stands of 200 to 250 plants m^{-2} (Zubr, 1997; Meakin, 2007; Urbaniak et al., 2008). Camelina may be direct harvested or swathed at maturity (Zubr, 1997) with yields typically ranging between 1500 and 2500 kg ha^{-1} (Plessers et al., 1962; Meakin, 2007; Urbaniak et al., 2008). Higher seeding rates increase crop competitiveness and reduce the time to maturity (Johnson et al., 2008).

Currently, in-crop herbicide options are limited in camelina production. Camelina is competitive and quick to emerge post planting but seedbed preparation by application of a non-selective herbicide is still recommended (Meakin, 2007). Camelina has been shown to be tolerant to dinitroalanine herbicides including trifluralin, pendimethalin, and ethafluralin (Crowley, 1999).

Camelina volunteers are relatively easy to control due to their herbicide sensitivity. In Canada, quizalofop-p-ethyl has received a User Requested Minor Use Label Expansion and in the United States, sethoxydim for the control of perennial and annual grassy weed control in camelina (CFIA, 2011c).

2.5.4 Pests and diseases of camelina

Camelina shows tolerance to many insect pests of the Brassicaceae family. Camelina is a non-preferred host to several insects including the crucifer flea beetle [*Phyllotreta cruciferae* (Coleoptera: Chrysomelidae: Alticinae)] (Henderson et al., 2004; Onyilagha et al., 2012), the cabbage root fly [*Delia brassicae* (Wiedemann) (Diptera: Anthomyiidae)] (Finch, 1978), and the cabbage

seed pod weevil *Ceutorhyncus obstrictus* (Marsh.) (Cárcamo et al., 2007). Additionally, *C. microcarpa* has exhibited tolerance to the swede midge [*Contarinia nasturtii* Kieffer (Diptera: Cecidomyiidae)] (Hallett, 2007).

A detailed investigation of camelina diseases was conducted by Séguin-Swartz et al. (2009). They found that camelina is robust and considered to be resistant to a number of diseases that infect canola including blackleg [*Leptosphaeria maculans* (Desmaz.) Ces. & de Not.] and *Alternaria* black spot [*Alternaria brassicae* (Berk.) Sacc.]. Genotypes resistant to sclerotinia stem rot [*Sclerotinia sclerotiorum* (Lib.) de Bary], brown girdling root rot (*Rhizoctonia solani* Kühn, *Fusarium* ssp.), and downy mildew (*Peronospora camelinae* Gäum.) suggest that breeding efforts may produce resistant cultivars. Camelina is susceptible to club root (*Plasmodiophora brassicae* Woronin), white rust (*Albugo candida*) and aster yellows (‘*Candidatus* Phytoplasma asteris’ Lee et al.; Séguin-Swartz et al., 2009). A deeper understanding of camelina is key in the development of cultivation practices that contribute to pest and disease control, increased yields, and seed bank management.

2.5.5 Camelina products and applications

Camelina oil is versatile and has potential applications in cosmetics, industry, human nutrition, and biofuel (Budin et al., 1995; Zubr, 1997; Frohlich and Rice, 2005). Analogous to flax oil, camelina is high in polyunsaturated fatty acids but, the presence of tocopherols increases its relative oxidative stability

(Zubr, 1997; Shukla et al., 2002; Szterk et al., 2010). Fuel properties of camelina oil are well within specifications but performance and viscosity in low temperature climates may be disadvantageous (Frohlich and Rice, 2005). Further improvements in the quantity and fatty acid profile of camelina oil may improve its utility and broaden its applications.

The protein content of camelina seed meal is suitable for animal feed (Zubr, 1997). The meal is approximately 28 to 33% protein with a favorable balance of amino acids, making it a potentially valuable feed for poultry, swine and ruminants and fish (Aziza et al., 2010). The US Food and Drug Administration (FDA) has approved use of camelina meal up to 10% in feed for cattle in confinement for slaughter, broiler chickens, and laying hens (CFIA, 2011c). The FDA additionally approved camelina meal up to 2% in the diet of growing swine. Currently, camelina meal has not been approved for use as animal feed in Canada. The presence of glucosinolates, sinapine, and phytic acid in camelina seed may limit utility in animal feed, causing reduced bioavailability of proteins and minerals (Matthaus and Zubr, 2000). A German study measured the glucosinolate content of 10 camelina genotypes and reported variability, citing that environment was an evident influence (Schuster and Friedt, 1998). Schuster and Friedt (1998) further stated that reduction of glucosinolates may be an aim of future breeding efforts.

2.5.6 Genetic engineering of camelina

Genetic engineering of camelina, in combination with conventional breeding, may enhance the agronomy and utility of this crop. Research and development of camelina bio-industrial oils (Linnaeus Plant Sciences, 2007) and bio-plastics (Metabolix Oilseeds, 2012) is ongoing. Lu and Kang (2008) employed *Agrobacterium*-transformation by floral dipping along with vacuum infiltration to introduce a CDNA encoding castor bean (*Ricinus communis* L.) hydroxylase. The novel gene permits *in planta* synthesis of ricinoleic acid, a valuable bio-industrial oil used in greases, lubricants, varnishes and hydraulic fluids (Naughton, 1974). Subsequently, Liu et al. (2012) optimized camelina *Agrobacterium*-mediated transformation by floral dipping, requiring no vacuum infiltration, with transformation efficiencies of up to 0.8%. The agronomic characteristics of camelina and its amenability to genetic manipulation make it an attractive option for biofuel and bio-industrial applications (Putnam et al., 1993).

2.5.7 Species related to camelina in North America

Camelina ssp. are reported to be interfertile within the *Camelina* genus (Tedin, 1922; Séguin-Swartz et al., 2011), but compatibility within the larger Camelineae tribe is unknown. *Camelina microcarpa* Andrz. Ex DC.*a* and *Camelina alyssum* (Mill.) Thell. are present throughout Canada and the United States, while *Camelina rumelica* Velen. has been found only in the United States and is yet to be reported in Canada (USDA, 2013). *C. alyssum* has limited distribution in Canada, found only in Alberta, Saskatchewan and Manitoba in

prairie fields and on roadsides (Francis and Warwick, 2009). In contrast, *C. microcarpa* has been identified in all of the provinces and the Yukon Territory (USDA, 2013). Within the larger Camelineae tribe, *Arabidopsis lyrata* L. and *A. thaliana* (L.) Heynh., *Capsella bursa-pastoris* (L.) Medik. (shepherd's purse), *Neslia paniculata* (L.) Desv., *Erysimum* spp., and *Turritis glabra* L. are also present in Canada (USDA, 2013).

Shepherd's purse, of the camelineae tribe, is most abundant in distribution and has been identified in every province, territory and state in North America (USDA, 2013). Shepherd's purse is native to southwest Asia and has been naturalized elsewhere as a cosmopolitan weed (Zhou et al., 2001). It is regarded as the second most common weed worldwide (Zhou et al., 2001) and is distributed throughout the earth's temperate region excepting the hot and wet tropics (Hurka and Neuffer, 1997). In agricultural surveys conducted post in-crop herbicide application in western Canadian fields for the 1990's, shepherd's purse occurred in 9.2% of all of the 3806 fields surveyed and was among the 25 most abundant weed species (Leeson et al., 2005).

Shepherd's purse is an annual to winter annual with a high degree of phenotypic plasticity. The fruits are siliques capable of producing up to 39 seeds each; up to 40,000 per plant with thousand TKW of 0.15 g (Aksoy et al., 1998). This weed is a primarily self-pollinating species attributed to protogyny, however, pollen remains viable for several hours and thus a 1-2% outcrossing rate may be observed (Aksoy et al., 1998). Shepherd's purse is a tetraploid with chromosome numbers generally 2n=32 (Aksoy et al., 1998).

2.6. Allelopathy

Allelopathy refers to the stimulatory or inhibitory effect through chemicals released into the environment from one plant on growth and development on another living organism (Inderjit et al., 2011). Chemicals that mediate interspecific interactions, otherwise known as allelochemicals, are primarily plant secondary metabolites including benzoxazinoids, glucosinolates, phenolics, and sesquiterpenoids (Mizutani, 1999). Potentially allelopathic compounds are liberated from plants by (a) leaching of foliage by rain, (b) abscission and litter fall, (c) volatilization from foliage, and (d) root exudation (Tukey, 1969). Allelochemicals may enter the environment naturally through decaying plant residues, root exudates, or leaf washings (Rice, 1974). Negative allelopathic interactions may provide a fitness advantage to the donor plant in competition for limiting resources, whereas the role of stimulatory allelopathy may be an important component of seed bank depletion and population regulation. The evolutionary significance of a stimulatory effect between species is yet to be established.

Allelopathy has been extensively investigated in the Brassicaceae family. Activity has been attributed, in part, to the presence of glucosinolates and their corresponding degradation products (Malik et al., 2010a; 2010b). Glucosinolates are sulfur containing compounds produced by all organs of plants belonging to the *Brassica* and *Sinapis* genera (Evenari, 1949). Upon infection, mechanical damage or pest attack, cellular break down exposes the stored glucosinolates to

degradative enzymes (Bones and Rossiter, 1996). Hydrolysis is then catalyzed by an intrinsic β-thioglucosidase called myrosinase (EC 3.2.3.1.) (Poulsen et al., 2008). Glucosinolate breakdown products include thiocyanate, isothiocyanate or nitrile (Bones and Rossiter, 1996).

Incorporation of plant residues into the soil may liberate allelochemicals through decomposition of organic litter by soil microorganisms. Petersen et al. (2001) observed reduced germination at a field scale when canola fresh residues were incorporated into soil, but no difference was observed when residues were solely on the surface. Reduced germination was attributed to the release of isothiocyanates upon biomass mulching. Soil borne isothiocyanates were found to be only temporary; >90% dissipation was observed within 24 hours that was presumably due to volatilization. In a natural environment, leaf material is most likely to remain on the soil surface. The potential for allelochemicals to enter the soil at concentrations large enough to influence neighboring plants is unknown and will likely vary between species.

In the laboratory, plant tissue extracts are often used for initial identification of allelopathic effects and isolation of potential allelochemicals. Mustard (Brassicaceae) oils are strong germination inhibitors (Evenari, 1949). Gressel and Holm (1964) observed that aqueous seed extracts of *Brassica juncea* (L.) Czern. significantly reduced the germination of alfalfa, radish and turnip (*Brassica rapa* var. *rapa* L.). Evenari (1949) found that aqueous seed extracts of *Brassica nigra* L. reduced the wheat germination index to 6%. Moreover, Evenari (1949) attributed prevention of germination to the presence of a halogen atom,

stating that a sulphur group makes inhibition five times more effective than without. Bell and Muller (1973) found that large quantities of allyl isothiocyante liberated from macerated *B. nigra* leaves potently inhibited germination, but the activity could not be demonstrated at a field level. Evidence suggests that low levels of isothiocyanates induce secondary dormancy (Petersen et al., 2001) and inhibition of germination is linked to lipid mobilization (Baleroni et al., 2000). It is apparent that there is a clear connection between glucosinolate content and germination inhibition, although more evidence demonstrating this activity in a natural environment is needed.

Root exudates result solely from the presence of living roots (Rice, 1974; Bertin et al., 2003). The rhizophere (root–soil interface) is the site of greatest activity within the soil matrix representing the largest fraction of root exudates (Bertin et al., 2003). The relative concentration of exudates can be dependent on external factors including photoperiod and temperature (Pramanik et al., 2000). Roots generally contain less allelochemicals than the leaves, which are less potent and present in smaller amounts (Rice, 1974).

Leaves appear to be the most consistent source of allelopathic inhibitors and have been the focus of many investigations (Rice, 1974). Allelochemicals contained in leaf washings are released by rainwater or moisture present on the leaf tissue. Bell and Muller (1973) found that *Brassica nigra* was capable of suppressing growth of selected grass species, including wild oat. They attributed the suppression to toxic substances from the previous year's dead *B. nigra* stocks leached from rainwater.

Lovett and Jackson (1980) documented a stimulatory allelopathic effect of camelina leaf washings on flax. They attributed the effect to camelina phyllospheric bacteria *Enterobacter cloacae* (Jordan) Hormaeche and Edwards. Lovett and Duffield (1981) subsequently determined that benzylamine may be responsible for the stimulatory effect. At low concentrations, benzylamine was found to be stimulatory yet inhibitory at high concentrations (> 200 µg L^{-1}) (Lovett and Duffield, 1981). However, Grümmer and Beyer (1960) reported that the presence of *Camelina alyssum* reduced the yield of flax more than 80% in field experiments with similar results for different camelina species. The effect was found to be due to leaf-bound kolines liberated by rain water. Results obtained from leaf leachate experiments lack consistency, which may be due to environment-specific factors.

2.6.1. Allelopathy in agriculture

Allelopathy is a naturally occurring ecological phenomenon among organisms that may be utilised to manage weeds, plant pathogens, and insects in field crops. Allelopathy may be used following rotation, using cover crops, mulching, and plant extracts for natural pest management as reviewed by Farooq et al. (2011). While allelopathic extracts may not provide complete control of weeds, they recommend augmentation with herbicide to reduce dose as an environmentally friendly alternative. Laboratory allelochemical identification may enable the manufacture of bio-herbicides or synthesis of novel crop management

tools. Although allelopathy has potential agricultural applications, it can only be an element of an integrated pest management strategy, not a solution.

Interest in breeding crops with enhanced weed suppressive ability is growing in response to herbicide resistant weeds and the need for alternative, sustainable, weed control methods (Belz, 2007; Bertholdsson, 2010). Mardani and Yousefi (2012) assessed the allelopathic potential of several wheat cultivars against wild barley (*Hordeum spontaneum* C. Koch.) under laboratory conditions. They reported allelopathic activity of two wheat cultivars, Falat and Azar2, capable of inhibiting root elongation of wild barley seedlings by 57 and 47% respectively. Bertholdsson et al. (2012) carried out a search for allelopathic sources among accessions of wheat, rye, triticale, and wheat-rye substitution and translocation lines to be used in breeding programmes to improve the weed suppression ability of wheat. Most of the triticale lines and some of the wheat-rye substitution and translocation lines showed allelopathic potential. They suggested that *in vitro* selection of potentially allelopathic accessions with a bioassay with mustard target plants could be used to improve the weed suppressive ability of wheat. Alternatively, Xu et al. (2011) used gene knock-out in rice to identify potentially allelopathic genes when compared to a wild type.

2.6.2. Challenges to identifying allelopathy

Allelopathy is separated from competition involving the removal or reduction of a given factor required by another plant sharing the environment

(Rice, 1974). Macías *et al.* (2007) further proposed that any allelopathic report must fit the following guidelines:

- Plant predominance/frequency/distribution cannot be explained solely on the basis of physical or biotic factors.
- The allelopathic donors (plants) should synthesize and release into the environment chemicals that must be or become bioactive.
- Soil permanence and concentrations should be high enough to produce effects on the germination and/or growth of neighboring plants, bacteria, and/or fungi.
- Uptake by the target plant (allelopathic recipient) and evidence of the detrimental/beneficial effects caused by the chemical(s).

The nature of allelopathic interactions within plant populations remains poorly understood. Although allelochemicals may enter the environment by residue incorporation, root exudates, and leaf leachates, it is difficult to separate the effects of each due to the complex nature of interactions.

Laboratory assays serve as an initial evaluation tool to identify a potential allelopathic interaction between two species. Laboratory assays may be criticized due to their artificiality (Inderjit and Dakshini, 1995), but enable researchers to eliminate confounding factors through controlled experimental conditions. Positive results in the laboratory indicate the need for further research in an ecological context. Even upon tentative identification of allelochemicals, it is exceedingly difficult to prove its role in plant to plant interactions.

2.7. Summary

The propensity for PMGF and SMGF is a key component of environmental risk assessment and should be examined on a case-by-case basis. Characterization of gene flow is an important determinant of a novel crop's ability to co-exist with conventional and organic agriculture. Camelina's strong agronomics and potential for genetic manipulation may enable the creation of novel varieties with broad utility but, a complete environmental risk assessment is required prior to unconfined release and commercialization. Characterization of the allelopathic potential will contribute to our understanding of the biology of this species. The research in this thesis is intended to contribute knowledge to a growing body of information on camelina. It is also expected that these results may aid in regulatory decision making relevant to development of GE camelina in Canada.

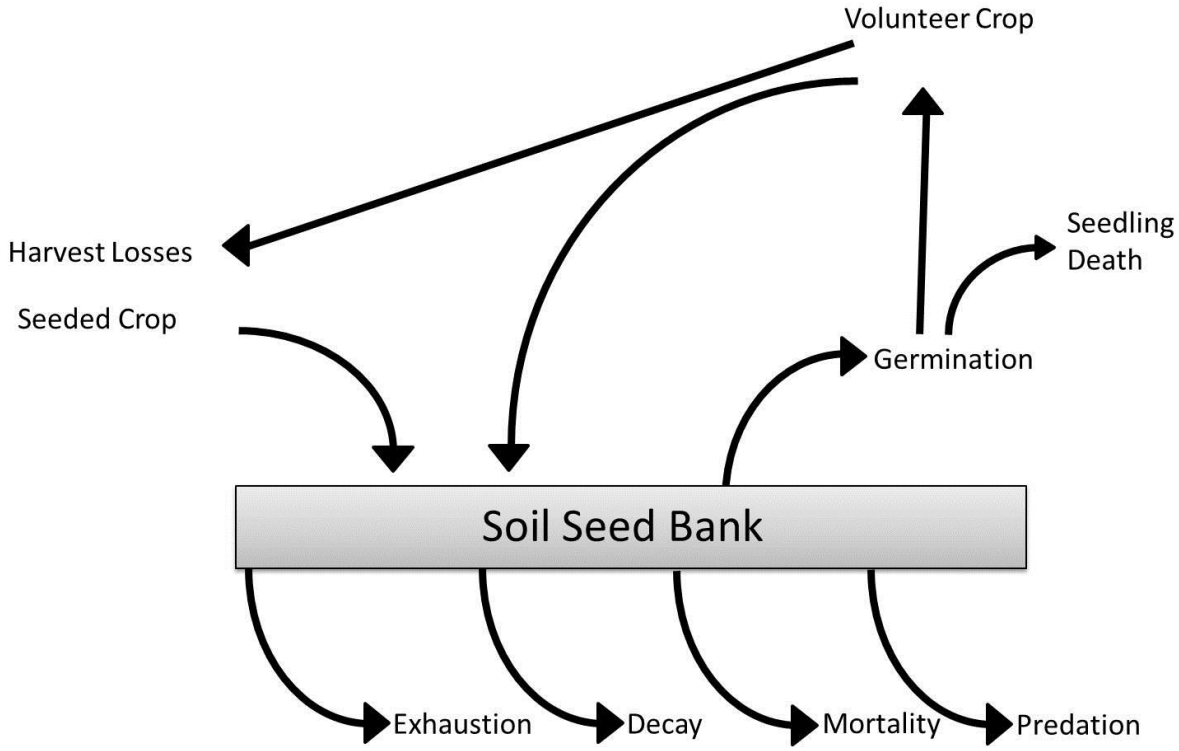

Figure 2-1 Crop seed in the soil seed bank. Seeds enter the seed bank primary through harvest losses and lie either on the soil surface or become buried. Surface weeds are exposed to predation and adverse environmental conditions whilst buried seeds are vulnerable to exhaustion and disease. Seeds emerge from the seed bank via germination, however, 'fatal germination' or seedling death may occur. Any plants that reach maturity may contribute additional propagules to the seed bank or be harvested and constitute a source of adventitious presence in the current crop. Figure adapted from Nielson et al., 2009.

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Chapter 3. Intraspecific pollen-mediated gene flow in camelina [*Camelina sativa* (L.) Crantz][1]

3.1. Introduction

Camelina [*Camelina sativa* (L.) Crantz] is a European old world oilseed crop of the Brassicaceae family (Francis and Warwick, 2009) being developed for cultivation in Canada. Camelina is well suited to some western Canadian environments due to early maturity, high yield potential, drought tolerance and resistance to some common pests of Brassicaceae oilseeds (Gugel and Falk, 2006). Rapid adoption of this novel crop may be aided by its compatibility with conventional farming practices. Camelina oil is similar to flax oil; high in unsaturated oleic, linoleic and linolenic acids (Budin et al., 1995; Shukla, 2002; Johnson et al., 2010). Currently, camelina is being used as a feedstock for the production of biodiesel (Moser and Vaughn, 2010; Fröhlich and Rice, 2005), however, genetic engineering (GE) may expand its uses for production of industrial oils. Prior to introduction of any transgenic crop in Canada, an environmental biosafety assessment must be conducted (CFIA, 2010) that includes evaluation of the potential for pollen-mediated gene flow (PMGF). Quantification of PMGF informs decisions about the suitability of the crop for GE applications as well as precautions such as isolation distances that may be required during variety development of transgenic crops. Transgenic plants may outcross to conventional varieties of the same species (intraspecific gene flow), or

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to other species (interspecific gene flow) including crop, wild and weedy relatives (addressed in chapter 5).

Outcrossing (OC) rates vary widely between species, from obligate OC species such as maize (Van De Wiel et al., 2009), to predominantly self-pollinated species such as wheat (Beckie et al., 2011) and flax with low levels of OC (Jhala et al., 2011). Moreover, OC rates are affected by crop variety (Hucl 1996), the presence of pollinators, and by the environment (Beckie and Hall, 2008). Camelina is considered to be a predominantly self-fertilizing species (Francis and Warwick, 2009); however, no published research has quantified the level of OC. Outcrossing rates may influence whether camelina is a suitable platform for bioproduct synthesis in the Canadian western prairies. The objective of this study was to ascertain, under field conditions, the short distance intra-specific OC rate for camelina. Data from this study will be used to plan the appropriate size of medium field scale experiments to better characterize camelina OC rates.

3.2. Materials and methods

Camelina from Linnaeus Plant Sciences Inc., genetically engineered with single-copy insertions of the *bar* and *DsRed* genes, was used as the pollen source (CsDsRed). The *bar* (bialaphos resistance) gene encodes a phosphinothricin acetyl transferase enzyme conferring resistance to glufosinate ammonium (Thompson et al., 1987), the active ingredient in herbicides such as Basta® and Liberty®. The DsRed gene encodes a red fluorescent protein derived

from reef coral and enables transgenic seed to fluoresce (Clontech Laboratories, 2005). The conventional European cultivar ‘Calena’ (Lethbridge, Alberta, CDA) was used as the pollen receptor.

Outcrossing was measured between rows with 20, 40 and 60 cm distances from the pollen source at the University of Alberta St. Albert Research Farm in 2010. Rows were planted by handwith a target density of ~100 plants/linear meter with 8 rows, 7 m in length, with row spacing of 20 cm and west/east orientation. Calena was planted on two dates to maximize flowering synchrony. On planting date 1 (June 2, 2010) two rows of DsRed and 3 rows of Calena were planted by hand. On the second planting date (June 8, 2010) three rows of Calena were planted. Planting dates were randomly assigned to the north or south of the pollen source. The experimental design was a split plot with distance being the main effect; the sub-plots were planting date and direction with 15 replicates.

The percentage of flowering CsDsRed and Calena was recorded 3-4 times weekly during flowering periods (data not shown). Following flowering, CsDsRed was removed from the field site. The remaining Calena was harvested by hand at maturity on September 22, 2010, ~16 to 17 weeks after planting. Confinement conditions were followed as detailed by the CFIA (2010) including weekly monitoring and removal of weedy relatives within the trial site.

Hybrid seeds with the DsRed trait were identified using fluorescence (330/570nm). The sample size was determined using power analysis with Type I error of alpha=0.05; Type II error of beta=0.20 and the frequency of non-hybrid

seeds p=0.95 (Zar, 1999; Jhala et al., 2011). Frequency of OC was calculated as the number of putative hybrids detected divided by the total number of seeds screened. Differences between treatments were determined using a generalized linear mixed model implemented in SAS PROC GLIMMIX (SAS Institute Inc., 2007) and data were expressed as the mean number of hybrid seeds. Since OC events were a rare occurrence, we employed a Poisson distribution to approximate OC occurrence. Data failed to converge when fit to negative binomial or geometric distributions. Despite a less optimal model fit, a Poisson distribution best represented the experimental design. Distance from the pollen source (20, 40 and 60cm) is a quantitative measure and thus was treated as a covariate to examine linear and quadratic trends. It is expected that there would be a non-linear decrease in the amount of OC as distance from the pollen source increased (Warwick et al., 2009). All statistical tests were evaluated using a significance level of $p \leq 0.05$.

Molecular confirmation using PCR amplification with *bar* specific primers was used to confirm hybridity (Figure 3-1). A sub-sample of hybrid, non-hybrid, parental Calena and CsDsRed plants were assayed. Leaf tissue was sampled at the 4 leaf stage. DNA was extracted from all tissue samples as described by Edwards (1991). DNA (5 µL; approximately 20-100 ng) was amplified by PCR in a total volume of 30 µL utilizing Qiagen Taq polymerase cat# 201205 (Toronto, Ontario, CDA) using *bar* specific primers (5'TCTGCACCATCGTCAACCACTACA3' and 5'AGAAACCCACGTCATGCCAGTT3') which amplifies a 428 bp fragment in tissue containing the *bar* gene. A positive control (wild type Calena DNA with

20 pg plasmid with the *bar* gene; p0308DSredBAR35SGUS from the University of Nebraska, Lincoln, USA) and negative controls (wild type Calena DNA and water only) were included in the PCR reactions. PCR conditions for the iCycler™ Thermocycler (BioRad; Mississauga, Ontario, CDA) were: 1 cycle for 2 min at 95˚C followed by 30 cycles of 30 s at 95˚C, 30 s at 55˚C and 30 s at 72˚C with a final extension of 2 min at 72˚C. Following PCR, 10 μL of each PCR reaction was run on a 1.0% agarose gel along with a 1 Kb marker cat# 15615-016 (Invitrogen, Burlington, Ontario, CDA). Forty putative non-hybrid seeds were screened and did not contain the *bar* gene.

To quantify false negatives, approximately 200 presumed non-hybrid seeds for each of 90 samples were screened for glufosinate ammonium resistance conferred by the *bar* gene. Calena and CsDsRed seed were included as controls. All seeds were planted on Sunshine Mix L4 in glasshouse, and at the two-leaf stage samples were sprayed with 0.19 ml m^{-2} glufosinate ammonium. Six days later the plants were screened and survivors re-sprayed to confirm resistance. False negatives occurred at a frequency of 0.0012 and there was no relationship between treatments and occurrence. The low rate of false negatives verified that visual detection is an accurate method of determining hybridization.

3.3. Results and discussion

A total of 7, 987, 500 seeds were screened (Table 3-1). There were significant ($p \leq 0.05$) differences in OC for distance, planting date and direction.

Significant ($p \leq 0.05$) interaction was detected between planting date and distance as well as direction and distance (Figure 3-2). For planting date 2 and south planted rows OC declined with distance, however planting date 1 and north planted rows OC increased marginally at the 60 cm distance. Rates of OC were significantly ($p \leq 0.05$) higher in rows planted north of the source (0.12 to 0.28%) compared to the south (0.01 to 0.15%). Planting date 1 (0.08 to 0.24%) had significantly ($p \leq 0.05$) higher OC than planting date 2 (0.06 to 0.19%) which was congruent with flower synchrony (data not shown). Distance was significant ($p \leq 0.05$) and found OC highest at 20 cm. Contrary to our expectations, OC rates were not lowest in entirety at the 60 cm distance. Camelina OC rates are lower than that of flax (1.85% measured at 1 m distance), which is generally classified as a self-pollinating species (AJ Jhala et al., 2011). Though significant differences are small, the impact is large when considered at a field scale. Rates of 0.28 and 0.01% correspond to 2,800 and 100 seeds respectively in 1,000,000 camelina seeds (1 kg). Preliminary findings confirm that *Camelina sativa* is a primarily self-fertile species (Francis and Warwick, 2009). Data collected in small-scale experiments cannot be easily extrapolated to a field scale because the relative size of the pollen source and sink populations and distances between sink and source affect OC rates (Beckie and Hall, 2008).

3.4. Conclusions

This is the first report of OC rates in camelina. Results from this initial, small scale, fifteen replicate split-plot trial found camelina OC rates to be less

than 0.30% at a 20 cm distance; less than or equal to maximum recorded soybean OC rates (Abud et al., 2007; Ahrent and Caviness, 1994). While PMGF is low, it is only one avenue for adventitious presence. Other routes of gene flow may include seed-mediated gene flow and interspecific gene flow to wild and weedy relatives. The detection of PMGF, albeit low, in camelina demonstrates that stewardship and due diligence should be employed to mitigate transgene movement from GE camelina. The data from this experiment will be used to plan medium scale PMGF experiments using larger pollen sources and sink populations.

Table 3-1 Outcrossing (%); mean number of hybrid seeds detected in each sample; the number of seeds screened and the total hybrids in all samples as influenced by direction, planting date and distance from the pollen source.

Sub-plot effect†	Distance‡	Outcrossing	Mean number of hybrids§	Seeds screened	Total putative hybrids¶
	(cm)	%			
North	20	0.28	69.00 ± 10.83	905000	1035
North	40	0.12	40.33 ± 14.84	1265000	605
North	60	0.26	49.53 ± 17.19	1135000	743
South	20	0.15	39.33 ± 10.21	1645000	590
South	40	0.06	27.27 ± 9.45	1307500	409
South	60	0.01	8.33 ± 5.72	1660000	125
Date 1	20	0.24	54.13 ± 11.35	1375000	812
Date 1	40	0.08	31.80 ± 10.98	1300000	477
Date 1	60	0.22	45.13 ± 15.56	1165000	677
Date 2	20	0.19	54.20 ± 11.15	1175000	813
Date 2	40	0.12	35.80 ± 13.94	1342500	537
Date 2	60	0.06	12.73 ± 10.46	1630000	191

†Planting dates 1 and 2 correspond to June 2, 2010 and June 8, 2010

‡Distance measured from CsDsRed camelina

§Means are presented ± the standard error

¶All data obtained from detection of false negatives was incorporated into tabular data

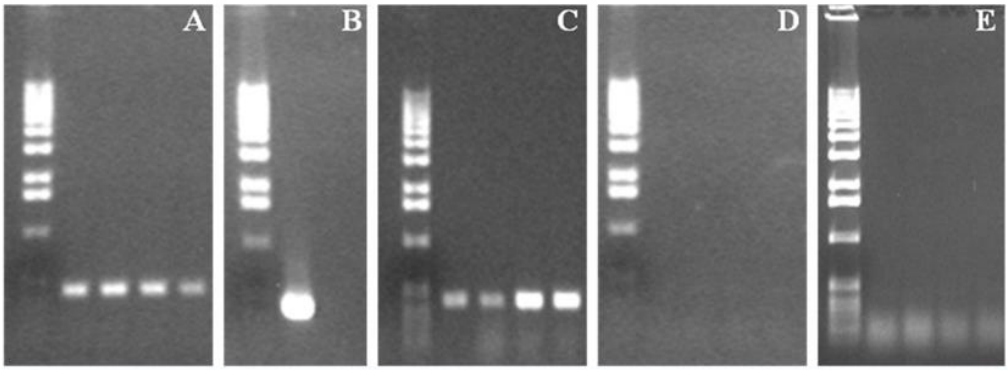

Figure 3-1 Confirmation of putative hybrids; agarose gels showing the amplified *bar* gene accompanied by 1kb ladder (Invitrogen, Burlington, Ontario). A. Four tissue samples from putative hybrid seed B. Positive control; p0308DSredBAR35SGUS containing the *bar* gene C. Four tissue samples from CsDsRed parental seed D. Four tissue samples from putative non-hybrid seed E. Four tissue samples from wild type Calena seed.

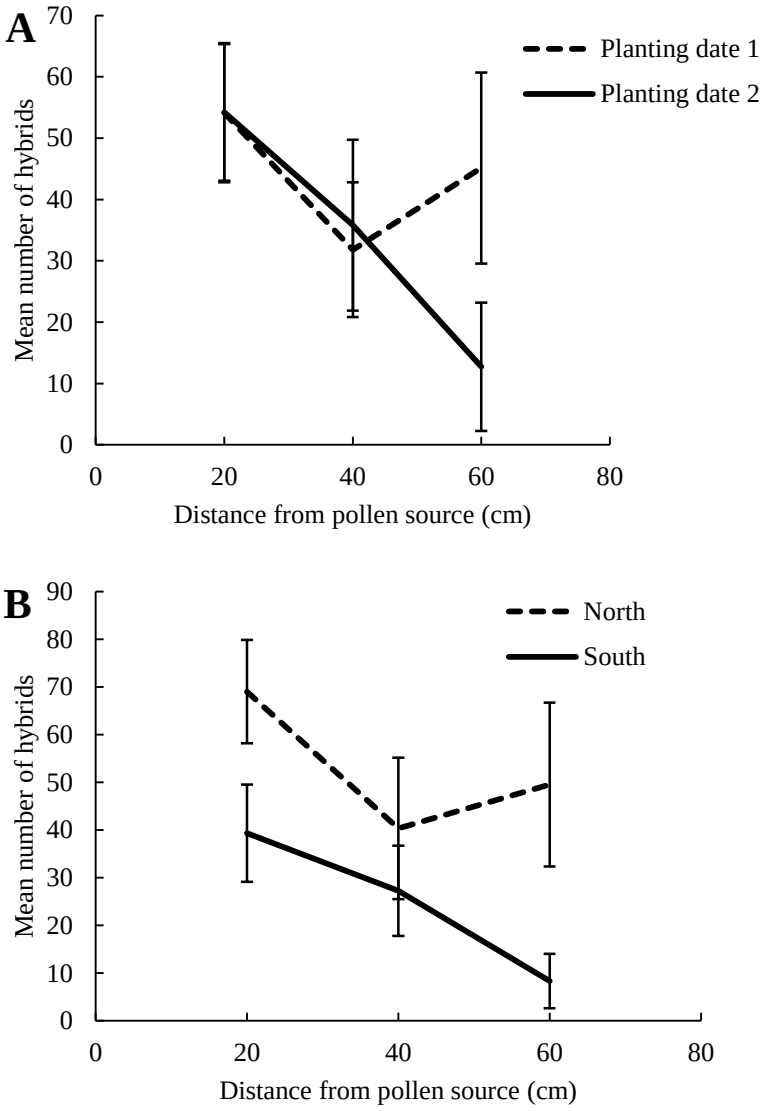

Figure 3-2 Mean number of hybrid seeds collected at varying distances from pollen source. Significant ($p \leq 0.05$) interactions are presented for planting date and distance from pollen source (a); direction and distance from pollen source (b). Bars indicate ±1 standard error of the mean.

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Chapter 4. Pollen-Mediated Gene Flow in *Camelina sativa* (L.) Crantz.

4.1. Introduction

Camelina, [*Camelina sativa* (L.) Crantz.], an oilseed of the Brassicaceae family, is being developed for cultivation in Canada. It is well suited for cool, arid climates and grows well in the more northerly regions of Asia, Europe, and North America. Adoption of this crop has been attributed to early maturity, high yield potential, cold hardiness, and minimal input requirements (Ehrensing and Guy, 2008). Moreover, flexibility in seeding date, compatibility with existing farm equipment, and resistance to several common pests of the Brassicas support cultivation of camelina as an alternative oilseed crop (Gugel and Falk, 2006; Séguin et al., 2009; Schillinger 2012). Presently, camelina is a minor use crop and cultivation area remains low and is unreported at a national level. Camelina oil is versatile and has applications in cosmetics, human nutrition, and industry (Budin et al., 1995; Zubr, 1997); however, north American production is devoted mainly to biodiesel feedstocks. Recently, its oil has been qualified as a biomass-based diesel and advanced biofuel in the US (United States EPA, 2013). Camelina is currently being evaluated as a genetically engineered (GE) crop platform for the production of bio-industrial oils.

Prior to commercial use of GE camelina cultivars, environmental and economic risks must be considered. Genetically engineered camelina may become a weed of agriculture, be invasive of natural habitats, or outcross to wild and

weedy relatives whose hybrid offspring may be more weedy or more invasive (CFIA, 2011). Economic risks include the potential for GE camelina seeds to inadvertently become mixed with conventional camelina or other crops, otherwise known as adventitious presence (AP) (Kershen and McHughen, 2005). This may result as the consequence of seed comingling during transport, storage or processing, through the unintentional harvest of persistent camelina volunteers, or by means of pollen-mediated gene flow (PMGF) from GE camelina to nearby conventional crop cultivars (Demeke et al., 2006). There is a zero tolerance for seed shipments containing unapproved experimental traits, yet conventional products with minimal approved GE content are in demand in selected regions, particularly the European Union (EU). The EU has established a threshold of 0.9% for products containing approved GE content to be labelled as genetically modified (EC, 2003). Adventitious presence testing has become routine to ensure seed purity according to country specific standards.

Pollen-mediated gene flow may be the main source of AP for outcrossing crops such as sugar beet (*Beta vulgaris* L.) (Darmency et al., 2007) and maize (*Zea mays* L.); however, the frequency of PMGF is reduced in primarily self-fertile crops including flax (*Linum usitatissimum* L.) (Jhala et al., 2011) and wheat (*Triticum aestivum* L.) (Hanson et al., 2005). In such crops, seed-mediated gene flow contributions may be more significant to AP (Beckie and Hall, 2008). Characterization of PMGF at increasing distances from the pollen donor will help in the development of regulatory measures intended to reduce AP. Additionally,

PMGF rates will assist in establishing isolation distances for seed growers and impact co-existence of GE and conventional camelina.

Camelina is primarily self-fertile (Francis and Warwick, 2009; Walsh et al., 2012). The flowers are protogynous (stigma ripens prior to anthers) and exhibit perfect sexual reproduction with stamens and pistil present within the same flower (Francis and Warwick, 2009). Selfing occurs as the flowers begin to close towards the evening, directing stamens towards the stigma where pollen is deposited (Schultze-Motel, 1986; Tedin, 1922). There are no recent reports on pollen vectors, although Tedin (1922) noted that bees or insects may account for less than 3% of pollination events. Although bee visitation has been observed, their role in pollen transfer has not yet been established. Camelina PMGF has been reported to range from 0.09 to 0.28% when measured at close proximity (up to 0.6 m) with a 0.2 by 7.0 m pollen donor (Walsh et al., 2012).

The research objective of this study is to document camelina PMGF under a medium-distance, field scale experiment conducted in western Canada. Gene flow was measured at up to 20 m from the pollen donor and modelled using a regression analysis. A transgenic camelina cultivar containing the dominant *Discosoma* ssp. red fluorescent protein (DsRed) was used to determine PMGF between two camelina cultivars. DsRed is a variant of the well-studied green fluorescent protein (Stuitje et al., 2003) and has been previously described as an effective marker that enables rapid visual screening of individual camelina seeds (Lu and Kang, 2008). Due to the monetary and temporal cost associated with transgenic research, only two site years were conducted.

4.2. Materials and methods

4.2.1. Plant Materials

Transgenic CsDsRed camelina (Linnaeus Plant Sciences Inc.) was utilized as the pollen donor and *Camelina sativa* cv. Calena, a spring-sown conventional cultivar, as the pollen receptor. CsDsRed is genetically engineered with single-copy insertions of the *bar* and DsRed genes. The *bar* (bialaphos resistance) gene encodes a phosphinothricin acetyl transferase enzyme conferring resistance to glufosinate ammonium (Thompson et al., 1987).

4.2.2. Field Trials

Field trails were conducted in 2011 and 2012 at St. Albert, Alberta, Canada (53°69’92”N, 113°62’6”W) and Edmonton, Alberta, Canada (53°64’66”N, 113°35’63”W) respectively. St. Albert soils are a silty clay and Edmonton, soils a sandy clay loam with silt. Prior to seeding, glyphosate was applied at 2 L ha^{-1} at both sites. For Edmonton 2012, an additional granular application of ethafluralin was applied at 21.9 kg ha^{-1} using a Valmar 1255 Twin Roller (Valmar Airflo Inc.). The trial design was a modified Nelder wheel (Nelder, 1962); a 10 by 10 m octagonal CsDsRed center (donor) surrounded by Calena (receptor) 23 m in all directions (Figure 1). CsDsRed seed was planted using a single row cone seeder (Craftsman Machine Co.) and the Calena using a Fabro air seeder with atom-jet openers (Fabro Enterprises Ltd.). Planting

specifications were ~1 cm depth at a rate of 5 kg ha^{-1}, with 20 cm row spacing for both receptor and donor seed. Zero and 75 kg ha^{-1} ammonium sulphate (21-0-0-24) was side banded for St. Albert and Edmonton, respectively as per soil fertility requirements. To synchronize flowering, CsDsRed was planted ~5 days prior to Calena. CsDsRed and Calena were planted on May 25 and June 1, and May 25 and 29 for St. Albert and Edmonton respectively (Table 4-1). Flowering synchrony was assessed as the number of days the receptor cultivar flowered together with the donor cultivar divided by the total number of flowering days, multiplied by 100 (Table 4-1). Following flowering, the CsDsRed camelina was removed to minimize the risk of CsDsRed admixture in the Calena receptor.

At maturity, the Calena receptor was subdivided into eight elongated trapezoidal arms 23 m in length in each of the four cardinal and four ordinal directions; the crop in between the arms was removed prior to pollen dispersal (Figure 4-1). Sampling began distal to the pollen source and progressed inward to reduce cross-contamination. The pollen receptor was harvested every 5 m from 20 m to 10 m; every 1.5 m up to 3 m; every 0.4 m up to 1 m; and every 0.2 m up to 0.2 m adjacent to the pollen source for each of the eight directions. Using a Monarch Jari Sickle Mower (Jari USA), 3.25 to 20 m samples swathed and threshed in-field using a Large Vogel Plot Thresher (ALMACO Manufacturing Co.). Samples 0.2 to 2.8 m were hand harvested as whole plants then threshed and cleaned in the laboratory and stored at room temperature (~20 °C) until further analysis. Harvesting was completed on September 20 and 5 in 2011 and 2012, respectively.

4.2.3. Weather Data

The seasonal and 30 year temperature and precipitation averages were obtained from the National Climate Data and Information Archives website maintained by Environment Canada (http://www.climate.weatheroffice.gc.ca) (Figure 4-2).

4.2.4. Environmental Biosafety Compliance

For all the of the transgenic field experiments, confinement protocols were adhered to as outlined by the Canadian Food Inspection Agency. This included maintenance of a 30 m isolation distance between CsDsRed and other, non-*C. sativa*, vegetation, and 200 km from commodity camelina. Field trials were monitored weekly to remove camelina volunteers and weedy relative shepherd's purse (*Capsella bursa-pastoris* L. Medikus) during the growing season. All equipment employed in the trial was thoroughly cleaned after used to prevent off-site movement of GE materials. Secure, appropriately labelled containers were used to transport all GE materials and a record of all movement was maintained.

4.2.5. Seed Screening

Hybrid seeds with the DsRed trait were identified using fluorescence (330/570 nm). This detection method has been previously published (Walsh et al., 2012) and verified to be accurate at determining hybridity. Sample sizes for seed

screening were established with power analyses using binomial probabilities as described by Zar (1999) and reported in Jhala et al. (2011). This method determines the sample size required to detect one transgenic seed for different theoretical frequencies based on degrees of confidence.

4.2.6. Statistical Analysis

Pollen-mediated gene flow was calculated as the number of detected hybrid seeds divided by the total number of seeds screened, multiplied by 100. Exponential decay curves were generated for each replicate (direction) at all sites and years using non-linear regression with PROC NLMIXED (SAS Institute Inc., 2007; McPherson et al., 2009; Jhala et al., 2011; Kavanagh et al., 2012). Estimation of slopes was completed using a binomial distribution and fitting the data to the exponential decay function reported by Hanson et al. (2005):

$$P = ae^{-bd}, \quad [1]$$

where P is the predicted frequency of PMGF; a is the intercept; b is the curve parameter; and d is the mean distance from the source (m). Parameter estimates for the intercept (a) and the rate of decline of outcrossing (b) were highly significant ($p < 0.0001$), indicating that the model was not over-parameterized. Standard errors and 95% confidence intervals were calculated for each parameter estimate. Distances where PMGF decreased by 50% (O_{50}) and 99% (O_{99}) were estimated using the exponential decay function and equations first reported by McPherson et al. (2009):

$$O_{50} = [\ln(0.5) - \ln(a)]/ -b, \quad [2]$$

$$O_{99} = [\ln(0.01) - \ln(a)]/ -b, \quad [3]$$

where a is the intercept and b is the slope.

Differences in PMGF between sites were evaluated by using the rate of PMGF decline or slope (b) generated by the exponential decay curves as a new variable. An ANOVA was performed on the slopes to determine whether there were site differences. Distance was considered to be fixed while direction(site) were random effects. To assess whether there were directional effects between replicates, the mixed-model analysis based on PROC MIXED (SAS Institute Inc., 2007) was used to perform pre-planned orthogonal contrast statements. More than 20 covariance structures were tested (data not shown) and no significant directional effects could be identified.

4.3. Results and discussion

All sites had plant stands of good quality with flowering synchronies of 73 and 100% for St. Albert and Edmonton, respectively (Table 4-1). Site years did not significant differ ($p = 0.64$) from each other, therefore, St. Albert 2011 and Edmonton 2012 data was combined for further analysis.

Temperatures in the early growing season were generally lower for St. Albert 2011 and higher for Edmonton 2012, relative to the 30 yr normal (Figure 4-2). The temperature range during flowering for St. Albert was 8.8 to 27.7°C and

was 6.4 to 31.7°C for Edmonton (Table 4-2). Precipitation during the flowering period was 16 mm for St. Albert and 99 mm for Edmonton with average humidities of 70 and 72% respectively (Table 4-2). West winds prevailed during the flowering period for both sites, however, Edmonton winds were less uniform (Table 4-2).

Over 19.5 million total seeds were screened via fluorescence (Table 4-3). Sample size (minimum 340,000 and maximum 1,600,000) was generally increased as PMGF events decreased to achieve a higher statistical confidence. Increasing the intensity of sampling improved the accuracy of the estimations and the likelihood that rare outcrossing events would be identified. Outcrossing frequency at increasing distances from the pollen source was modelled using an exponential decay function.

A total of 6904 hybrids were identified by screening 19,505,000 seeds (Table 4-3). The slope for PMGF was 0.4608 with an intercept of 0.0013 (Table 4-4). A maximum gene flow of 0.783% was observed at a minimum distance of 0.2 m. Outcrossing declined rapidly with distance. Average PMGF declined by 50% at 1.5 m from the source and 99% at 9.99 m from the pollen source. At 20 m from the pollen source, PMGF was 0.001%; 1,600,000 seeds were screened at this distance and 18 hybrids were detected.

Maximum camelina PMGF was 0.783% (Table 4-3), approximately three times higher than the tier II reported 0.28% (Walsh et al., 2012). Pollen movement is dependent on the size of the donor population, therefore, small-scale studies

may underestimate PMGF (Mallory-Smith and Zapiola, 2008). For example, Kavanagh et al. (2012) measured PMGF in triticale (X *Triticosecale* Wittm. Ex A. Camus) and found an average of 0.76% at up to 1.6 m when the pollen donor was 50 by 1.4 m, but when the donor was increased to 20 by 20 m PMGF ranged from 0.45 to 5.07% at up to 1.6 m.

Pollen-mediated gene flow of numerous self-pollenated crops has been studied. McPherson et al. (2009) quantified PMGF in safflower (*Carthamus tinctorius* L.) and found frequencies of 0.48 to 1.67% up to 3 m with rare events detected (0.01%) at 100 m from the pollen source. Similarly, soybean [*Glycine max* (L.) Merr.] mean PMGF was reported as 0.52% immediately adjacent to the pollen source, with rare events (0.01%) at 9 m (Abud et al., 2007). Camelina outcrossing appears to be higher than soybean yet similar to safflower, but it is difficult to directly compare gene flow due to differences in experimental design.

4.4. Conclusions

Pollen-mediated gene flow of camelina was found to be < 1%, immediately adjacent to the pollen donor, when measured under a medium-distance field scale. Camelina PMGF was influenced by the size of the pollen donor and acceptor populations. It is expected that outcrossing will be elevated at a commercial scale, however, given the presently low cultivated area it seems that camelina PMGF would be a minor contributor compared to seed-mediated gene flow significantly contribute to AP. Based on our exponential decay model, we

recommend an isolation distance of at least 10 m from camelina crops to reduce PMGF by up to 99%.

Pollen-mediated gene flow should not constrain development of novel camelina cultivars, however, PMGF is only one avenue of AP. For self-fertile small-seeded crops, seed-mediated gene flow may be more critical (Beckie and Hall, 2008). A comprehensive assessment of gene flow will consider seed-mediated gene flow, the persistence of volunteer populations, and transgene movement into wild and weedy populations (Mallory-Smith and Zapiola, 2008). While zero AP tolerance is unrealistic, recognition of attainable AP levels is essential for co-existence of GE camelina and conventional crops.

The conclusions drawn in this study are reflective of a medium distance camelina PMGF evaluation. Further assessment of intraspecific PMGF may be required prior to commercialization including experimental replication at a medium scale or quantification of PMGF at a commercial scale, the potential variation of reproductive traits among camelina varieties, and the impact of insect pollinators on short to long distance pollination events.

Table 4-1 Seeding, flower initiation, synchrony and harvest dates for camelina cultivars CsDsRed and Calena for trials 2011 at St. Albert and 2012 at Edmonton, Alberta.

Year	Site	Cultivar	Seeding date	Flowering dates	Flowering synchrony†	Harvest date
2011	St. Albert	CsDsRed	25 May	25 July-4 Aug	-	-
2011	St. Albert	Calena	1 June	21 July-4 Aug	73%	September 20
2012	Edmonton	CsDsRed	25 May	2-19 July	-	-
2012	Edmonton	Calena	29 May	2-19 July	100%	September 5

† Flowering synchrony was assessed as the number of days the main raceme of the receptor flowered together with the donor cultivar divided by the total number of flowering days, multiplied by 100.

Table 4-2 Meteorological data for the camelina flowering periods by site year and location. Mean, maximum, and minimum temperature (°C), total precipitation, prevailing wind direction, and average humidity are reported.

Year	Site	Temperature		Total precipitation	Prevailing wind direction†	Humidity
		Min	Max			
		-------(°C)-------		(mm)		(%)
2011	St. Albert	8.8	27.6	16.0	S, SW, and W (18, 18, and 64%)	70
2012	Edmonton	6.4	31.7	99.5	E, SE, S, SW, and W (33, 11, 17, 11, and 28%)	72

† Percent duration of prevailing wind is reported in parentheses

Table 4-3 Percent camelina pollen-mediated gene flow (PMGF) from 0.2 to 20.0 m distance from the pollen donor. The number of seeds screened, number of hybrids detected, and the power for each distance sampled is included.

Distance	PMGF	Seeds screened†	Hybrids	Power
(m)	(%)			(1 – β); α = 0.05%‡
0.2	0.783	340,000	858	0.95
0.4	0.451	385,000	675	0.95
0.6	0.520	655,000	767	0.95
0.8	0.522	830,000	661	0.95
1.0	0.220	815,000	439	0.95
1.2-1.4	0.149	1,150,000	760	0.95
1.6-1.8	0.198	1,145,000	412	0.95
2.0-2.2	0.288	1,160,000	442	0.95
2.4-2.6	0.330	1,180,000	448	0.95
2.8-3.0	0.156	1,080,000	465	0.95
3.25-4.45	0.160	1,350,000	460	0.95
4.75-5.95	0.038	1,485,000	281	0.95
6.25-7.45	0.023	1,530,000	131	0.95
7.75-8.95	0.003	1,600,000	45	0.8
10.0-11.2	0.002	1,600,000	29	<0.8
15.0-16.2	0.001	1,600,000	13	<0.8
20.0-21.2	0.001	1,600,000	18	<0.8

† Total number of seeds screed from all directions (replicates).

‡ Power was calculated using a 95% confidence interval (Power was calculated using a 95% confidence interval (α = 5%).

Table 4-4 Parameter estimates, O_{50} and O_{99} values with their respective standard errors and confidence intervals as calculated by regression analysis.

				95% confidence interval	
Parameter†	Estimate‡	Standard error	*df*	Lower	Upper
O_{50}	1.5042	0.0277	271	1.4496	1.5588
O_{99}	9.9937	0.1841	271	9.6312	10.3562
A	0.0013	0.00003	271	0.0013	0.0014
B	0.4608	0.0085	271	0.4441	0.0051

† Parameters *a* and *b* were estimated using Eq. [1]. The distances (O_{50} and O_{99}) where outcrossing was reduced by 50 and 99% were estimated using Eq. [2] and [3], respectively.

‡ Estimates of the parameters for intercept (*a*), slope (*b*), and the estimates of the distances where outcrossing was reduced by 50 and 99%.

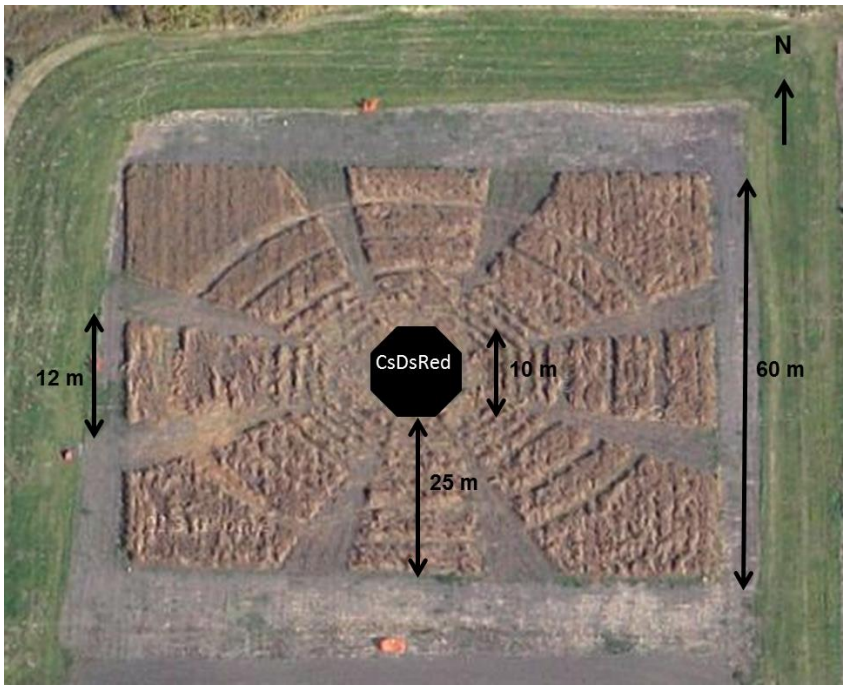

Figure 4-1 Design of pollen-mediated gene flow experiment in camelina for 2011 St. Albert and 2012 Edmonton trials. The pollen source camelina cultivar (CsDsRed) was planted in the 10 by 10 m octagon in the center of the field. The pollen receptor camelina cultivar (Calena) was planted in the surrounding 60 by 60 m area. After flowering, prior to crop maturity, the pollen receptor was divided into eight elongated trapezoids, and the remainder crop was removed. Camelina samples were harvested at maturity at specific distances from the CsDsRed centre; seen as arcs where crop has been removed in each of the eight directional arms (Source Google Maps, 2012)

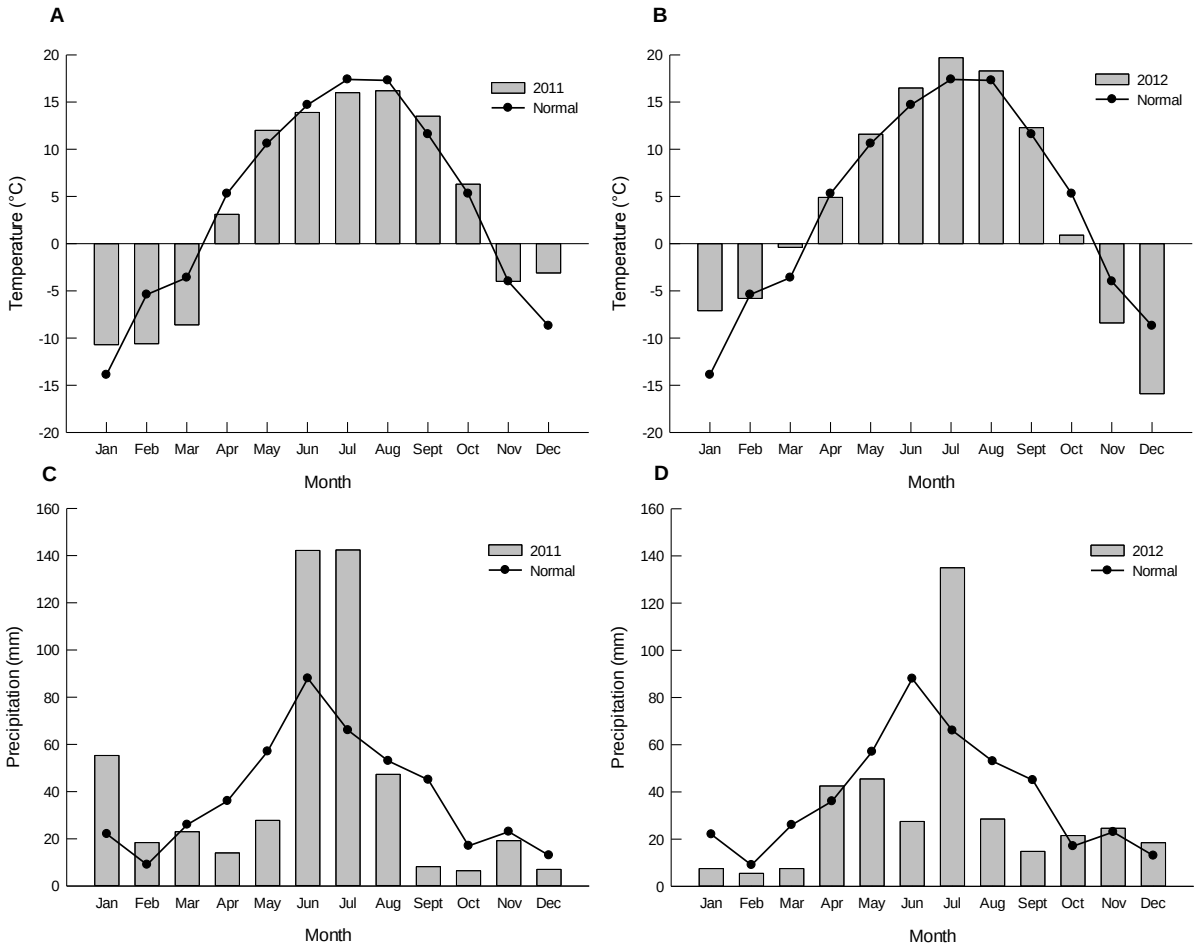

Figure 4-2 Average monthly temperatures (°C) for St. Albert, 2011 (A) and Edmonton, 2012 (B) and total monthly precipitation (mm) for St. Albert, 2011 (C) and Edmonton, 2012 (D) with 30 year averages presented.

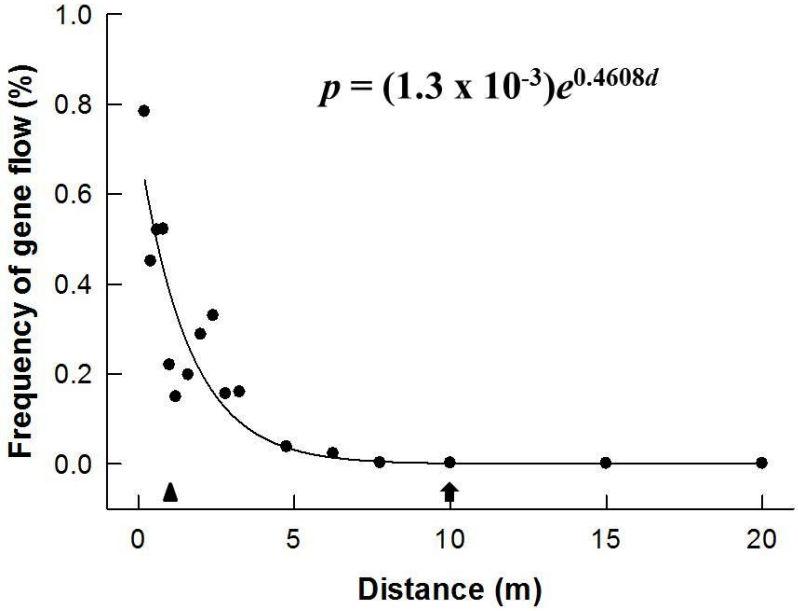

Figure 4-3 Pollen-mediated gene flow (PMGF) in camelina as a function of distance from the CsDsRed camelina donor to Calena. The line is the fitted model and points are the PMGF means at each distance. The triangle indicates the estimated distance at which 50% (O_{50}) reduction in the frequency of gene flow and the arrow indicates the estimated distance at which 99% (O_{99}) reduction in gene flow from the pollen donor.

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Chapter 5. Potential for interspecific gene flow between *Camelina sativa*(L.) Crantz. and its weedy relative *Capsella bursa-pastoris* (L.) Medik (shepherd's purse).

5.1. Introduction

Camelina [*Camelina sativa* (L.) Crantz.], an oilseed of the Brassicaceae family, is being evaluated as a genetically engineered (GE) crop platform for the production of bioindustrial oils. Currently, North American production occurs on a small scale, devoted to use as a feedstock for the production of biodiesel. In 2013, camelina oil was qualified as a 'biomass-based diesel and advanced biofuel' in the US (United States EPA, 2013). Camelina grows well in cool, arid climates and is adapted to the more northerly regions of Asia, North America, and Europe. Adoption of this novel crop has been attributed to early maturity, high yield potential, low input requirements, and compatibility with existing farm equipment (Gugel and Falk, 2006). Additionally, resistance to several common pests of the Brassicas demonstrates camelina's utility as a rotational crop (Séguin-Swartz et al., 2009; Johnson et al., 2010). Camelina has been transformed with several novel genes (Lu and Kang, 2008; Liu et al., 2012); however, presently no GE cultivars are commercially grown.

Transgene introgression into wild and weedy relatives is a potential risk of cultivating GE crops (Ellstrand and Hoffman, 1990; Raybould and Cooper, 2005; Kavanagh et al., 2010). Gene flow between crops and their wild relatives is well documented, occurs naturally, and is not unique to GE crops (Ellstrand et al., 1999). However, the primary concern is that a transgene-conferred fitness

advantage may result in weedy hybrids with increased weediness or invasiveness (CFIA, 2011a). While the potential for a fitness benefit is dependent on the GE trait as well as the ecology of the recipient populations (Snow et al., 2005), such an outcome is predicated on an initial successful introgression event. The potential influence of a transgene on hybrid fitness will have a reduced risk if transgene introgression is infrequent. Therefore, a detailed assessment, including potential for interspecific gene flow, must be conducted prior to GE commercialization.

Camelina sativa is able to interbreed with its congeners, *Camelina alyssum* (Mill.) Thell. and *Camelina microcarpa* Andrz. Ex DC., in the *Camelina* genus (Séguin-Swartz et al., 2011; Al-Shehbaz, 1987). Given that distribution is limited and that camelina and *C. alyssum* are self-fertile species (Walsh et al., 2012; Mulligan, 2002), the potential for gene flow is reduced.
Camelina microcarpa has been identified in every Canadian province and can be found in grain, flax, corn and hay fields, prairies, pastures, grasslands, rangeland, low slopes, flats and sloughs as well as along roadsides, railways and wharves and in waste places (USDA, 2013; Warwick et al., 2003). *Camelina alyssum* is present only in Alberta, Saskatchewan and Manitoba and can be found in prairie fields and on roadsides (USDA, 2013; Warwick et al., 2003). Prairie weed surveys conducted over a 30 year period starting in the 1970's found *Camelina* ssp. to be scarce in agricultural fields, each being ranked as the 100th most common weed or lower (Leeson et al., 2005) and there has been no indication that they were increasing in abundance.

Other members of the large Brassicaceae family are more distantly related and therefore less likely to interbreed. Narasimhulu et al. (1994) conducted protoplast fusion of camelina and Ethiopian mustard (*Brassica carinata* A. Braun) and found that minimal shoot regeneration occurred but roots fail to form. Similarly, protoplast fusion of camelina and *Brassica oleracea* L. reported that shoots regenerated at a 0.5% frequency but root establishment in soil was unsuccessful (Hansen 1998). The inability for interspecific hybrids to form under laboratory (artificial) conditions indicates that introgression under natural conditions will be less likely. Gene flow to the Brassicas is not considered to be a significant concern due to substantial reproductive barriers, however, the propensity for gene flow between intermediary Camelineae tribe members has not yet been established. Species found in Canada include *Arabidopsis lyrata* L. and *A. thaliana* (L.) Heynh., *Capsella bursa-pastoris* (L.) Medik. (shepherd's purse), *Neslia paniculata* (L.) Desv., *Erysimum* spp., and *Turritis glabra* L. (Warwick et al., 2003).

Shepherd's purse has been identified in every province, territory and state in North America (USDA, 2013). Native to southwest Asia, shepherd's purse is regarded as the second most common weed worldwide (Zhou et al., 2001). It is distributed throughout the earth's temperate region excepting the hot and wet tropics (Hurka and Neuffer, 1997). Shepherd's purse is an annual to winter annual with a high degree of phenotypic plasticity. Fruit is a silique capable of producing up to 39 seeds each, with up to 40,000 per plant (Aksoy et al., 1998). Shepherd's purse is a tetraploid with chromosome numbers generally 2n=32 (Aksoy et al.,

1998). Ubiquitous abundance and high seed output make this species a significant weed of agricultural and non-agricultural ecoregions.

Shepherd's purse, similar to camelina, is primarily self-fertile attributed to protogyny (Aksoy et al., 1998). The duration of pollen viability is several hours, consequently 1 to 2% outcrossing may be observed (Aksoy et al., 1998). Séguin-Swartz et al. (2011) performed laboratory pollinations on emasculated pistils of camelina and shepherd's purse to investigate the potential for interspecific hybrid formation. Of the 50 camelina pistils that were pollinated by shepherd's purse, 68% had germinated pollen grains but there was no evidence of pollen tube growth. Forty-nine pistils were examined in reciprocal (camelina to shepherd's purse) crosses; 82% of the stigmas showed germinated pollen grains and 55% showed pollen tubes. Of the shepherd's purse pistils that had pollen tubes, 31% had pollen tube growth near the ovules. This study did not determine seed set and warranted further evaluation.

The potential for gene transfer within the Camelineae tribe is a significant data gap identified by the Canadian Food Inspection agency in their recent 'Biology of Camelina' document (CFIA, 2011c). If interspecific gene flow can occur, it draws regulatory commercialization of GE camelina into question. Therefore a risk assessment strategy that addresses the relevant concerns is essential to regulatory approval. Tiered testing is a series of experiments with decreasing severity intended to increase confidence, reduce type II errors and limit the collection of superfluous data (Raybould 2006; Raybould and Cooper, 2005).

The primary research objective of this study was to assess the potential for introgression from GE camelina to camelineae tribe members, specifically shepherd's purse. In order to quantify the propensity for interspecific gene flow, tier II and III experiments were conducted. Tier I, the reciprocal emasculated crosses of camelina and shepherd's purse, was conducted by Dr. Sara Martin in Ottawa, Ontario, Canada. The assessments examined hybrid formation resulting from: (II) open pollination between camelina and shepherd's purse with insect pollinators present in contained greenhouse conditions, and (III) open pollination between camelina, shepherd's purse, *C. alyssum*, *C. microcarpa*, and *N. paniculata* under small-scale field conditions. A transgenic camelina cultivar containing a dominant *Discosoma* ssp. red (DsRed) fluorescent protein was used as the pollen source. Outcrossing events were identified using the dominant DsRed trait expressed in seed of the F_1 generation (Stuitje et al., 2003).

5.2. Materials and methods

5.2.1. Plant materials

Camelina sativa cv. Calena, a spring-sown European cultivar, was utilized as the positive control, and CsDsRed (Linnaeus Plant Sciences Inc.) genetically engineered with single-copy insertions of the *bar* and DsRed genes, as the pollen donor. The *bar* (bialaphos resistance) gene encodes a phosphinothricin acetyl transferase enzyme conferring resistance to glufosinate ammonium (Thompson et al., 1987). The DsRed gene encodes a red fluorescent protein derived from reef coral and enables transgenic seed to fluoresce (Clontech Laboratories, 2005).

Shepherd's purse and *N. paniculata* seeds were collected from Edmonton, Alberta and Stony Plain, Alberta, Canada. For camelina weedy relatives *C. microcarpa* and *C. alyssum* seed bank accessions Pl 650132 and PL 650137 were used.

5.2.2. Tier II

To ensure flowering synchrony, seed of CsDsRed, Calena and shepherd's purse was planted in five, once-weekly intervals. Four seeds of camelina (Calena or CsDsRed) were planted to 13 x 13 cm pots and subsequently thinned to two plants per pot. Approximately 1000 shepherd's purse seeds were planted in 25 x 50 cm flats. At the rosette stage, single plants were then each transplanted to 10 x 10 cm 1 L pots. All seeds were planted in soilless medium (Sunshine Mix L4, Sun Gro Horticulture, Vancouver, BC, Canada), watered and placed in a greenhouse at 16 hours light at 18^{o}C and 8 hours dark at 12^{o}C. Approximately 2 days prior to flowering, plants were moved to 40 by 40 by 80 cm cages screened with nylon silk screen material. Eight treatment cages contained two CsDsRed and eight shepherd's purse plants; two control cages with two CsDsRed and ten Calena. Pollinators were introduced to simulate field conditions where pollen transfer may be facilitated by an insect vector. Native to western Canada brassicaceae fields (Dosdall and Mason, 2010), cabbage root maggot flies (*Delia* ssp.)(Doptera: Anthomyiidea) were introduced to facilitate pollen transfer. Groups of 100 puaria, each in vermiculite filled Petri dishes with ~1 mL distilled water, were introduced to every treatment and to only one of the control cages. Full emergence of *Delia* was apparent 2 days after trial initiation.

Following CsDsRed flowering, shepherd's purse plants were removed and transferred to a greenhouse bench. Any unopened flower buds were excised and shepherd's purse plants were harvested at maturity. Each cage was considered an experimental unit therefore all shepherd's purse seed from each cage was bulked into one sample. The experimental design was a randomized complete block with four replicates.

Harvested seeds were then screened using fluorescence (330/570nm) to detect the DsRed transgene as described by Stuitje et al. (2003). Frequency of gene flow was expressed as the number of putative hybrids divided by the total number of seeds screened.

5.2.3. Tier III

Similar to tier II methods, sequential plantings of Calena, shepherd's purse, *C. alyssum*, *C. microcarpa*, and *N. paniculata* were established in the greenhouse to ensure flowering synchrony. Up to four seeds per pot of each species were planted in soilless medium (Sunshine Mix L4, Sun Gro Horticulture, Vancouver, BC, Canada) in 10 x 10 pots. Plants were watered and grown in greenhouse conditions at 16 hours light at 18^{o}C and 8 hours dark at 12^{o}C. For flowering to occur, *C. microcarpa* required vernalization for six weeks at 4 °C in the dark.

Field experiments were conducted in 2011 at St. Albert, Alberta, Canada (53°69'92"N, 113°62'6"W). Soil texture was silt clay and soil analysis indicated that no additional fertility additions were required. Prior to seeding, a

recommended rate of glyphosate was applied. CsDsRed was planted on 2011 May 24 at a rate of 5 kg ha^{-1} in a 10 by 10 m block with 20 cm row spacing using a single row cone seeder (Craftsman Machine Co.). On July 27, CsDsRed initiated flowering at which time potted Calena, shepherd's purse, *C. alyssum*, *C. microcarpa*, and *N. paniculata* were placed between CsDsRed field rows. Flowering data was taken every two to three days to assess flowering synchrony (data not shown), and pots were watered as required. CsDsRed flowering was complete on August 4. Potted Calena, shepherd's purse, *C. alyssum*, *C. microcarpa*, and *N. paniculata* were transferred back to greenhouse conditions and any unopened flower buds excised. At maturity plants were harvested individually.

The mature, harvested seeds were then screened using fluorescence (330/570nm) to detect the DsRed transgene. Frequency of gene flow was expressed as the number of putative hybrids divided by the total number of seeds screened.

5.2.4. Data Analysis

The frequency of interspecific hybridization was reported as the number of putative hybrids divided by the total number of seeds screened. Percent outcrossing was then reported according to sample size and statistical confidence as determined by power analysis (Jhala et al., 2011).

5.2.5. Compliance with environmental biosafety

For all the transgenic experiments, confinement protocols were adhered to as outlined by the federal government of Canada (CFIA, 2011b). This included double containment of all transgenic seed and plant materials as well as any potentially introgressed materials. All experiments were conducted under confined conditions to prevent accidental release. The field study was appropriately isolated from commodity camelina and related species not directly included in the study. Moreover, field trials were frequently monitored during the growing season and in post-harvest years.

5.3. Results and discussion

Zero interspecific shepherd's purse hybrid seeds were identified in 103,000 and 30,000 seeds for tiers II and III, respectively (Table 5-1). Plants grown for tiers II and III were grown under high humidity greenhouse conditions, therefore, production of seed was limited and plant disease susceptibility was elevated. Percent of interspecific introgression was at or below 0.1% (α=2.5) for tier II and 0.025% (α=5) for tier III with a power of 0.85 for both. Screening via fluorescence is relatively facile and permits rapid hybrid identification but may lack accuracy.

The DsRed trait has been used previously to identify intraspecific outcrossing (Walsh et al., 2012); however, it may not be a reliable indicator of interspecific events. Kavanagh et al. (2013) measured interspecific introgression

from triticale (x *Triticosecale* Wittm. Ex A. Camus) to spring wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* L.) using a blue aleurone as a visual marker in conjunction with molecular markers. They found that interspecific hybrid seeds were shriveled and lacked the blue aleurone trait despite its documented success in measuring intraspecific outcrossing in triticale and wheat (Kavanagh et al., 2012; Hanson et al., 2005b). The DsRed trait alone may not be sufficient to confirm interspecific gene movement, therefore screening for herbicide resistance (HR) using the *bar* gene should be included in subsequent analyses.

This multi-tier study is a component of a camelina crossability risk assessment through measurements of interspecific outcrossing frequency. Tier II tests examine the propensity for hybridization under greenhouse conditions, where cross pollination is encouraged by synchronous flowering, insect pollinators, and minimal distance between pollen source and sink populations. Tier III tests used less conservative short-distance field conditions and encouraged pollination events likewise, however no pollinators were introduced. Based on the information provided by tier III, the risk may be determined to be "acceptable" or "unacceptable" and testing may terminate (Raybould and Cooper, 2005). However, if the results of tier III full assessment indicate potential risk, needing further characterization, a tier IV experiment should be conducted.

Transgene movement to a wild or feral relative is well documented and has been reported in many crops. Simard et al. (2006) documented hybridization between HR canola and *Brassica rapa* L. (bird rape) in Quebec, Canada. They

detected the transgene in the eight *B. rapa* populations sampled, PMGF ranged from 1.1 to 17.5%; however, gene flow was dependent on density, sympatry, and spatial arrangement of the two species. In HR rice (*Oryza sativa* L.), experimental outcrossing to the weedy relative red rice was conducted in Arizona, USA (Shivrain et al., 2007). Pollen-mediated gene flow was low and was found to be dependent on cultivar where combined plot outcrossing (0 to 5 m from the pollen source) ranged from 0.003 to 0.008%. Field experiments conducted by Hanson et al. (2005a) measured the transfer of HR from wheat to jointed goatgrass (*Aegilops cylindrical* L.) in Washington and Idaho, USA. Resistant hybrids were identified 40.2 m from the pollen source; however, the overall incidence of interspecific hybrids was similar to the predicted, naturally-occurring, acetolactate synthase resistance in weeds.

Transgene movement must overcome several hurdles prior to stable gene introgression into a novel plant population. First generation hybrids are often unstable and require the formation of backcross generations for fit progeny. Hills et al. (2007) performed laboratory crosses to evaluate the crossability of triticale with relatives wheat, durum wheat, and rye (*Secale* ssp.). They found that a lack of self-fertile interspecific F_1 hybrids produced from triticale male parent pollinations suggested that transgene movement via pollen was unlikely. Conversely, Warwick et al. (2008) reported the six-year persistence of HR weedy *B. rapa* hybrids containing an introgressed transgene from *B. napus* commercial fields in Quebec, Canada. Although *B. rapa* hybrids persisted in the absence of

herbicide selection, of the 200 plants surveyed, the population declined from approximately 85 plants to less than 5 over a three year period.

5.4. Conclusions

Results from this small scale, initial assessment, suggest that interspecific hybridization between camelina and shepherd's is unlikely to prevent commercialization of GE camelina. However, gene flow to wild and weedy relatives is only one component of an environmental risk assessment. Details on the environmental fate of hybrids and specific mechanisms of introgression are still to be addressed in order to properly assess risk.

Table 5-1 Tier II and III interspecific outcrossing for camelina and weedy relatives with camelina as pollen donor. The species, number of plants, number of seeds screened and the percent outcrossing is included.

Experimental tier	Species	Plants	Seeds screened†	Hybrids detected‡
Tier II	*Camelina sativa*	38	-	-
	Camelina microcarpa	73	-	-
	Camelina alyssum	29	-	-
	Capsella bursa-pastoris	51	30,000	0
	Neslia paniculata	30	200	0
Tier III	*Capsella bursa-pastoris*	256	103,000	0

† Seed numbers are approximate values

‡ Outcrossing percentages are based on fluorescence

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Chapter 6. Transient seed bank of *Camelina sativa* (L.) Crantz. contributes to a low weedy propensity in western Canadian cropping systems[2]

6.1. Introduction

As the plant-based bioeconomy grows, a substantial amount of research and development will be devoted to creating feedstocks to meet the demand for specialized bioproducts derived from genetically engineered (GE) technologies. Most projections assume widespread deployment of novel feedstocks yet seldom consider all the challenges involved with the use and implementation of GE crops, which include regulatory hurdles, market adoption, and public acceptance (Chapotin and Wolt, 2007). By producing industrial products in a crop not currently used for human or animal consumption, food and feed safety concerns can be reduced. However, non-traditional crops pose other challenges including the need to optimize agronomic practices, inclusion in rotations, and potential weediness of crop volunteers. Weediness of novel GE crops would increase agronomic difficulties, enhance the potential for pollen- and seed-mediated gene flow and increase the risk of adventitious, or unintended, presence of GE seed in following and non-GE crops (Kershen and McHughen, 2005). Weedy species may also pose a risk of invasiveness in disturbed or natural areas.

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Crop seed may be lost to the seed bank prior to and at harvest by shatter losses or mechanically from field equipment (Willenborg and VanAcker, 2008). The seed bank can be either transient, in which most seeds germinate or expire within one year of plant maturation, or persistent (Thompson and Grime, 1979; Cavers, 1983). Most domesticated crops have transient seed banks because they lack dormancy or have short term primary dormancy (often referred to as after ripening) and lack secondary dormancy (Warwick and Stewart, 2005). After-ripening occurs as seeds dry and are released from primary dormancy and is influenced by air temperature, seed moisture content, time, and plant species (Murdoch and Ellis, 2000; Foley, 2001). As after-ripening develops, seeds become able to germinate over a widening range of conditions (Baskin and Baskin, 2004). Some crops, including canola (*Brassica napus* L.), can have inducible secondary dormancy (Gulden et al., 2004a) and form persistent seed banks (Simard et al., 2002). Seed persistence, volunteer competiveness, and the fecundity of volunteer populations will determine the density and recurrence of volunteer crop populations in subsequent crops, along roadsides, or within natural areas (Warwick et al., 2009).

Crop seed lost prior to and during harvest may contribute to high seed densities in the soil seed bank. Additions appear to be negatively correlated with seed weight; harvest losses have been reported for wheat (*Triticum aestivum* L.) at ~300 seeds m^{-2} (Clarke, 1985); 22 to 1,986 seeds m^{-2} for flax (*Linum usitatissimum* L.) (Dexter et al., 2011); and ~3,000 seeds m^{-2} for canola (Gulden et al., 2003), which correspond to approximately 35, 5.5 and 4 g average thousand

kernel weights (TKW), respectively (Alberta Agriculture and Rural Development, 2007). Seed loss, density, and distribution vary with type of harvesting equipment, environmental conditions, and crop maturity (Willenborg and Van Acker, 2008). In the absence of dormancy, germination of annual spring crop seed occurs after seed shed at harvest and early in the following spring (Warwick et al., 2009; Dexter et al., 2010). Volunteers are usually controlled by management practices including herbicide application however, those that survive and set seed in subsequent crops may be harvested with the crop or replenish the seed bank and perpetuate volunteer populations (Raatz, 2012).

Camelina is a relatively new brassica crop being developed as an alternative feedstock for biodiesel and bio-jet fuel on the Canadian prairies. Camelina is currently a minor crop grown in the US and Canada and has been promoted as a low-input crop capable of thriving on marginal lands, particularly in sandy soils (Putnam et al., 1993; Meakin, 2007). Breeding and agronomy research is ongoing with new cultivars and best management practices being developed (Gugel and Falk, 2006; Urbaniak et al., 2008; Johnson et al., 2010; Steppuhn et al., 2010). Genetic engineering may permit the synthesis and accumulation of novel high value bio-industrial fatty acids in camelina seed oil (Lu and Kang, 2008), therefore opportunities for conventional as well as GE cultivars exist. In order for new crop rotations and market opportunities to be realized, research is required to determine whether conventional and GE camelina can coexist and whether they will become weedy in uncultivated areas.

The purpose of this study was to assess the propensity for camelina to be persistent and was conducted in four parts. Camelina persistence was assessed by quantifying: (1) conventional camelina seed bank inputs via harvest losses under typical field conditions, (2) the time dependant germinability of 20 different camelina cultivars, (3) the longevity of camelina seed banks, and (4) volunteer camelina population dynamics in subsequent crops. Data obtained will be used to determine if camelina is persistent or invasive within agricultural areas and predict potential adventitious presence in subsequent crops.

6.2. Materials and methods

6.2.1. Harvest losses from commercial fields

Three commercial camelina fields in Alberta, Canada were surveyed for three consecutive years (2008-2010) to quantify camelina seed lost at harvest. Fields selected had not been seeded to camelina at least 5 years previous. All fields were harvested using commercial harvesting equipment without prior swathing. To determine the distribution of seeds in each field, seed samples were collected soon after harvest from three transects oriented perpendicular to the direction of combine travel. Within each transect, 25 by 25 cm quadrats were established per meter of combine header width; transect length ranged from 7 to 11 quadrats between fields. Seedlings (if present) were counted from within each quadrat, then crop residue, non-harvested seed, and soil were removed using a wet-dry vacuum cleaner. Samples were allowed to air dry at ~25˚C and stored until further analysis.

Samples were sieved through a 10/64" round hole screen (3.97 mm hole perforation equivalent) (CanSeed Equip. Ltd., Saskatoon, SK) to remove crop residue and to break up soil aggregates. Screened samples were spread over 32 by 26 by 6 cm aluminum trays filled with soilless medium (Sunshine Mix L4, Sun Gro Horticulture, Vancouver, B.C., Canada.), watered and placed in a greenhouse at 16 hours light at 18^{o}C and 8 hours dark at 12^{o}C. Camelina emergence was quantified and germinated seedlings removed every 48 hours for six days (at which time no further germination was evident). Field and greenhouse seedling numbers were combined to determine viable seed lost per m^{2}. Experimental design was a two-level nested design where year was considered a random effect and fields were nested in sites and considered fixed.

The data met the assumptions of normality and homogeneity of variances for ANOVA. PROC MIXED procedure in Statistical Analysis Software (SAS Institute Inc., 2007) was used to determine differences in camelina seed bank additions between fields (seed lost from harvesting equipment in seeds m^{-2}) and total seed loss (average kg ha^{-1} seed loss) among sampled fields. Least squared means are reported where means separations are based on Bonferroni-adjusted *p*-values ($p < 0.006$) using PDMIX800 (Saxton, 1998).

6.2.2. After-ripening

Plots were established in spring 2012 to quantify the time dependent germinability of camelina for 20 cultivars from flowering to post-harvest.

Experiments were conducted at Edmonton Research Station, AB, Canada (53°29′19″ N, 113°34′8″ W), an Eluviated Black Chernozemic soil (Udic Boroll). Cultivars were broadcast seeded at a rate of 1.0 g m^{-2} followed by light soil disturbance to improve seed to soil contact. Plots measured 1 by 1 m and were maintained weed free by hand hoeing. Cultivar blocks were not replicated; however, seeds were harvested as three subsamples per cultivar starting approximately 10 days after flowering had initiated (50 days post planting). Fifty seeds were harvested per subsample from siliques randomly chosen from plants across the length of the plot. Siliques chosen were borne on the mid-stem therefore representing the average seed maturity of the harvested plant. Sampling was conducted weekly until harvest on September 10, 2012. Following harvest, seed germinations were performed for three subsamples of 50 seeds removed from the harvested portion of each plot. Sampling and germinations continued for 2 weeks post-harvest.

Germination was assessed by placing 50 seeds into 100 by 15 mm polystyrene Petri dishes lined with Whatman 90 mm #1 filter paper and 5.0 mL of water. Petri dishes were sealed to retain moisture and stored in the dark for 7 days at ambient temperature (~25˚C). Germination was defined as radicle shoot > 1 mm. Germinated seeds were recorded and expressed as a percent of the total seeds.

Prior to ANOVA the germination data was checked to ensure the residuals were random, and homogeneous with a normal distribution. The analysis of variance (ANOVA) was carried out using the PROC MIXED procedure in SAS

(SAS Institute Inc., 2007) to determine after ripening differences between camelina cultivars. Cultivar and time of sampling were considered fixed effects and subsample was considered a random effect. Cultivar, time of sample and their interaction were highly significant ($p < 0.00005$). Weekly germination data were subject to ANOVA using Proc MIXED with Bonferroni-adjusted mean separations to communicate differences between the cultivars at every week of sampling.

6.2.3. Seed bank persistence

In order to quantify the viability and longevity of spring seeded camelina, seed burial experiments were established at two sites: Edmonton research station ($53^{o}29'19"N$, $113^{o}34'8"W$) and Lethbridge research station ($49^{o}41'2"N$, $112^{o}37'41"W$) in Alberta, Canada in fall 2008 and 2009. Edmonton had Eluviated black Chernozemic soils (Udic Boroll) and Lethbridge had an Orthic dark brown Chernozemic soil (Typic Boroll). Soil characteristics were determined for composite 0 to 15 cm soil samples prior to establishing the trials. Prior to seed burial the annual precipitation was 64 and 85% of thirty year averages in 2008 and 61 and 100% of 30 year averages in 2009, at Edmonton and Lethbridge, respectively. Monthly precipitation and temperature data was compiled per site for the duration of the experiment (Figure 6-1). Sites were kept free of weeds using a recommended rate of glyphosate at least one growing season prior to experiment initiation. Seeds of camelina cultivars, Calena and CN101985 (Lethbridge, AB), were both grown and harvested in both 2008 and 2009 just

prior to experiment establishment to emulate the physiological state of seed lost at harvest. Initial germination rates were determined (data not shown) by placing seeds into 24 by 16 by 4 cm acrylic germination boxes (Hoffman Manufacturing, Inc.) lined with non-toxic white filter paper (15 by 23 cm No. 601 Whatman #1 equivalent, Hoffman Manufacturing, Inc.) and 10 ml distilled water.

Experiments were established as a split-plot design with three replicates, where burial depth was the main plot, and camelina cultivar was the split plot. Two hundred seeds of two cultivars (Calena and CN101985) were sewn into 10 by 10 cm compartments of bags constructed from nylon silk screen mesh. Experiments were initiated in fall 2008, on September 25 (Edmonton) and October 1 (Lethbridge) and fall 2009 on September 18 (Edmonton) and September 30 (Lethbridge). Bags were placed on the soil surface, or buried to 3 or 10 cm (Burnside et al., 1996) to simulate seed loss on surface and shallow to moderate seed placement through tillage. Although tillage is not common to the Canadian Prairies, deep burial was included because it has been demonstrated to induce dormancy in canola (Gulden et al., 2004a, 2004b). Each treatment was enclosed within a 100 by 90 by 22 cm galvanized steel cage (65 mm mesh) to minimize vertebrate seed predation. Packets of seed were extracted five times throughout the growing season at approximately four week intervals beginning May and concluding in September. Seed germination and viability were assayed at each extraction period. Camelina seed that had germinated (radicle > 1 mm) or decayed was considered to have exited the seed bank. Ungerminated seeds were placed in polystyrene Petri dishes 100 by 15 mm lined with Whatman # 1, 90 mm

filter paper and ~5mL of distilled water. Petri dishes were sealed to retain moisture and stored in the dark for seven days at ambient temperature (~20˚C). Seeds that germinated were considered viable.

The number of seeds which germinated from extracted samples were considered to be viable and a part of the seed bank. Viable seeds, expressed as a frequency of initial viable seed (y), from each of the two years, two sites, three depths, and two cultivars were regressed over time using a non-linear regression mixed model (NLMIXED) (SAS Institute Inc., 2007). The binomial distribution (y ~binomial (n, P)) was used to approximate the dependent variable where n is the total number of buried seeds in each sample and P is the probability of viable seeds estimated as a ratio of viable over total seeds withdrawn at each sample date (P = y/n). Previous seed longevity studies report that volunteer crop seeds in the agricultural seed bank decline exponentially (McPherson et al., 2009; Nielson et al., 2009; Raatz et al., 2012), therefore data were fit to a simple exponential decay curve given in Eq. [1]

$$P = ae^{-bd}, \quad [1]$$

where P is the probability of viable seeds, a is the intercept, b is the slope of the curve and the parameter describing the rate of viability decline and d is the number of days following seed burial.

The rate of viability decline (b) generated by regression was used as a new parameter and analyzed with ANOVA using a using a mixed model (SAS Institute Inc., 2007) within four environments, Edmonton 2008, Lethbridge 2008,

Edmonton 2009, and Lethbridge 2009, in which burial depth was the main plot and cultivar was the split plot. Depth and cultivar were considered to be fixed effects while environment, replicate (environment), and depth x replicate (environment) were random effects. Significant differences between main effects and their interaction of burial depth, cultivar, and depth x cultivar were tested.

The slope (*b*) or rate of decline parameter was inverse square root transformed to meet the assumptions of normality and homogeneity of variances for ANOVA. After testing whether depth, cultivar, or their interaction were significant, the initial data set was regressed again with combined data where appropriate. The days to 50% and 99% seed extinction were estimated (EX_{50} and EX_{99}; Eqs. [2, 3]),

$$EX_{50} = [\ln(0.5) - \ln(a)]/ -b, \quad [2]$$

and

$$EX_{99} = [\ln(0.01) - \ln(a)]/ -b, \quad [3]$$

where *a* is the intercept (set as 1) and *b* is the slope of the curve and the parameter describing the rate of decline. Estimates of seed viability the spring (May 1) and fall (Sept 1) following seed burial were also made.

6.2.4. Volunteer camelina presence and persistence

To quantify the presence of volunteer camelina, 11 fields in Alberta and Saskatchewan were selected for surveying that had been seeded to conventional camelina in spring of 2009. Survey fields were chosen to reflect a variety of

growing environments and farming practices (Table 6-1) and ranged in size from 20 to 65 ha. Fields were surveyed for the presence of volunteer camelina five times per growing season from April 2010 to August 2011 (Table 6-2). Surveys were conducted before pre-seed herbicide application (PRESEED); prior to an in-crop herbicide application (PREHERB); following an in-crop herbicide application (POSTHERB); prior to harvest (PREHARV); and after harvest (POSTHARV).

Camelina volunteers were surveyed using a modified W-pattern of sampling as previously described (Thomas, 1985). In each field the surveyor walked 100 paces along the field edge, turned at a right angle, and an additional 100 paces into the field. Sampling began at this point and followed an inverted W-pattern. Five locations, each 20 paces apart, were sampled along each arm of the pattern, giving a total of 20 locations per field. Density and growth stage of volunteer camelina were determined from within 50 by 50 cm quadrats. Experimental design was a two-level nested design where sample period and year were considered random and fields, which were nested within sites, were considered fixed.

Volunteer camelina density (plants m^{-2}) data met the assumptions of normality and homogeneity of variances and were subject to ANOVA using mixed model analysis (PROC MIXED) (SAS Institute Inc., 2007). To determine differences in camelina densities between sites, a one-way ANOVA was conducted where year and survey time were considered random and site was fixed. Years were analyzed separately because of differences in the number of

elapsed days between survey sample times (Table 6-2). Least squared means per survey period for each grower are reported. For each year and survey period time (PRESEED, PREHERB, POSTHERB, PREHARV, or POSTHARV) volunteer camelina data were summarized: frequency is the percentage of surveyed fields in which volunteer camelina occurred; field uniformity (all) is the percentage of quadrats in which volunteer camelina occurred in all fields; field uniformity (occurrence) is the percentage of quadrats in which volunteer camelina occurred in occurrence fields; field density (all) is the average density in all fields surveyed; field density (occurrence) is the average density of volunteer camelina in fields where populations were found. The highest density and most advanced growth stages of the volunteer camelina across fields were recorded.

6.3. Results and discussion

6.3.1. Harvest losses from commercial fields

Camelina seedlings were present at high densities in-field shortly after harvest in 2008 and 2010, where densities ranged from 853 to 3004 seedlings m^{-2} (Table 6-3). In-field germinated seedlings were absent in 2009, presumably due to dry environmental conditions following harvest and prior to sampling. High camelina seedling densities support previous observations of a lack of primary seed dormancy (CFIA, 2011). The camelina seed bank was rapidly depleted when moisture was sufficient for germination. Fall germination of major crops lacking seed dormancy is commonly observed and generally fatal, but rarely quantified under Western Canadian conditions. It has been reported that camelina seedlings

may overwinter (Crowley, 1999), but conditions suitable for winter survival have not yet been established.

Density of viable camelina seed recovered from the soil surface was quantified by spreading the sifted residual sample onto moist soilless medium and counting all germinated seedlings. Seed densities were high, ranging from 349 to 40,635 plants m^{-2} for 2008 and 2010, and 2973 to 31,257 plants m^{-2} for 2009 (Table 6-3).

Camelina seed lost at harvest (seeds and seedlings) was variable between fields, ranging from 1202 to 43,430 seeds m^{-2} (Table 6-3) and averaging 11,907 seeds m^{-2}. Within and between fields and transects, camelina densities were also highly variable (data not shown). Total harvest losses were estimated using total seeds per sample and an assumed conservative TKW of 1.0 g; losses varied from 12 to 434 kg ha^{-1} and averaged 111 kg ha^{-2}. Camelina yields typically vary between 1500 and 2500 kg ha^{-1} (Plessers et al., 1962; Meakin, 2007; Urbaniak et al., 2008), therefore quantified harvest losses account for 0.7 to 25.5% of an average 1700 kg ha^{-1} yield. Fields 08-1 and 09-1, belonging to the same grower, were harvested using a non-standard harvest method (stripper-header combine), and had significantly ($p < 0.006$) higher loss of camelina seed than all other fields sampled. Excepting fields 08-1 and 09-1, yield loss ranged from 12 to 74 kg ha^{-1} (0.7 to 4.3% total yield loss), similar to 3.3 to 9.9% total yield loss quantified in canola (Gulden et al., 2003). Large seed loss density is attributed, in part, to small seed size which may negatively affect seed retention at harvest; camelina TKW is 1-2 g (Meakin, 2007), approximately one third that of canola (Alberta Agriculture

and Rural Development, 2007). All fields sampled were direct harvested (without swathing), however harvest equipment, harvest speed, crop maturity and environmental conditions at harvest influences the amount and distribution of seed dispersal (Gulden et al., 2003). It is likely that grower practices may improve as experience with the crop increases and the economic consequences of yield loss become apparent. In addition to seed germination, viable seed densities will be reduced by seed loss from insect, mammalian, and avian seed predation, disease and abiotic stress, environmental conditions, as well as seed intrinsic factors including secondary seed dormancy, if present (Dalling et al., 2011).

6.3.2. After-ripening

Camelina cultivars varied in primary dormancy (or pre-harvest sprouting tolerance) prior to harvest, but all cultivars rapidly after-ripened prior to harvest. All cultivars became germinable at or before 99 days post-planting (1 week pre-harvest) and remained so, into the post-harvest time period (Table 6-4). Moreover, there were no significant differences in germinability between cultivar immediately following harvest (Table 6-4). Experimental findings suggest that camelina exhibits brief primary dormancy and completes after-ripening prior to harvest.

6.3.3. Seed bank persistence

Rate of seed viability decline was significantly different between years of trial initiation (2008 or 2009; $p = 0.0228$), trial site (Edmonton or Lethbridge; $p = 0.0093$), and depths of seed burial ($p < 0.0001$), however there were no significant differences between camelina cultivar (Calena or CN101985; $p = 0.93$) (Table 6-5) and therefore data were combined. Within year of trial initiation, site was not significant in 2008 ($p = 0.2243$), but was highly significant in 2009 ($p = 0.0038$) (Table 6-5). Burial depth significantly affected seed bank longevity in all years and sites (2008, $p < 0.0001$; 2009 Edmonton, $p = 0.0118$) except 2009 Lethbridge ($p = 0.3682$) (Table 6-5; Fig. 6-2). For both trials initiated in 2008, buried seeds (3 and 10 cm) were less persistent than those on the surface ($p < 0.0001$); seeds buried at 3 and 10 cm were not significantly different from each other ($p = 1.0$) (Table 6-5; Fig. 6-2A). For buried seeds, the estimated days to EX_{50} and EX_{99} seed decay were 24 and 161 days, respectively. Surface placed seeds persisted longer with EX_{50} and EX_{99} estimates at 66 and 438 days, respectively (Table 6-5; Fig. 6-2A). For trials initiated in 2009, Edmonton surface and 3 cm samples were not significantly different ($p = 1.0$), but seeds buried to 10 cm were depleted from the seed bank more rapidly than seeds buried at 3 cm ($p = 0.0174$) and those on the soil surface ($p = 0.0422$) (Table 6-5; Fig. 6-2B). For Edmonton 2009, seed persistence was short; surface seeds and those buried to 3 cm depleted rapidly within the seed bank. EX_{50} estimates were 27 and 25 days for surface and 3 cm, respectively and EX_{99} estimates were 180 and 170 days for surface placed seeds and those buried to 3 cm, respectively. Seeds on the soil surface and those buried

to 3 cm were depleted more rapidly than those buried to 10 cm (EX_{50} of 37 days and EX_{99} of 247 days) (p = 0.0422) (Table 6-5; Fig. 6-2B). For Lethbridge 2009, depth was not significant and seed bank depletion was rapid. When all depths were combined, EX_{50} and EX_{99} estimates were 25 and 168 days, respectively and are comparable to estimates for 2008 buried samples (24 and 161 days, respectively) (Table 6-5; Fig. 6-2C).

Environmental differences between years and sites influence seed bank persistence. Precipitation in October 2009 was higher than normal (Fig. 6-1) and likely contributed to rapid seed germination and removal from the seed bank at both sites. Surface persistence may be more variable because it is most influenced by intermittent drying. Buried seeds of domesticated crops are usually less persistent than seed on the soil surface. Similar findings have been reported for wheat (Harker et al., 2005; Nielson et al., 2009), triticale (× *Triticosecale* Wittm. ex A. Camus) (Raatz et al., 2012), and safflower (*Carthamus tinctorius* L.) (McPherson et al., 2009). Estimated days to seed bank extinction (EX_{50} and EX_{99}) for camelina seed on the soil surface and buried seed are comparable. Most domesticated crops, with the exception of canola (Gulden et al., 2004b), differ from many weed species which can have seeds exhibiting primary and secondary dormancy and form persistent seed banks.

Seed bank longevity is often studied using artificial seed banks due to ease of retrieval, however, they have several drawbacks. Natural and artificial seed banks may have differential responses to biotic (predation, pathogenic and non-pathogenic microbial populations) and abiotic (moisture and temperature, physical

weathering) factors that influence germination, emergence, and exhaustion from the seed bank (Leon and Owen, 2004; Raatz et al., 2012). Data from artificial seed bank studies should be viewed with caution and results should be validated using alternative methods.

6.3.4. Volunteer camelina presence and persistence

Selected camelina fields covered a wide geographical area and represented several ecoregions that are best suited for agricultural production (Table 6-1). The volunteer camelina density in spring following crop production was high and variable within and between fields. In 2010, PRESEED densities ranged from 9 to 4839 plants m^{-2} with an average of 1208 plants m^{-2} (Tables 6-6 and 6-7). The observed variability is reflective of variable seed bank inputs via harvest losses and weather dependent fall germination.

Camelina volunteer densities dropped sharply with conventional farming practices, including routine herbicide applications. Volunteer plant populations were lower in 2011 than in 2010 indicating a significant population decline ($p <$ 0.0001). Densities declined over the course of the 2010 growing season from an average of 1208 plants m^{-2} for PRESEED to 3.9 plants m^{-2} POSTHARV (Table 6-7). A similar trend was observed in 2011; densities were highest for PRESEED ranging from 0 to 69 plants m^{-2} with a mean of 16 plants m^{-2}, but declined to 0 to 18 plants m^{-2} with a mean of 1 plant m^{-2} POSTHARV (Tables 6-6 and 6-7). The population increase from POSTHARV 2010 to PRESEED 2011 is likely

attributed to seed bank replenishment by mature camelina volunteers or viable seeds remaining in the soil.

While this study quantified the emergence of volunteer camelina in commercial fields, it did not quantify the role of seed replenishment from camelina volunteers. However, estimates generated from artificial camelina seed banks suggest seed will be almost entirely depleted by the May following harvest with only 0.2 to 10.6% of viable seeds remaining (Table 6-5).

Most fields showed an abrupt decline in volunteer densities by the PREHERB survey excepting Field 9 (Table 6-6). In Field 9, camelina was not harvested and all seed remained in the field resulting in unusually large seed bank inputs. Additionally, chemical fallow was applied relatively late in 2010 (mid-June), therefore volunteer densities were uncharacteristically high in this field (Tables 6-1 and 6-6).

Volunteer crop population recruitment occurs mainly in the year post dispersal followed by a sharp population decline in other crops including wheat (Harker et al., 2005; Nielson et al., 2009), safflower (McPherson et al., 2009), and flax (Dexter et al., 2010). Camelina volunteer field uniformity and density decreased over two years to near extinction in the eleven fields surveyed. Camelina plants with stem elongation or "bolting" were observed in the initial 2010 PRESEED survey suggesting that some volunteers had overwintered as rosettes. For 2010 and 2011, PRESEED field uniformity of camelina was 100 and 73% of surveyed fields, but declined to 9.1% (frequency) in 2011 PREHARV

(Table 6-7). Camelina was found in 93, 29 and 2% of all 2010 PRESEED, 2011 PRESEED and 2011 PREHARV quadrats surveyed, respectively (Table 6-7). The largest reduction in density occurred between the PREHERB and POSTHERB survey period where field density (occurrence) was reduced from 441 to 57 plants m^{-2} in 2010 and from 14 to 3 plants m^{-2} in 2011, respectively, which corresponds to an 87 and 80% reduction in plant density (Table 6-7).

From the initial PRESEED 2010 to the final PREHARV 2011 survey periods, the total reduction in field uniformity (all) and field density (all) was from 93.2 to 1.8% which corresponds to an initial density of 1208 plants m^{-2} dropping to 0.6 plants m^{-2} (Table 6-7).

In agricultural fields, camelina has high fecundity and seed losses incurred at harvest contribute to large but variable seed bank inputs. While yield losses between 0.7 to 4.3% are comparable to other oil seed crops such as canola and flax (Gulden et al., 2003; Dexter et al., 2011), camelina's small seed size results in a high density of individuals with reproductive potential. The seed bank is transient and viable seeds are depleted, primarily through germination, to near extinction within two years post-production. Volunteers are initially abundant, but populations rapidly diminish, presumably due to crop competition and routine herbicide application. Davis et al. (2013) reported a low competitive ability, citing that canola and *Bromus tectorum* (L.) (downy brome) were superior competitors relative to camelina. Camelina plants are controlled by most broadleaf herbicides but show tolerance to soil applied herbicides ethalfluralin (*N*-ethyl-α,α,α-trifluoro-

N-[2-methylallyl]-2,6-dinitro-*p*-toluidine) and trifluralin (2,6-Dinitro-*N*,*N*-dipropyl-4-[trifluoromethyl] aniline) (Johnson et al., 2008).

Camelina shows most of the characteristics of a domesticated crop including seed retention at maturity, limited seed dispersal, synchronous germination (loss of secondary dormancy), reduced competitive ability, and short-lived seeds (Warwick and Stewart, 2005). Observations in all aspects of this study support the previous observations of a lack of primary and secondary seed dormancy (CFIA, 2011). In-field after harvest, greater than 50% of the seeds had germinated when conditions were conducive for germination, seed bank depletion was rapid and high densities of volunteers were present in the first year following the camelina crop.

Crop volunteers are common and abundant agronomic weeds (Leeson et al., 2005). Volunteers do not preclude the release of GE crops if the crop has been approved as safe for food and feed consumption. While the use of non-food crops as platforms for the production of bio-industrial oils avoids concern about product mixing, the public perception of adventitious presence of GE camelina seed grown for industrial purposes in other crops has not been tested.

Mitigation of volunteer survival and seed production may be possible by modifying current agronomic practices. Optimizing harvest practices can minimize seed loss, increase grower profitability and decrease potential volunteer populations. While tillage operations following harvest may reduce camelina seed banks by stimulating germination and allow growers to control volunteers pre-

seeding, this practice is not consistent with current soil and water conservation practices. Rather, we recommend application of a short residual broadleaf herbicide followed by seeding of a competitive cereal crop, careful application of in-crop herbicides and monitoring following application to verify control. Early and consistent control of volunteer camelina will prevent seed bank replenishment and reduce persistence. The adventitious presence of camelina seed in subsequent crops is possible, however removal of small seeds during commercial harvest of most larger seeded cereal crops is likely.

6.4. Conclusions

Despite high fecundity and the reported ability of camelina to tolerate abiotic stress, camelina has limited ability to be weedy or invasive of agricultural areas when coupled with conventional farming practices. Competitive crops and the use of herbicides for weed control reduced camelina density and fecundity. Previous studies have examined invasiveness in disturbed and undisturbed native range. Davis et al (2011) reported camelina had low populations growth rates (λ), ranging from 0.017 to 0.001. In these non-agricultural environments, both seedling emergence and plant survival limited population longevity. Emergence was low, with the exception of seed spread in spring, and emergent seedlings had low survival (0 to 16 %) and camelina was reported to be uncompetitive (Davis, 2010). Populations established in two sites in two years were unable to reach replacement ($\lambda = 1$) and would be become extinct without further seed inputs.

While camelina SMGF can be mitigated with stewardship and segregation of equipment and land, complete segregation of GE and organic camelina is unrealistic and impractical. Should unconfined environmental release be realised, the establishment of attainable thresholds for adventitious presence, will be key to co-existence.

Seed persistence, dormancy, and population dynamics of the crop are factors to consider when choosing a platform for an industrial bioproduct. Weediness in agricultural areas or invasiveness of natural areas should not prevent the release of GM camelina as an industrial crop.

Table 6-1 Crop type and location for 11 commercial fields surveyed in 2010 and 2011 to quantify camelina persistence.†

			Crop type	
Field	Location	Ecoregion	2010	2011
1	Blackie, AB	Aspen parkland	Barley	‡
2	Claresholm, AB	Moist mixed grassland	Barley	Barley
3	Coaldale, AB	Moist mixed grassland	Canola	Canola
4	Delia, AB	Aspen parkland	Canola	Canola
5	Lethbridge, AB	Moist mixed grassland	Flax	Canola
6	Rumsey, AB	Aspen parkland	Barley	Canola
7	Skiff, AB	Mixed grassland	Winter wheat	Barley
8	Swift Current, SK	Mixed grassland	Barley	Peas
9	Tompkins, SK	Cypress upland	Chemical fallow	Barley
10	Viking, AB	Aspen parkland	Canola	Canola
11	Wilkie, SK	Moist mixed grassland	Barley	Lentils

† All crop type information was collected from producer.
‡ Field was not surveyed.

Table 6-2 Survey dates to examine camelina volunteer persistence.

	Year	
Survey period†	2010	2011
PRESEED	May 10, 11, 12, 13	May 9, 10, 11
PREHERB	June 7, 8, 9, 10, 15, 16	June 13, 14
POSTHERB	July 13, 14, 15	July 12
PREHARV	Aug 16, 23, 24, 25, Sept 1, 2	Aug 11
POSTHARV	Oct 6, 7, 8, Nov 2	‡

† PRESEED, before seeding; PREHERB, after seeding but before in-crop herbicide application; POSTHERB, after in-crop herbicide application; PREHARV, before harvest; POSTHARV, after harvest.
‡ POSTHARV 2011 survey was not conducted due to lack of camelina volunteers.

Table 6-3 Camelina seed losses in direct combine harvested fields quantified as seedlings counted in the field and germinated under greenhouse conditions, expressed as average density of propagules (seeds m^{-2}) and the seed weight (kg ha^{-1}) based on a conservative thousand kernel weight of 1.0 g.

		Camelina seedling density			Camelina seed weight		
Year	Field	In-field†	Greenhouse†	Total	In-field	Greenhouse	Total
		----------seeds m^{-2}----------			----------kg ha^{-1}----------		
2008	08-1	2795 ± 498a	40,635 ± 3,048a	43,430 ± 3,062a	28.0	406.4	434.3
	08-2	2938 ± 475a	2,030 ± 2,906b	4,967 ± 2,920b	29.4	20.3	50.0
	08-3	1382 ± 475a	683 ± 2,906b	2,065 ± 2,920b	13.8	6.8	20.7
2009	09-1	0b	31,257 ± 2,906a	31,257 ± 2,920a	0	312.6	312.6
	09-2	0b	2,973 ± 2,906b	2,973 ± 2,920b	0	29.7	29.7
	09-3	0b	7,372 ± 2,906b	7,372 ± 2,920b	0	73.7	73.7
2010	10-1	3004 ± 595a	1,032 ± 3,643b	4,037 ± 3,660b	30.0	10.3	40.4
	10-2	853 ± 595a	349 ± 3,643b	1,202 ± 3,660b	8.5	3.5	12.0
	10-3	1230 ± 595a	1,314 ± 3,733b	2,571 ± 3,750b	12.3	13.1	25.7

† Least square means from mixed model ANOVA using PDMIX800 (Saxton, 1998) with adjusted $P < 0.005$.

Table 6-4 Percent germinations for 20 camelina cultivars at intervals of days post planting for pre-harvest and post-harvest periods at Edmonton, Alberta, Canada in 2012 and results of ANOVA.

	Days post planting									
	Pre-harvest†								Post-harvest	
Cultivar	50‡	57‡	64	72	78	85	92	99	106	113
	----------Germination (%)----------									
CN113715	0	0	0a¶	5a	7a	0c	24abc	94a	94a	100a
CN113756	0	0	0a	0a	18a	29abc	75ab	92a	100a	99a
CN113704	§	0	0a	0a	40a	25bc	7c	93a	99a	99a
CN113673	§	0	0a	0a	54a	39abc	61abc	93a	96a	95a
CN113707	0	0	2a	2a	34a	37abc	59abc	93a	99a	95a
CN113746	0	0	0a	2a	17a	18bc	74ab	97a	97a	99a
CN113690	0	0	1a	0a	29a	44ab	79a	93a	96a	98a
CN113758	§	0	1a	0a	2a	9bc	11c	89a	97a	99a
CN113738	0	0	0a	0a	22a	25bc	81a	89a	98a	97a
CN113701	§	0	0a	0a	53a	69a	66abc	92a	94a	95a
CN113729	0	0	0a	9a	7a	8bc	17bc	97a	99a	98a
CN113681	§	0	0a	1a	5a	11bc	5c	91a	97a	97a
CN113685	0	0	0a	4a	1a	12bc	29abc	93a	98a	99a
CN113710	§	0	0a	0a	15a	37abc	53abc	95a	97a	96a
CN113749	§	0	0a	0a	2a	2bc	28abc	92a	91a	99a
CN113752	§	0	0a	0a	61a	6bc	52abc	95a	99a	99a
CN113723	0	0	0a	3a	28a	33abc	52abc	96a	94a	97a
CN113716	§	0	0a	1a	15a	2bc	46abc	94a	93a	97a
CN113728	0	0	0a	0a	5a	9bc	15bc	95a	97a	100a
CN113696	§	0	0a	0a	30a	2bc	64abc	92a	97a	98a

† Germination for each cultivar was tested weekly starting approximately 10 days post flowering initiation. Cultivars were harvested at 106 days post planting.

‡ Data did not converge due to lack of non-zero data points.

§ Cultivars were too immature to be sampled.

¶ Means followed by the same letter are not significantly different (Saxton, 1998). Mean separations are based on Bonferroni-adjusted *p*-values, $p < 0.00005$.

Table 6-5 Summary of results of seed burial experiments conducted in 2008 and 2009 at Edmonton and Lethbridge, including slope (*b*) from regression analysis of the exponential loss of seed viability ($P = ae^{-bd}$), estimated days to 50% and 99% seed extinction (EX_{50} and EX_{99}), and estimated frequency of viable seed the following spring and fall.

Year	Location	Depth	Slope estimate†			Frequency of viable seed‡	
			B	EX_{50}	EX_{99}	Spring (May)	Fall (Sept.)
		--cm--		-----d-----			
2008	Edmonton and Lethbridge	0	0.0105 ± 0.0001	66.0 ± 0.8	438.4 ± 5.2	0.1056 ± 0.0028	0.0290 ± 0.0012
		3 and 10	0.0286 ± 0.0010	24.3 ± 0.9	161.3 ± 5.6	0.0022 ± 0.0005	0.0001 ± <0.0001
2009	Edmonton	0	0.0256 ± 0.0016	27.0 ± 1.7	179.7 ± 11.3	0.0041 ± 0.0014	0.0002 ± 0.0001
		3	0.0270 ± 0.0019	25.6 ± 1.8	170.3 ± 12.1	0.0031 ± 0.0013	0.0001 ± 0.0001
		10	0.0186 ± 0.0007	37.2 ± 1.3	247.1 ± 85.6	0.0185 ± 0.0026	0.0019 ±0.0004
	Lethbridge	0, 3, and 10	0.0274 ± 0.0012	25.3 ± 1.0	168.3 ± 6.6	0.0029 ± 0.0007	0.0001 ± <0.0001

† Parameter estimate ± SE for *b* (the rate of seed viability decline), where intercept $a = 1$.
‡ Frequency of viable seed ± SE estimates for spring (day 211, roughly 1 May to 23 May) and fall (day 334, roughly 1 Sept. to 23 Sept.) following seed burial.

Table 6-6 Mean volunteer camelina density at five different survey periods over two years in eleven commercial fields in Alberta and Saskatchewan, Canada to quantify volunteer camelina persistence.

Survey period‡	Field										
	1	2	3	4	5	6	7	8	9	10	11
	----------plants m^{-2}----------										
2010											
PRESEED	43	92	37	26	3512	228	865	9	4839	447	253
PREHERB	40	8	12	19	618	19	640	17	2863	61	28
POSTHERB	4	4	8	0	13	12	0	0	264	8	20
PREHARV	0	0	10	0	4	0	4	0	80	9	8
POSTHARV	0	0	8	0	6	0	0	0	32	0	0
2011											
PRESEED	§	6	32	0	5	5	4	0	69	4	5
PREHERB	§	0	§	0	21	6	0	0	36	0	0
POSTHERB	§	0	§	§	0	§	0	§	12	0	0
PREHARV	§	§	0	§	0	0	§	§	18	§	§

† All values represent the least squared means from the mixed model ANOVA.
‡ PRESEED, before seeding; PREHERB, after seeding but before in-crop herbicide application; POSTHERB, after in-crop herbicide application; PREHARV, before harvest; POSTHARV, after harvest.
§ Field was not surveyed

Table 6-7 Mean volunteer camelina frequency, field uniformity, density and growth stage at five different survey periods in 11 commercial fields in Alberta and Saskatchewan, Canada in 2010 and 2011 for volunteer camelina persistence study.

Survey period§	Field uniformity†			Field density‡			Most advanced growth stage
	Frequency	All	Occurrence	All	Occurrence	High	
	----------%----------			--------plants m^{-2}--------			
2010							
PRESEED	100	93.2	93.2	1208	1208	10,112	Main stem elongating
PREHERB	100	88.6	88.6	441	441	6,592	First flowers open
POSTHERB	81.8	30.9	57.5	41.2	56.6	1,280	Mature plant
PREHARV	63.6	15.5	28.3	8.8	16.1	172	Mature plant
POSTHARV	36.4	10.9	40	3.9	14.2	296	Mature plant
2011							
PRESEED	72.7	28.6	39.4	16.0	22.0	644	Main stem elongating
PREHERB	30	9.5	31.7	4.4	14.2	252	First flowers open
POSTHERB	9.1	1.8	20.0	0.3	2.8	24	Mature plant
PREHARV	9.1	1.8	20.0	0.6	6.2	80	Mature plant

†Frequency is the percentage of surveyed fields in which volunteer camelina occurred; All is the number of quadrats in which volunteer camelina occurred of total number of quadrats; Occurrence is the number of quadrats in which volunteer camelina occurred of quadrats sampled in occurrence fields.
‡All is the average density in all fields surveyed; Occurrence is the average density of volunteer camelina in fields where insofar occurred; High is the highest camelina density observed per survey period.
§PRESEED, before seeding; PREHERB, after seeding but before in-crop herbicide application; POSTHERB, after in-crop herbicide application; PREHARV, before harvest; POSTHARV, after harvest.

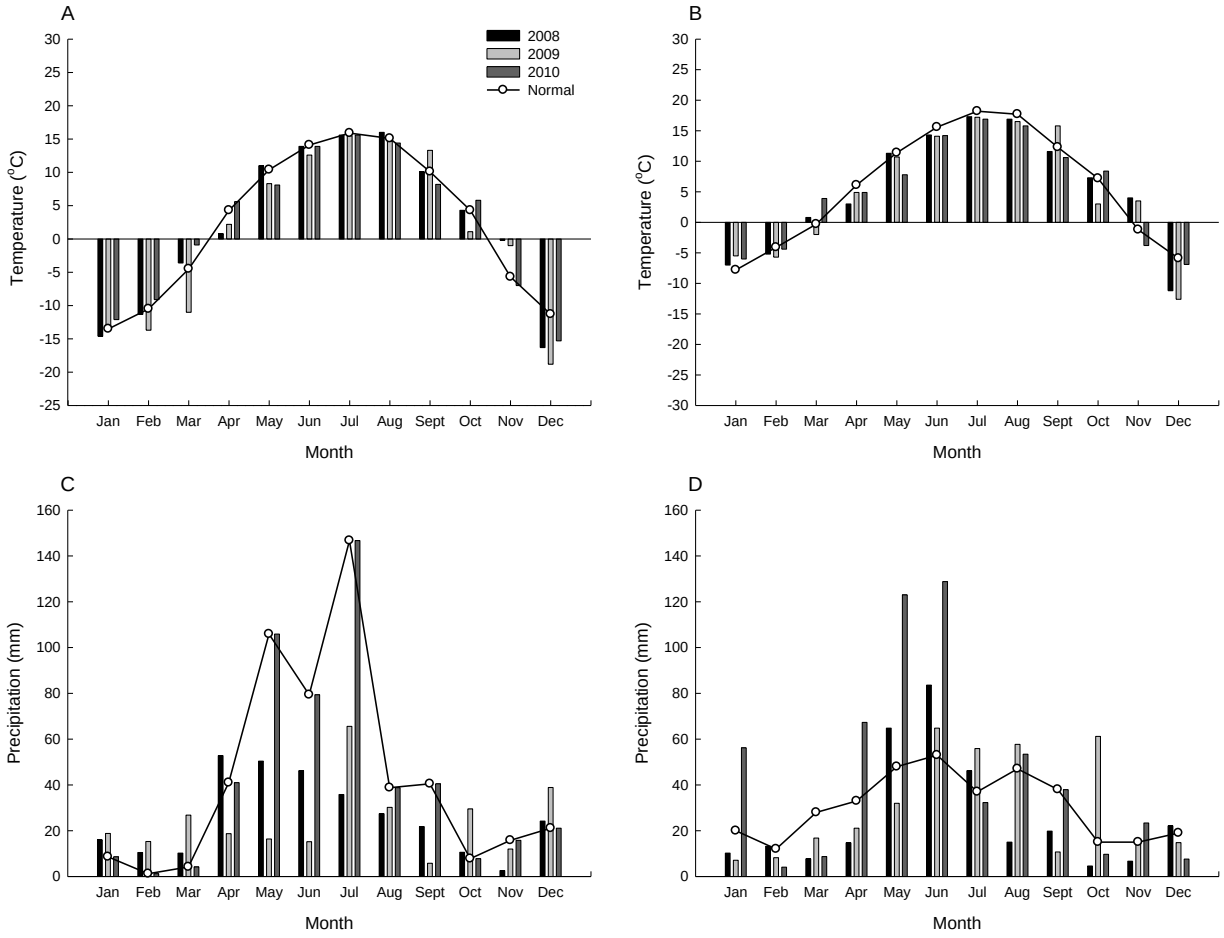

Figure 6-1 Average monthly temperatures (^{o}C) for Edmonton (A) and Lethbridge (B) and total monthly precipitation (mm) for Edmonton (C) and Lethbridge (D) for years 2008 to 2010 with 30 year averages presented.

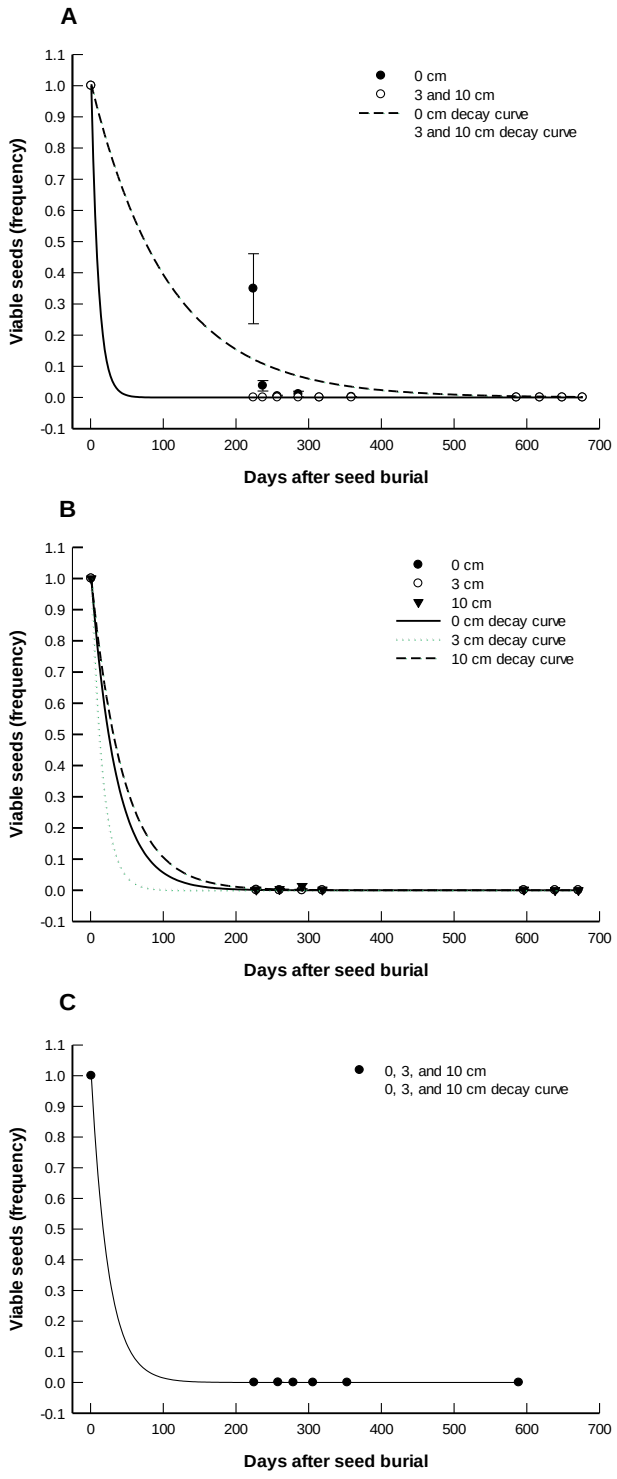

Figure 6-2 Seed survival curves for camelina A) on the soil surface and buried (pooled 3 and 10cm depths) at Edmonton and Lethbridge trials initiated in 2008, B) on the soil surface and buried to 3 and 10 cm at Edmonton trial initiated in 2009, and C) pooled 0, 3 and 10 cm depths at Lethbridge trial initiated in 2009. Seed survival curves are a representation of pooled data for cultivars Calena and

CN101985. Seed survival is estimated from the exponential decay, $P = ae^{-bd}$. Symbols are the observed mean frequency of viable seeds ± standard error of the mean. Standard error bars may be obscured by symbols.

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Chapter 7. Allelopathic effects of Brassicaceae members camelina and canola on germination and growth of wild oat, flax and radish

7.1. Introduction

Allelopathy is the stimulatory or inhibitory effect of chemicals released into the environment from one plant on the growth and development on another living organism (Inderjit et al., 2011). Members of the Brassicaceae family have been frequently cited as being allelopathic (Evenari, 1949; Lovett and Sagar, 1978; Lovett and Jackson, 1980; Lovett and Duffield, 1981; Petersen et al., 2001; Jafariehyazdi and Javidfar, 2011) and include crops canola (*Brassica napus* L.) and camelina [*Camelina sativa* (L.) Crantz.], as well as vegetable crops broccoli (*Brassica oleracea* L.) and radish (*Raphanus sativus* L.). Glucosinolates, sulfur containing compounds characteristic of Brassicaceae members, have been attributed with allelopathic activity (Poulsen et al., 2008; Malik et al., 2010; Yasumoto et al., 2010). Glucosinolates themselves may possess limited biological activity, however, enzymatic degradation *via* thioglucoside glucohydrolase (EC 3.2.3.1) results in the formation of allelochemicals including isothiocyanates and nitriles (Brown and Morra, 1996).

Camelina is an oilseed crop of European origins that has renewed interest for its potential to serve as a feedstock for biofuel production (Frohlich and Rice, 2005; Johnson et al., 2008). Known commonly as 'false flax', camelina was once considered a weedy species in association with flax crops, an opportunity that may have afforded crop-weed evolution (Francis and Warwick, 2009; Lovett and Sagar, 1978). Currently, no herbicides have been registered for broadleaf weed

control in camelina, thus limiting weed control options. Early observations by Grümmer and Beyer (1960) noted that *Camelina* ssp. in flax (*Linum usitatissimum* L.) reduced flax yield up to 80% in glasshouse experiments. The observed suppression was attributed to leaf-bound kolines. In contrast, subsequent research reported a stimulatory allelopathic effect of camelina leaf washings on flax growth credited to the presence of phyllospheric bacteria *Enterobacter cloacae* (Jordan) Hormaeche (Lovett and Sagar, 1978; Lovett and Jackson, 1980). Incongruent results are indicative of the innate complexity of allelopathic interactions.

Utilization of canola for green manure or cover cropping can be used to suppress weeds and maintain soil structure (Boydston and Hang, 1995; Haramoto and Gallandt, 2005; Yasumoto et al., 2010), however, the significance of allelopathy in this activity has yet to be conclusively determined. Yasumoto et al. (2011) conducted laboratory assays and reported the auto-toxic effects of canola tissue, aqueous extracts and root exudates on seed germination and plant growth. Furthermore, they observed reduced sunflower (*Helianthus annuus* L.) growth in the field following canola cultivation and suggested root exudates were responsible. Likewise, Jafariehyazdi and Javidfar (2011) documented inhibition of sunflower germination and growth from the aqueous extracts of several *Brassica* ssp. including canola in laboratory assays. Further research is required to determine the mechanism of suppression observed in these studies.

Laboratory assays serve as an initial evaluation tool to identify a potentially allelopathic interaction. A four-part laboratory and glasshouse

experiment was designed to investigate the effects of camelina and canola: (I) leaf washings; (II) aqueous plant extracts; (III) soil incorporated fresh plant residues; and (IV) root exudates. The effects were evaluated on three recipient plant species; wild oat, an abundant weed in the Canadian prairies (Sharma and Born, 1978); flax, a species historically associated with camelina; and radish, a fellow Brassicaceae member. Identification of allelopathic activity may enable development of cultivars with enhanced allelopathic activity and contribute to the development of integrated weed management systems.

7.2. Materials and methods

Camelina (cultivar 'Calena'), canola (variety 72-55 Roundup Ready®), flax (cultivar 'Bethune'), radish (cultivar 'Cherry Belle') and wild oat seeds harvested from wild plants near Edmonton, Alberta, Canada, were used for these assays. Camelina and canola aqueous extracts were prepared from plants grown in the field, maintained without chemical controls, in 2011 at Ellerslie, Alberta, Canada. Field grown plants were harvested for extract preparation at the stem elongation to early flowering growth stages. The remaining assays were performed with camelina and canola grown from seed in glasshouse conditions. All glasshouse propagated plants for the assays outlined below were grown in 15 cm pots in soilless potting medium (Sunshine Mix 4, Sun Gro Horticulture) and fertilized bi-weekly using 24-8-16 all-purpose plant food (Scotts Miracle-Gro).

7.2.1. Effects of leaf washings

Camelina and canola were tied into 76.0 g shoot bundles, cut stems were sealed with Parafilm® (Pechiney Plastic Company), and bundles were agitated at 135 rpm for 10 min in 1.0 L distilled water. Washings were stored in the dark at 4 ˚C. Fifty recipient seeds (wild oat, flax or radish) were placed in individual acrylic germination boxes (Hoffman Manufacturing, Inc.) between two sheets of non-toxic white filter paper (15 x 23 cm No. 601 Whatman #1 equivalent, Hoffman Manufacturing, Inc.) and saturated with 25 mL of the appropriate washing. An additional 5 mL was added on day 6 to avoid desiccation. Three treatments per recipient species were performed as follows: camelina leaf washings, canola leaf washings, and distilled water. Seeds were placed in Adaptis A1000 (Conviron Ltd., USA) growth chamber; 23°C, 16 hr light/8 hr dark cycles for 10 days when individual seedling root and shoot fresh weights were recorded. Samples were dried at 65°C for 96 hrs and dry weights recorded. Experimental design was a randomized complete block with three replicates, where treatment was the allelopathic donor.

7.2.2. Effects of aqueous extracts

Camelina and canola root and shoot aqueous extracts were prepared individually by homogenization of 150 g fresh material and 300 mL distilled water for 2 minutes in a Waring blender, filtered through cheesecloth, and stored in the dark at 4°C. pH was recorded for each extract (data not shown). Fifty recipient seeds (wild oat, flax or radish) were placed in individual acrylic

germination boxes (Hoffman Manufacturing, Inc.) between two sheets of non-toxic white filter paper (15 x 23 cm No. 601 Whatman #1 equivalent, Hoffman Manufacturing, Inc.) saturated with 25 mL of extract. An additional aliquot of 5 mL extract was added on day 6 to avoid desiccation. Each recipient species received one of five extracts: camelina shoot, camelina root, canola shoot, canola root or distilled water. Boxes were stored at 23°C (Adaptis A1000, Conviron Ltd., USA) with 16 hr light/8 hr dark cycles. The number of germinated seeds was quantified daily for 12 days after which individual seedling root and shoot fresh weights were recorded. Germination rate was calculated:

$$Germination\ rate = N_1/D_1 + N_2/D_2 + \ldots + N_i/D_i \quad [1]$$

Where: *Ni* means the number of seeds that germinated in days *Di* (Jafariehyazdi and Javidfar, 2011). Samples were dried at 65°C for 96 hrs and weighed. The experiment was a randomized complete design, replicated three times, blocked in time where donor species was the main effect and root vs. shoot was the sub-effect.

7.2.3. Effects of incorporated fresh residues

Glasshouse grown camelina and canola root and shoot biomass was chopped into ~1 cm fragments, mixed with soilless medium and divided evenly between 24 pots. An additional 12 pots were filled with medium only. To separate out the effects on germination, 6 pre-germinated seedlings per recipient were planted in 12 pots (4 control, 4 camelina residues, 4 canola residues) and thinned to 4 seedlings 2 weeks post planting in glasshouse conditions. Five weeks after

planting, individual recipient height, and shoot fresh weight were recorded; an additional tuber fresh mass was taken for radish plants. Samples were desiccated at 65°C for 96 hrs and the resultant dry weight recorded. The experiment was a randomized complete design blocked and was performed a total of three times.

7.2.4. Effects of root exudates

Glasshouse grown camelina and canola were removed from pots at the stem elongation to early flowering stage of development (at approximately 5-6 weeks growth). The media was hand sifted to remove all root fragments, bulked, and then redistributed into clean pots; and additional (control) pots filled with new media. The remainder of the experiment was executed according to 'effects of incorporated fresh residues' assay methods outlined previously. The experiment was a randomized complete design and was performed three times.

7.2.5. Statistical analysis

Data were subjected to analysis of variance (ANOVA) by employing a mixed model (PROC MIXED) in SAS (SAS Institute Inc., 2007). Prior to ANOVA the data was reviewed to ensure residuals met the assumptions for normality and homogeneity of variance. All data did not significantly differ from that of a normal population with the exception of 'effects of incorporated fresh residues' and 'effects of root exudates', which were square root transformed. The data were analyzed with a mixed model as a one-way ANOVA where block

(replicate) was considered random. Least squared means generated by the model with Bonferroni adjusted alpha values and means separation were carried out using PDMIX800 (Saxton, 1998).

7.3. Results and discussion

7.3.1. Effects of leaf washings

Where a change was observed, camelina and canola leaf washings generally increased recipient seedling fresh weight (Table 7-1). Leaf washings had no significant effect on wild oat root or shoot fresh weight compared to controls ($p > 0.05$). Flax root and shoot fresh weight increased in response to canola ($p < 0.05$), but not to camelina ($p > 0.05$) (Table 7-1). Radish root, and shoot fresh weight increased in response to both camelina and canola leaf washing ($p \leq 0.001$). Although leaf washings of both donors intermittently increased seedling fresh weight, canola was effective on radish and flax recipients, while camelina only affected radish.

Leaves appear to be the most consistent source of inhibitors and thus have been the focus of many allelopathic investigations (Rice, 1974). Lovett *et al*. (Lovett and Sagar, 1978; Lovett and Jackson, 1980; Lovett and Duffield, 1981) observed a positive effect on flax growth in response to camelina leaf washings. We observed no increase in flax root or shoot weight in response to camelina leaf washings. Wild oat was similarly unaffected while radish was affected and weight was increased relative to the control. Canola leaf washings resulted in an

increase in both flax and radish weights. Lovett and Sagar (1978) attributed the stimulation of flax growth to the presence of the native camelina phyllospheric bacteria, *E. cloacea*. They also demonstrated that *E. cloacea* metabolized glucosinolate benzylisothiocyanate to benzylamine, an allelochemical stimulatory at low concentrations. Glucosinolate content in camelina seed ranges from 18.7 to 36.2 μmol g^{-1} (Schuster and Friedt, 1998), well below levels 39.94 to 53.01 μmol g^{-1} documented in canola seed (Yasumoto et al., 2010), however, reports of glucosinolate content in leaf tissue or washings have not yet been established. Future research may be directed towards identification, quantification and comparison of benzylamine, or other potential allelochemicals, in camelina and canola leaf washings.

7.3.2. Effects of aqueous extracts

Aqueous extracts suppressed seed germination and treatment was highly significant (Table 7-2). However, the effects of canola root and shoot extracts did not significantly vary from controls on flax and wild oat. Additionally, canola extracts were generally less inhibitory than camelina extracts on seed germination. Variability was observed in the effect of plant extracts on seedling fresh weight. Due to the low total seed germination of wild oat and flax, the ability to interpret the effect of camelina and canola extracts on fresh weight was limited. No significant differences were observed for flax shoot fresh weight. For radish, root fresh weight generally decreased, whilst shoot fresh weight increased. Despite

decreased germination rates, total seedling fresh weight of radish was increased by canola root and shoot extracts.

The suppression of germination by camelina and canola extracts observed in this study is reflective of previously published research. Evenari (1949) reported that glucosinolate containing mustard (Brassicaceae) oils are strong germination inhibitors. Aqueous seed extracts of *Brassica juncea* (L.) Czern. significantly can reduce germination of alfalfa (*Medicago sativa* L.), radish and turnip (*Brassica rapa* var. *rapa* L.) (Gressel and Holm, 1964). Similarly, aqueous seed extracts of *Brassica nigra* L. reduced wheat (*Triticum aestivum* L.) germination indices to 6% (Evenari, 1949) and allyl isothiocyante liberated from macerated *B. nigra* leaves inhibited germination of *Bromus rigidus* (Roth) seeds (Bell and Muller, 1973). Germination inhibition *via* allelochemicals has been connected to lipid mobilization (Baleroni et al., 2000), and low levels of isothiocyanates, a glucosinolate break-down product, have also been shown to induce secondary dormancy (Petersen et al., 2001). The use of plant extracts to elicit allelopathic responses has been justifiably questioned as it is not an accurate representation of plant activity in a natural setting (Inderjit and Dakshini, 1995). The identification of active compounds present in extracts with the ability to suppress seed germination or seedling growth is still of interest as it may give credence to alternative weed control methods.

7.3.3. Effects of incorporated fresh residues

The incorporation of canola and camelina residues significantly increased wild oat dry weight and seedling height (Table 7-3). Flax was not significantly affected. Radish height, fresh and dry weights were significantly increased by camelina and canola residues.

Brassica species are important North American crops and have potential for green manuring as part of an integrated crop management strategy (Boydston and Hang, 1995; Brown and Morra, 1996; Krishnan et al., 1998). A green manure crop is planted in the fall to prevent soil erosion and improve moisture retention. Brassica green manuring efficacy has been previously attributed to the presence of the glucosinolate degradation product isothiocyanate (Vaughn and Boydston, 1997; Petersen et al., 2001; Gimsing et al., 2007; Poulsen et al., 2008). Isothiocyanates are volatile which may limit the allelopathic potential of donor residues. Petersen *et al.* (2001) observed that following incorporation of turnip-rape mulch the amount of isothiocyanates in the soil remaining after 24 hours had declined by >90%. The cause and effect linkage between the observations and allelopathic response must be viewed with caution.

We observed an increase in the fresh weight of wild oat and radish, but not flax recipients, in response to incorporation of camelina or canola fresh plant residues. In contrast, research by Boydston and Hang (1995) reported that spring-incorporated canola reduced weed biomass up to 96 % in potato (*Solanum tuberosum* L.) fields while Krishnan *et al.* (1998) reported reduction of 49 % weed biomass in soybean (*Glycine max* L.) crops. Although reduced weed

biomass was attributed to secondary metabolites (AlKhatib et al., 1997), the mechanism has not been well characterized. Weed biomass accumulation in field situations is confounded by many factors, including environmental effects that influence seed germination such as temperature, light, soil nitrate levels and timing of emergence. While many studies investigate reduction in weed biomass without eliminating seed germination and emergence as variables (Boydston and Hang, 1995; Krishnan et al., 1998; Kumar et al., 2009; Uremis et al., 2009), we utilized pre-germinated recipient seedlings to specifically test the allelopathic effects on seedling growth and biomass accumulation in wild oat, flax and radish. Under these conditions, soil incorporated canola and camelina residues stimulated growth of wild oat and radish. While our results are discordant with many studies, it is possible that the historically observed weed biomass reduction is the result of reduced or delayed seed germination.

7.3.4. Effects of root exudates

Canola significantly decreased ($p > 0.05$) wild oat and radish fresh and dry weights, while camelina root exudates only significantly decreased ($p = 0.0079$) flax dry weight (Table 7-4). Camelina significantly reduced ($p = 0.0477$) flax fresh mass however dry mass was increased relative to the control. Similarly, canola reduced fresh mass in wild oat but increased radish dry mass.

Camelina and canola root exudate assays revealed no highly significant effects on recipients. Where effects were observed, wild oat and flax fresh shoot

mass decreased. Root exudates are considered products of living roots where no leaf washings, volatiles or residues from the above ground biomass are present (Rice, 1974), consequently isolation of the root exudates and observation of their effects can be difficult. Roots generally contain less allelochemicals than leaves, and root allelochemicals may be less potent and present in smaller amounts, though this is not always the case (Rice, 1974). Additionally, the amount of exudates present can be dependent on external factors including photoperiod and temperature (Pramanik et al., 2000), yet certain phenolics exuded from roots may be inhibitory or stimulatory depending on the concentration (Bertin et al., 2003). The rhizosphere is the site of greatest activity within the soil matrix and therefore represents the largest fraction of root exudates (Bertin et al., 2003). Rhizospheric soil is ≤ 2 mm from the root surface (Bertin et al., 2003). Therefore, we examined the effects of both rhizosphereic and non-rhizospheric medium in this allelopathic study. Yasumoto *et al.* (2011) observed a reduction in yield and quality of crops following canola cultivation that they attributed to the presence of juvenile volunteer canola seedling root exudations. The observed reduction in shoot weight may be associated with the presence of allelochemicals; however, volunteer canola can be frequently found in high densities and the effects on soil fertility or moisture and cannot be dismissed.

7.4. Conclusions

Selective breeding efforts to improve the allelopathic activity of some crop species including rice, wheat and barley have been published (Bertholdsson,

2007; Khanh et al., 2007; Bertholdsson, 2010; Kong et al., 2011). This study demonstrated evidence for stimulatory as well as inhibitory potential allelopathic activity in camelina and canola. Observed variability between the responses of the three recipient species and in the results of the four different assays emphasizes challenges associated with identifying allelopathic responses and separation of confounding factors. Laboratory assays serve as a useful initial screening method to identify potential allelopathy. Future experimentation will be required to identify potential allelochemicals and test their activity in an ecological context.

Table 7-1 Effects of camelina and canola leaf washings on wild oat, flax, and radish seedling root and shoot fresh weight and root to shoot ratio.

Treatment	Wild oat				Flax				Radish			
	Root weigh	Shoot weight	Total seedling weight	Root to shoot ratio	Root weight	Shoot weight	Total seedling weight	Root to shoot ratio	Root weight	Shoot weight	Total seedling weight	Root to shoot ratio
	----------(g)----------				----------(g)----------				----------(g)----------			
Control	0.042ab	0.036a	0.080a	1.4a	0.028a	0.009a	0.037a	4.4ab	0.049a	0.024a	0.076a	2.7a
Canola	0.046a	0.036a	0.085a	1.4a	0.034b	0.013b	0.047b	3.3a	0.081b	0.030b	0.113b	3.5a
Camelina	0.037b	0.034a	0.074a	1.2a	0.031ab	0.010a	0.040a	4.8b	0.069c	0.033c	0.105b	2.8a

Values displayed are least squared means of a mixed model ANOVA. Mean values within a column followed by the same letter are not significantly different ($p > 0.05$) (Saxton, 1998). Mean separations are based on Bonferroni-adjusted p-values, $p < 0.005$.

Table 7-2 Effects of camelina and canola aqueous root and shoot extracts on wild oat, flax, and radish seed germination rate and seedling root and shoot fresh weight.

Treatment	Wild oat				Flax				Radish		
	Germination rate	Root fresh weight	Shoot fresh weight	Sum total seedling fresh weights	Germination rate	Root fresh weight	Shoot fresh weight	Sum total seedling fresh weights	Germination rate	Root fresh weight	Shoot fresh weight
		----------(g)----------				----------(g)----------				----------(g)----	
Control	35.0a	0.020a	0.016a	4.30	61.3a	0.010	0.020a	4.29	139.6a	0.0276a	0.051a
Canola root	2.4b	*	0.018ab	0.25	28.7ab	*	0.017a	0.94	73.8bc	0.012b	0.112b
Canola shoot	12.4ab	0.007b	0.029b	1.84	21.5b	*	0.021a	1.04	57.9bc	0.012b	0.096b
Camelina root	0.2b	*	*	0.03	9.1b	n/a	n/a	n/a	87.9bc	0.004c	0.073c
Camelina shoot	4.1b	*	0.012a	0.51	8.0b	*	0.008a	0.04	42.0c	0.002c	0.053a

[n/a] Data failed to converge due to lack of germinated seedlings.

* Least squared estimates were unable to be generated due to highly non-normal data points.

Germination rate was calculated according to: $\text{Germination rate} = N_1/D_1 + N_2/D_2 + \ldots + N_i/D_i$ (Jafariehyazdi and Javidfar, 2011).

Values displayed are least squared means of a mixed model ANOVA excepting total seedling fresh weight. Mean values within a column followed by the same letter are not significantly different ($p > 0.05$) (Saxton, 1998). Mean separations are based on Bonferroni-adjusted p-values, $p < 0.005$.

Table 7-3 Effects of camelina and canola incorporated fresh residues on wild oat, flax, and radish seedling weight and height.

Treatment	Wild oat			Flax			Radish		
	Fresh weight (g)	Dry weight (g)	Height (cm)	Fresh weight (g)	Dry weight (g)	Height (cm)	Fresh weight (g)	Dry weight (g)	Height (cm)
Control	0.707a	0.145a	35.1a	0.637a	0.129a	23.1a	0.413a	0.077a	7.7a
Canola	1.145a	0.240b	39.3ab	0.558a	0.120a	23.1a	0.787b	0.171b	10.4b
Camelina	0.943a	0.187ab	40.9b	0.608a	0.121a	21.9a	1.004b	0.178b	10.4b

Values displayed are least squared means of a mixed model ANOVA. Mean values within a column followed by the same letter are not significantly different ($p > 0.05$) (Saxton, 1998). Mean separations are based on Bonferroni-adjusted p-values, $p < 0.005$.

Table 7-4 Effects of camelina and canola root exudates on wild oat, flax, and radish weight and height.

Treatment	Wild oat			Flax			Radish		
	Fresh weight (g)	Dry weight (g)	Height (cm)	Fresh weight (g)	Dry weight (g)	Height (cm)	Fresh weight (g)	Dry weight (g)	Height (cm)
Control	0.652a	0.0237a	46.1a	0.136a	0.010a	12.5a	0.679a	0.086a	4.9a
Canola	0.237b	0.0104a	43.9a	0.066ab	0.083ab	10.3a	0.550a	0.156b	4.6a
Camelina	0.431ab	0.017a	43.4a	0.023b	0.063b	10.2a	0.458a	0.089a	4.4a

Values displayed are least squared means of a mixed model ANOVA. Mean values within a column followed by the same letter are not significantly different ($p > 0.05$) (Saxton, 1998). Mean separations are based on Bonferroni-adjusted p-values, $p < 0.005$.

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Chapter 8. Conclusion

8.1. Summary of results

The research presented in this thesis supports the environmental biosafety risk assessment of genetically engineered (GE) camelina [*Camelina sativa* (L.) Crantz.] for use as a bio-industrial crop. Pollen- (inter- and intraspecific) and seed-mediated gene flow were characterized and quantified, a significant gap identified by the Canadian Food Inspection agency in the 'Biology of Camelina' document (CFIA, 2011). An additional investigation was conducted to assess camelina's potential for allelopathy. The results of this research will contribute to the development of risk mitigation strategies intended to reduce pollen- and seed-mediated gene flow from GE camelina and promote coexistence with conventional cultivars in an agroecosystem.

Short-distance outcrossing of transgenic to non-transgenic camelina was evaluated in Chapter 3. Small-plots (0.6 by 7 m) of receptor camelina were planted on two dates, situated north or south of the transgenic pollen donor (0.2 by 7 m). Pollen-mediated gene flow (PMGF) was measured up to 0.6 m and ranged from 0.09 to 0.28%, with the highest frequencies detected immediately adjacent to the pollen donor population. Direction (north or south) and planting date significantly influenced PMGF. These experiments confirmed that camelina is self-fertile, as previously reported by Francis and Warwick (2009). The experimental findings were used to design and execute medium- to large-scale camelina PMGF trials.

In Chapter 4, Medium-distance experiments were conducted to assess intraspecific PMGF from transgenic to non-transgenic camelina. Due to the temporal and monetary costs associated with transgenic research, only two trials were conducted in years 2011 and 2012 respectively. The experimental design was a modified Nelder (1962) wheel with a central pollen donor (10 by 10 m) and a concentric pollen receptor (56 by 56 m). Camelina outcrossing declined rapidly with increasing distance and was fit to an exponential decay model. The highest average PMGF (0.783%) was detected immediately adjacent to the pollen source, however, outcrossing was reduced by 99% in under 10 m.

In Chapter 5, tier II and III interspecific outcrossing experiments were conducted under greenhouse and short-distance field conditions to assess the potential for gene flow from camelina to weedy relative shepherd's purse [*Capsella bursa-pastoris* (L.) Medikus]. Zero interspecific shepherd's purse hybrid seeds were identified in 103,000 and 30,000 seeds for tier II and III examinations, respectively. Based on sample sizes established by power analysis, outcrossing is less than or equivalent to 0.025% (α=5) for tier II and 0.1% (α=2.5) for tier III with a power of 0.85 for both. This suggests that interspecific hybridization is unlikely, however, this study is a component of an ongoing investigation of interspecific gene flow.

In Chapter 6, the loss of camelina seed at harvest, its persistence in the soil at different depths, the potential for dormancy, and the volunteerism in commercial fields following cultivation was assessed. Harvest losses were numerous and variable, ranging from 1,202 to 43,430 viable seeds m^{-2}. However,

persistence was limited and viable seeds (buried or on the soil surface) became depleted within 15 months of dispersal. While surface seeds tended to persist slightly longer than those buried, presumably due to intermittent drying, the rapid seed bank extinction suggests that camelina seeds lack dormancy. The absence of dormancy did not appear to be cultivar specific as indicated when 20 cultivars were tested for their time dependant geminability. All cultivars rapidly after-ripened and became fully germinable prior to harvest and exhibited no subsequent seed dormancy. Camelina volunteer plant densities in commercial fields following cultivation were initially numerous and variable ranging from 9 to 4839 plants m^{-2}. Volunteer populations sharply declined and became near extinct after two years under conventional farming practices.

In Chapter 7, camelina allelopathy was evaluated relative to canola (*Brassica napus* L.) on allelopathic recipients wild oat (*Avena fatua* L.), flax (*Linum usitatissimum* L.), and radish (*Raphanus sativus* L.). Camelina and canola leaf washings increased radish seedling weight while flax weight increases were specific to canola. Aqueous extracts of camelina and canola reduced germination of wild oat, flax, and radish. Wild oat and radish seedlings had reduced root weight and increased shoot weight. Incorporation of camelina or canola fresh plant residues into growth medium increased radish weight, but only canola residues caused an increase in wild oat biomass. Canola root exudates decreased wild oat weight, but increased radish weight whilst camelina exudates decreased flax weight. Radish was the most prominently affected in these assays. Variable results between the four parts of the study are reflective of the challenges of

identifying allelopathic interactions between species and the need for rigorous and multi-part analyses requiring subsequent confirmation in an ecological context.

This biosafety risk assessment of camelina pollen- and seed-mediated gene flow provides insight into gene flow. Due to camelina's self-fertility and currently low cultivated area, intraspecific PMGF should not prohibit the co-existence of GE and conventional crops. Moreover, total elimination of gene flow is not realistic thus we recommend the implementation of attainable thresholds so that organic farming too may co-exist.

Interspecific outcrossing to weedy relative shepherd's purse appears to be a low risk. However, given the density and distribution of shepherd's purse further investigation is required to the findings presented in this thesis are part of an ongoing research effort. While camelina seed bank inputs may be numerous, a lack of dormancy and its rapid extinction under normal farming practices limit its agricultural weedy propensity.

This thesis supports the development of GE camelina when combined with the appropriate mitigation measures. Pollen represents a minor gene flow constituent but cannot be disregarded. Therefore we recommend that a buffer of at least 10 m be utilized to limit intraspecific PMGF and maintain purity of seed. While the potential for interspecific gene flow may be low, control of weedy shepherd's purse should be part of both GE and conventional regimes. This may include the application of a pre-seeding non-selective herbicide to reduce the number of winter annual shepherd's purse within field.

Camelina seed bank management is a key component of reducing seed-mediated gene flow. Maximizing seed retention by adjusting combine settings or harvesting immediately following maturity to minimize crop shatter losses may effectively decrease seed bank densities. Camelina seeds lack dormancy and rapidly exit the seed bank, therefore the next crucial step is to control volunteer plants that emerge primarily in the late spring to early summer following cultivation. This research is a foundation on which further risk assessments may be conducted to prior to the potential commercialization of GE camelina.

8.2. Future research

- We report camelina PMGF at short and medium-distance field conditions but have not quantified outcrossing at a commercial scale. Differences in PMGF have been reported between field- and commercial-scale cultivation in wheat, a self-fertile crop (Beckie et al., 2011). In the event of unconfined environmental release of GE camelina, it is important to understand the implications of commercial-scale PMGF in order to better assess risk.
- Numerous factors influence seed loss at the time of harvest including harvesting equipment, harvesting speed, crop maturity and environmental conditions as observed in canola (Gulden et al., 2003). This thesis quantified camelina seed lost at the time of harvest but did not consider factors influencing the density of seed bank entrants. Further characterization will contribute to the implementation of best management practices that limit SMGF.

- Identify if camelina's weedy propensity will be altered in environments outside of the western Canadian Prairies. Further research should be conducted to determine if variable moisture and temperature regimes, amongst other environmental factors, will enhance weedy or invasive behavior of camelina.
- Shepherd's purse was the primary focus of interspecific gene flow in this thesis. Prior to unconfined release of GE camelina, the propensity for fellow camelineae tribe members *Arabidopsis lyrata* L. and *A. thaliana* (L.) Heynh., *Neslia paniculata* (L.) Desv., *Erysimum* spp., and *Turritis glabra* L. will require assessment. If risk is found to be sufficiently low and deemed "acceptable", gene flow to wild and weedy relatives should not prohibit GE release.
- We report evidence for camelina allelopathy but cannot confirm the influence of an allelochemical under field conditions. Isolation and characterization of camelina allelochemicals will be required to confirm or refute allelopathic potential. In the likelihood that allelochemicals are identified, the effects specific to allelopathy may be deduced.

8.3. Literature cited

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