Feral Nature of Alfalfa (*Medicago sativa* L.): Implications for Novel Trait Confinement

By

Muthukumar Valayapalayam Bagavathiannan

A Thesis Submitted to the Faculty of Graduate Studies of
The University of Manitoba
In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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GENERAL ABSTRACT

Alfalfa is an important forage crop in North America which can also escape cultivation and establish in unmanaged habitats. Genetically modified (GM) alfalfa has been approved for environmental release in Canada and the United States and the occurrence of alfalfa in unmanaged natural and semi-natural habitats may compromise the successful co-existence of GM and non-GM alfalfa. To-date, little information has been available on the nature and dynamics of roadside alfalfa populations and their ability to become feral. Such knowledge is necessary to design efficient trait confinement protocols and to enhance the co-existence of GM and non-GM alfalfa within agricultural regions. The overall aim of this work was to characterize roadside alfalfa populations and to establish their role in novel trait movement. A roadside survey revealed the widespread occurrence of feral alfalfa populations in southern Manitoba. We described the seedbanks of roadside alfalfa populations, seedling recruitment and adult reproductive success, indicating that alfalfa is capable of establishing self-perpetuating feral populations in unmanaged habitats. We also noted the successful establishment of alfalfa in a grass sward representing roadside vegetation. Roadside mowing can reduce (and perhaps prevent) seed production in roadside alfalfa; however, mowing failed to drive the populations to extinction in the short-term. Herbicide (2,4-D) applications controlled alfalfa plants but seeds in the seedbank may still contribute to new seedling recruitment. The roadside alfalfa populations we worked with exhibited high levels of genetic diversity, indicating an absence of past population bottlenecks or genetic drift. In addition, phenotypic characterization provided evidence that roadside alfalfa populations were experiencing selection pressure for adaptive traits including winter survivability, rhizome production and prostrate growth habit; all traits that favor persistence in unmanaged habitats. We also noted the occurrence of high (>60%) levels of outcrossing in feral alfalfa populations, establishing their role as sources and sinks for novel traits. Our findings indicate that alfalfa populations occurring in unmanaged habitats need to be considered in trait confinement protocols in order to reduce the adventitious presence (AP) of novel traits and to enhance the successful co-existence of GM and non-GM alfalfa.

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DEDICATION

I dedicate this degree to my parents (Mr. and Mrs. Bagavathiannan), wife (Nithya) and son (Arun) for everything they sacrificed.

I acknowledge the remarkable contribution of the farming community to the subsistence of our species on this planet and would like to dedicate this work to all those who toil on their fields so that we can continue to survive.

…… Farmers are the linchpin of the world, for they support all those who take to other work, not having the strength to plough.

(Thirukkural 1032)

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This thesis is written in a manuscript style and the manuscripts have been prepared following the format provided by the journal- Ecological Research (Springer journal publishers). The thesis consists of eight manuscripts comprising of four data based papers, two review papers and two position papers. The position papers are included as appendices A and B. The work provided in Appendix A was an outcome of a visiting research fellowship at the Institute of Advanced Studies in Science, Technology and Society (IAS-STS), Graz, Austria. Appendix B is a theoretical analysis of possible gene flow between GM and non-GM alfalfa populations in the US-Canada international border region.

The publication status of each of the thesis chapter is given below:

Chapter 2: A general review on crop ferality – published

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Chapter 3: A comprehensive review on the biology and ecology of alfalfa - published

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Chapter 4: Characterizing the demography of feral alfalfa – in review

Bagavathiannan MV, Gulden RH, Begg GS, Van Acker RC (20xx) The demography of feral alfalfa populations occurring in roadside habitats in southern Manitoba, Canada. Environmental Science & Pollution Research.

Chapter 5: Alfalfa response to disturbances – in review

Bagavathiannan MV, Gulden RH, Van Acker RC (20xx) Establishment of alfalfa under different dispersal times and disturbance regimes in a semi-natural habitat. Ecological Research.

Chapter 6: Feral alfalfa survey and gene flow study – in review

Bagavathiannan MV, Gulden RH, Van Acker RC (20xx) Occurrence of feral alfalfa populations along roadside habitats in southern Manitoba, Canada and their role in intra-specific novel trait movement. Transgenic Research.

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Appendix A: Position paper on GM regulatory procedures – in review

Bagavathiannan MV, Van Acker RC (20xx). The deregulation of genetically modified alfalfa in the United States: mounting challenges for risk assessors and policy makers. Agricultural and Environmental Ethics.

Appendix B: Theoretical analysis of international gene flow – in press

Bagavathiannan MV, Van Acker RC (2009) Transgenes and national boundaries-The need for international regulations. Environmental Biosafety Research. DOI: 10.1051/ebr/2009011.

1.0 General Introduction

Recombinant DNA technology has allowed for the introduction of novel traits from sexually incompatible organisms into crop plants in order to address some of the challenging issues in crop production. These crops are commonly known as genetically modified (GM) crops and the common GM traits include herbicide resistance, pest resistance, disease resistance and traits that improve the nutritional quality of crops. Genetically modified crops have seen wide commercial acceptance since their introduction about one decade ago. In 2008, GM crops were cultivated on over 125 million hectares worldwide. Because these crops are released into the environment, there exist possibilities that the novel GM traits may be present adventitiously (AP = adventitious presence) in the environment. Adventitious presence of novel traits is a cause of concern because of potential market and ecological risks associated with them. Recently, recombinant DNA technology has been extended for the production of industrial enzymes including biopharmaceuticals from plants (i.e. second generation GM crops) and the AP of these traits may pose serious health concerns. Therefore, the next wave of GM crops may warrant more stringent confinement. The sustainable co-existence of GM and non-GM crops in the agricultural landscapes requires the establishment and maintenance of efficient trait confinement protocols and stewardship practices. To be effective, such protocols and practices require detailed knowledge on the ecology and biology of the plant species in question.

Crop ferality has implications for novel trait confinement. Crop ferality is the escape and establishment of domesticated crop plants in unmanaged habitats as self-perpetuating populations. Crop domestication often resulted in the loss of wild, adaptive traits in favor of yield traits. As a result, domesticated crops are generally less capable of surviving without managed cultivation. The degree of domestication varies among crops and some crops are more capable of surviving in unmanaged natural and semi-natural habitats than others

(Gepts 2002). In some crops, particularly in forage species, domestication traits included the traits of persistence, favoring their successful establishment in unmanaged areas, including roadsides and wastelands. Feral crops are considered as a potential barrier for the co-existence of GM and non-GM crops because the feral populations can act as a reservoir and bridge for novel traits; a place for the traits to move to and a place for the traits to come from to contaminate non-GM crops.

Alfalfa, known commonly as lucerne, is an important forage crop in North America. On the Canadian prairies, the introduction of winter-hardy cultivars facilitated the adaptation and widespread cultivation of alfalfa. Its extensive use and the challenges of establishment due to weed interference, led to the recent development of GM herbicide-resistant cultivars. A glyphosate-tolerant form of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) isolated from the soil bacterium *Agrobacterium tumefaciens* strain CP4 was introduced into alfalfa through genetic engineering. The EPSPS enzyme in the glyphosate-resistant (GR) alfalfa has a reduced affinity for glyphosate and thus the alfalfa plants survive the application of this herbicide. Glyphosate resistant alfalfa was approved for environmental release in US and Canada. This marks the advent of a new step in the development of plants with novel traits in that it is the first perennial GM crop plant to be given regulatory release.

Glyphosate resistant alfalfa was available for commercial cultivation in the US in 2005 but it is currently under a court moratorium. The moratorium was issued based on the grounds that GR alfalfa was deregulated without conducting sufficient environmental impact assessments. In Canada, approval for commercial cultivation of GR alfalfa has not yet been granted but is currently under way. Alfalfa is also considered as a valuable plant for the production of plant-made pharmaceuticals. Such second generation GM crops will warrant strict trait confinement protocols if they are to be cultivated under field conditions. As such, there is a growing need for developing efficient novel trait confinement protocols in alfalfa and effective trait confinement protocols would favor the success of co-existence programs. Although trait confinement and co-existence protocols were already developed for alfalfa, there exist some knowledge gaps in this regard and addressing these gaps may improve the efficiency of these protocols. In this regard, the

feral nature of roadside alfalfa populations and their role in novel trait movement is not well understood. Such knowledge will also have implications for other GM crops grown in this region that are capable of establishing roadside feral populations (e.g. canola).

The perennial nature, deep root system, symbiotic nitrogen fixation and adaptation to severe drought and cold conditions may favor the establishment and persistence of alfalfa in uncultivated areas such as roadsides, wastelands and railway verges. Since alfalfa is a highly cross-pollinated crop and pollination is often facilitated by bees, there is potential for the transfer of novel traits between cultivated and feral populations of alfalfa. This could facilitate the AP of novel traits in the environment and movement into non-GM cultivars and therefore, it is important to understand the nature of feral alfalfa populations. This knowledge is essential if we are to determine the potential capabilities of the feral alfalfa populations to serve as a genetic bridge facilitating the movement of novel traits among alfalfa populations. However, no study has yet investigated this in detail, anywhere in the world. The overall aim of this study is to characterize feral alfalfa populations occurring in roadside habitats in southern Manitoba, Canada.

The objectives of this study are:

i) To characterize the nature of roadside alfalfa populations and to assess their ability to establish self-sustaining feral populations;

ii) To determine the significance of roadside alfalfa populations in intra-specific novel trait movement; and

iii) To determine options for the management of roadside alfalfa populations

2.0 Crop Ferality: Implications for Novel Trait Confinement

2.1 Abstract

Ferality is observed in many crop species wherein individuals of the cultivated crop reproduce successfully and establish a self-perpetuating population in natural or semi-natural habitats. Feral populations can evolve to differ from their parent populations and lose traits associated with domestication including for example, a lack of seed dormancy. Hybridization between wild and cultivated forms of cropped species may facilitate ferality. If genetically modified (GM) plants become feral, they can establish populations in natural and semi-natural environments and act as both source and sink for novel traits. The presence of novel traits may facilitate the persistence of feral populations if the novel trait confers a selective advantage (e.g. drought tolerance, salinity tolerance, pest and disease resistance), but there is no evidence yet that transgenesis *per se* facilitates ferality. In some cases and in some jurisdictions the introduction of GM crops will require assurances of effective segregation and novel trait confinement. The existence of feral crop populations can make novel trait confinement more difficult. Monitoring and management of feral populations will be required for effective novel trait confinement.

2.2 Introduction

The introduction of new genetically modified (GM) crops becomes a greater concern as traits that are ever more extraordinary are introduced into crop plants, in particular pharmaceutical and industrial traits. Most risks associated with the release of crops with such extraordinary traits are related to trait movement (Marvier and Van Acker 2005). Trait confinement protocols will be required for the commercialization of some GM

crops (Demeke et al. 2006) and effective trait confinement is necessary to facilitate the co-existence between GM and non-GM crops (Damgaard and Kjellsson 2005; Jank et al. 2006). In this respect, feral populations of cultivated species can play an important role in novel trait escape and movement (Rabbani et al. 1998; Berville et al. 2005a; Snow and Campbell 2005; Devaux et al. 2007). Occurrence of feral populations has been reported for a number of crop species including oilseed rape (*Brassica napus*), sunflower (*Helianthus annuus*) and alfalfa (*Medicago sativa*) (Crawley and Brown 1995; Baki et al. 2000; Ellstrand 2003; Stewart et al. 2003; Bagavathiannan et al. 2006; Burger et al. 2006; Knispel et al. 2008, Table 2.1) and the occurrence of GM crop volunteers has been widely studied (Tølstrup et al. 2003; Willenborg et al. 2009). However, the nature and dynamics of roadside feral populations has been studied to a limited extent, particularly in the context of novel trait confinement (Gressel 2005a; Garnier et al. 2006; Devaux et al. 2007) yet there is an increasing interest in the nature of feral populations of cropped species (Ramsay et al. 2003; Cresswell and Osborne 2004; Massinga et al. 2005; Garnier et al. 2006). The objective of this review is to provide background on crop ferality, the nature of feral populations, the role of GM in ferality and insight into the problems associated with the occurrence of feral crops with respect to novel trait confinement.

2.3 Definitions

Gressel (2005b) defined feral plants as "plants derived fully or in part from crop plants that have become partially or fully de-domesticated". However, a cultivated crop species that has escaped and is growing in an unmanaged environment with a self-perpetuating population could still be considered feral even if it retains all of its original traits. The important characteristic feature of feral crop populations is that they are able to successfully reproduce without management intervention (White et al. 2006). Although there is a clear distinction between volunteer and feral crop populations, the terms are not used consistently. Volunteers are derived from seeds that the crop has released before and during harvest (Gressel 2005b). Warwick and Stewart (2005) defined volunteers as "crop plants that grow in the same field in subsequent crops or years from a seed bank formed from seed that either shattered from the crop prior to or as a result of harvesting

Table 2.1 Summary of reports on crop species ferality or potential ferality

Species	Probable origins	Potential regions	References
Oilseed rape	Seed spill during transportation, farm machineries, cross between *B. napus* and *B. campestris*	Europe (UK, France, Denmark), USA, Canada	Rich (1991); Crawley et al. (1993); Crawley and Brown (1995); Wilkinson et al. (1995, 2000); Gray and Raybould (1998); Pessel et al. (2001); Hails et al. (2002); Bond et al. (2004); Cresswell and Osborne (2004); Claessen et al. (2005a, b); Garnier et al. (2006)
Radish	Cross between cultivated radish (*Raphanus sativus*) and weedy relative (*R. raphanistrum*)	USA, Japan, Pakistan	Rabbani et al. (1998); Snow et al. (2001); Nature Conservancy (2005); Snow and Campbell (2005); Hedge et al. (2006)
Rye	Cross between cultivated (*Secale cereale*) and mountain rye (*S. strictum*), dedomestication	USA	Stump and Westra (2000); Berville et al. (2005b); Burger and Ellstrand (2005); WCO (2005); Burger et al. (2006) ; White et al. (2006)
Cotton	Seed escape, cross between cultivated (*Gossypium hirsutum*) and pima cotton (*G. barbadense*)	USA (Florida, Hawaii), U.S. Virgin Islands, Puerto Rico	Ellstrand et al. (1999); USEPA (2001)
Alfalfa	Seed spill during transportation, farm machineries, anthropogenic factors	USA, Canada	Fitzpatrick et al. (2003); Kendrick et al. (2005); Bagavathiannan et al. (2006, 2007)

Sugar beet	Cross between cultivated (*Beta vulgaris* ssp. *vulgaris*) and wild beets (*B. vulgaris* ssp. *maritima*)	Europe (France, Belgium, Germany), USA (California)	Bartsch et al. (1996); Desplanque et al. (1999); Ellstrand (2003); Sukopp et al. (2005)
Sunflower	Seed escape, cross between *H. annuus* x *H. tuberosus*	USA, Europe	Faure et al. (2002); Massinga et al. (2003); Stewart et al. (2003); Berville et al. (2005a); Massinga et al. (2005)
Wheat	Seed escape, dedomestication	USA, Canada, Europe, Tibet	Chen et al. (1998, 1999); Sun et al. (1998); Stewart et al. (2003)
Sorghum	Seed escape, dedomestication; cross between *Sorghum bicolor* and *S. halepense* or *S. Sudanense*	USA, Africa	Arriola and Ellstrand (1996, 1997); Stewart et al. (2003); Ejeta and Grenier (2005)
Ornamentals	Exotic introduction, dedomestication	USA, Europe, Australia	Levin (2001); Stewart (2004); Kowarik (2005); AIPC (2007)
Meadow fescue	Seed escape	Norway, Sweden	Rognli et al. (2000)
Rice	Dedomestication	South East Asia, China, USA	Baki et al. (2000); Lu and Snow (2005); Valverde (2005); Vidotto and Ferrero (2005)
Vigna group (cowpea, rice bean, azuki bean)	Seed escape, cross with wild relatives	Asia	Berville et al. (2005b)
Safflower	Seed escape	USA, Canada, Mediterranean	Berville et al. (2005b)

operations or from originally sown seed that did not germinate immediately after sowing". Gressel (2005b) suggested that crop volunteers could become feral within cultivated fields if they self-perpetuate but a more strict definition makes clear that feral populations exist in non-cropped areas. Claessen et al. (2005a) and Garnier et al. (2006) categorized ferals as populations occurring outside of an arable field and volunteers as populations inside an arable field. Likewise, Devaux et al. (2007) described volunteers as plants from previously grown cultivars in fields and ferals as plants that are widespread in field verges or roadsides. Volunteers of most crop species rarely persist for more than one or two cropping seasons. However, volunteers existing on field margins may contribute founding seeds for feral populations. One might term persistent volunteers as 'weedy volunteers' and volunteers that are found outside of cultivated fields, but are unable to sustain a population over time as 'escaped crops'. In this review we will assume that volunteers exist in managed or cultivated environments while ferals exist in unmanaged natural and semi-natural habitats. A practical definition of ferality, therefore, is where individuals of a cultivated crop escape a managed area, survive, reproduce successfully and establish a self-perpetuating population in either a natural or semi-natural habitat. The pathway of dedomestication in cultivated crops and related use of terminologies is described in (Fig. 2.1).

2.4 The establishment of feral populations

Knowing how feral populations establish and evolve is fundamental to understanding ferality. Generally, the occurrence of feral forms of cultivated crop plants is initiated by the dispersal of seed from cultivated fields to adjacent unmanaged ecosystems. Seed dispersal could be facilitated by farm machinery (Crawley and Brown 1995), seed spill during transport (Crawley and Brown 1995; Gray and Raybould 1998; Senior and Dale 2002; Claessen et al. 2005a; Yoshimura et al. 2006), dispersal by vehicles (Garnier and Lecomte 2006) and also by birds, rodents and other seed predators (Claessen et al. 2005a). Although the escaped populations are common for many cropped species (Crawley and Brown 1995; Bond et al. 2004; Claessen et al. 2005a), not many self

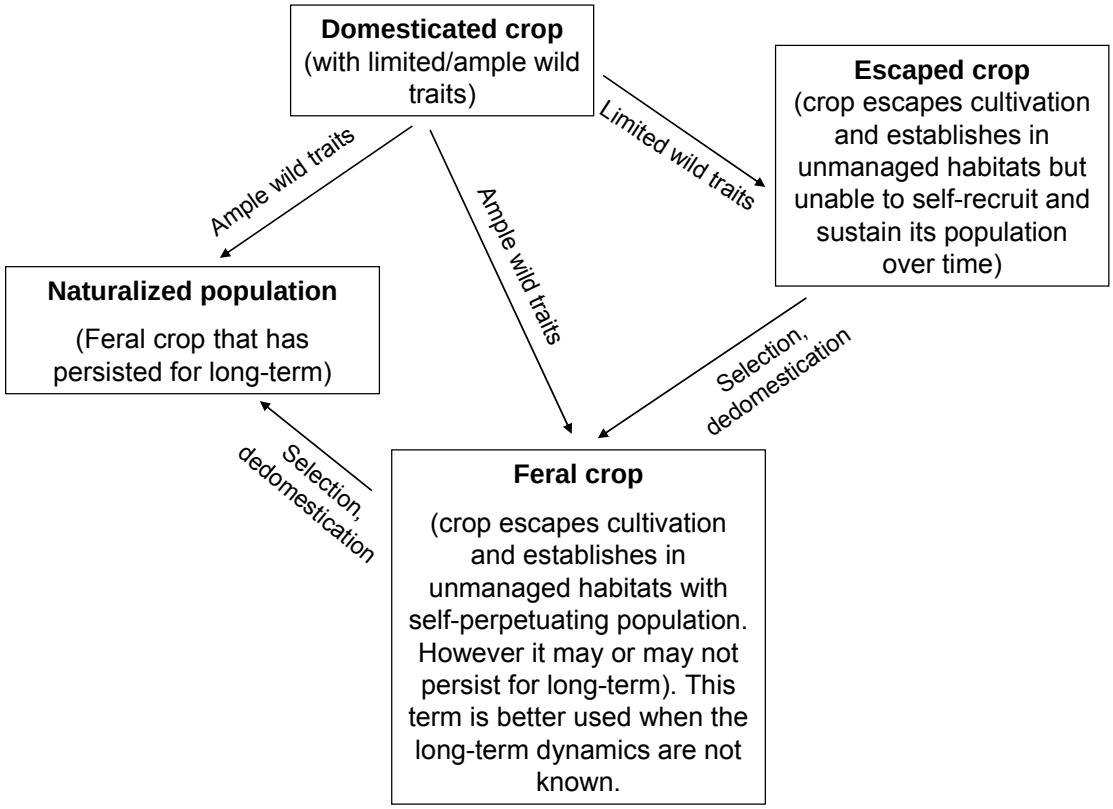

Fig. 2.1 Pathway of dedomestication in cultivated crops

perpetuate and become feral (Crawley et al. 1993; Pessel et al. 2001).

2.5 Reports on feral crop species

The majority of ferality reports and studies have been on annual crop species. However, perennial forage grasses [e.g. meadow fescue (*Festuca pratensis*)] and perennial legumes (e.g. *M. sativa*) have high ferality potential and many perennial ornamentals [e.g. creeping bellflower (*Campanula rapunculoides*), tall-baby's breath (*Gypsophila paniculata*), etc.] introduced from exotic locations have become feral and, in many cases, invasive (AIPC 2007).

Feral *B. napus* populations are commonly observed in roadside verges and they can persist for several years (Pessel et al. 2001; Ramsay et al. 2003; Crawley and Brown 2004). Soil adjacent to individual feral oilseed rape plants can contain significant quantities of seeds (Wilkinson et al. 1995; Simard et al. 2002). Similarly, Lutman (1993) observed the occurrence and persistence of feral oilseed rape in the United Kingdom (UK) and stated that these populations could have survived in the wild possibly for five years or more. Likewise, Pessel et al. (2001) reported that the feral forms of conventional oilseed rape plants have persisted for at least eight years in semi-natural road verges in France.

2.6 Feral traits

Seed dormancy and the ability to develop a seedbank appear to be a key trait for feral species including oilseed rape and alfalfa. Models developed to predict the persistence of feral populations demonstrate that seed persistence is a key trait driving population persistence (Crawley et al. 1993; Bullock 1999; Garnier and Lecomte 2006), especially when environmental stochasticity is considered (Hails et al. 2002; Claessen et al. 2005a, b). For oilseed rape, Garnier et al. (2006) predicted feral population persistence to be as long as five years in the absence of seed addition, and up to 10 years if there were a low level of seed addition.

Because traits associated with ferality are often wild type or weediness traits, it is not surprising that ferality is often observed in areas where crops and wild relatives co-occur. Hybridization between crop and wild populations can result in greater fitness in the progeny and selection pressure could lead to the persistence of these new genotypes (Campbell et al. 2006). Although gene flow and hybridization could be an important aspect in the evolution of feral plants, the extent to which this can happen depends on several factors including, but not limited to, flowering synchrony (Lu and Snow 2005), mode of inheritance (Massinga et al. 2005) and relative fitness; which includes plant vigor, biomass, seed production, seed dormancy and competitiveness (Dale 1994; Arriola and Ellstrand 1996). Key traits associated with most successful feral species are:

Variety of pollinators	Broad germination requirements
Continuous seed production	Discontinuous germination
Considerable seed output	Rapid vegetative growth
Seeds produced in several habitats	Ability to withstand competition
Seed dispersal over short and long distances	Tolerance to unfavorable biotic and abiotic conditions
Seed dormancy (ability to form seedbank)	Rapid flowering

Crop species may evolve feral characteristics over time once they are in unmanaged habitats. During the process of domestication, crop species are increasingly differentiated from wild forms and the increase in cropping suited traits are often equated with a reduction in environmental fitness (Wang et al. 1999). Levin (1990) proposed that any selection pressure imposed on feral populations will eventually lead to genetic divergence of the feral population from the original cultivar(s) and successful feral populations may result from evolutionary reversion to wild type traits. These may include the replacement of self-compatibility by self-incompatibility and non-dormancy in seed by prolonged seed dormancy (Levin 2001). Good examples of reversion away from domestication traits are

the occurrence of feral hexaploid wheat (*Triticum aestivum* L.) in Tibet (Chen et al. 1998, 1999; Sun et al. 1998) and feral rye (*Secale cereale* L.) in USA (Burger et al. 2006).

2.7 Ferality and transgenic crops

Transgenic crops that are hardy, perennial, open pollinating, prolific, have a wide range of relatives and are able to colonize natural and semi-natural habitats, have greater chances for persistence in unmanaged areas and if the transgenic modification enhances these native traits then it will enhance the feral potential. For example, Pessel et al. (2001) noted that the time to extinction of roadside feral populations might increase if the feral plants contain insect resistance or drought tolerance. Novel traits that favor invasiveness and ecological fitness (e.g. drought tolerance, biotic and abiotic resistance) will favor the founding and persistence of feral populations in unmanaged environments (Cummings et al. 2002). Claessen et al. (2005b) studied the role of seed dispersal and environmental variability on the dynamics of feral populations and revealed that seed dispersal lowers the decline of metapopulations and aids in the occurrence of long-lasting local feral populations. They also predicted that the persistence of feral populations could be enhanced by novel traits for oil modifications (high stearate or high laurate) and for insect resistance. Long-distance seed dispersal is another factor that could aid in the persistence of feral populations. For example, Snow and Campbell (2005) predicted that radish populations that became feral could disperse over greater distances and increase their chances for persistence and invasion.

When the environment is favorable, local seed production could significantly contribute to the seed bank and thus population persistence. The survival of feral crops in soil seed banks would aid in subsequent dispersal of novel traits even beyond the life time of a given variety (Linder and Schmitt 1994). In terms of feral potential, traits that confer biotic and abiotic tolerance are often more important than other traits including herbicide resistance especially in the absence of herbicide application (Lee and Natesan 2006). This helps to explain why herbicide resistance traits offer no advantage to escaped crops in non-cultivated areas. Crawley et al. (2001) planted several herbicide tolerant GM

oilseed rape cultivars in natural habitats and observed them over a 10-year period. They found no difference in persistence between the GM and non-GM cultivars. However, in the presence of herbicide applications there would have been great differences (Pilson and Prendeville 2004). Interestingly, although Klinger and Ellstrand (1994) suggested that the effects of heterosis resulting from hybridization between feral and cultivated populations could enhance the fitness of feral populations, Allainguillaume et al. (2006) studied the fitness of the progenies resulting from hybridization between cultivated oilseed rape and wild *B. rapa* and noted a reduction in the fitness of the hybrid, but they noted that GM traits conferring stress tolerance could readily offset this reduction in fitness resulting in a net enhancement of fitness. Comparative studies of the impact of GM traits on ferality are uncommon in crops other than oilseed rape. But one example is the study by Bartsch et al. (1996) who compared GM beets resistant to Beet Necrotic Yellow Vein Virus (BNYVV) to non-GM material from the same genetic line and found that this novel trait provided a clear additive ecological advantage.

Results of studies to-date on the impact of transgenesis on feral potential show that the establishment of GM crops as ferals may be more likely if they possess traits of ecological significance (Wolfenbarger and Phifer 2000) and that transgenesis *per se* may not affect feral potential. New perennial GM crops such as turf grasses and pasture species may be more likely to establish persistent feral populations than annual species due to the propensity of these species for ferality. This may be especially true if these perennial GM crops produce persistent seedbanks as well (Godfree et al. 2004; Watrud et al. 2004).

2.8 Role of feral crops in novel trait movement

Feral sub-populations established in semi-natural and natural habitats could act as a genetic bridge allowing novel traits to move among crops and perhaps to wild compatible relatives. Feral crops could act as repositories for engineered genes where the pollen source and pollen recipient are sexually compatible (Al-Ahmad et al. 2006; Ellstrand 2006). Claessen et al. (2005b) noted that feral populations of GM crops may also serve as

a stepping stone for gene flow from crop to wild relatives. In Canada, Knispel et al. (2008) showed that escaped roadside populations of oilseed rape can outcross with cultivated populations of GM oilseed rape and accumulate transgenic traits.

There have been numerous reports suggesting that feral oilseed rape could exchange genes with other feral oilseed rape populations, cultivated oilseed rape and wild relatives in the ecosystem via pollen flow (Brown and Brown 1996; Thompson et al. 1999; Simard et al. 2002; Ramsay et al. 2003; Chèvre et al. 2004; Cresswell and Osborne 2004; Garnier et al. 2006). The same has been observed for other cropped species. While studying the effects of distance and pollen competition on gene flow in *F. pratensis*, Rognli et al. (2000) observed that pollen from feral *F. pratensis* that are present on road verges and paths leading to cultivated fields could have entered into the fields and resulted in gene flow. They also showed that removing feral populations located up to 500m from the cultivated field did not prevent the feral pollen from fertilizing acceptor plants. The occurrence of gene flow between cultivated and feral forms has also been reported in a variety of crop species including *H. annuus* (Berville et al. 2005a; Massinga et al. 2005), *B. vulgaris* (Boudry et al. 1993; Desplanque et al. 2002), *G. hirsutum* (USEPA 2001), and *M. sativa* (Fitzpatrick et al. 2003).

Although feral populations are typically small compared to cropped populations and they contribute little to the pollen load in the environment (Devaux et al. 2005), they might still be significant in the dispersal of novel traits into nearby non-GM crop fields (Devaux et al. 2007). The ability of a range of crop species to form effective feral populations has been well documented (Table 2.1). And the ability of feral populations to facilitate novel trait movement from crop to crop and even from crop to wild type has been shown in several cases.

2.9 Feral transgenic crops and novel trait movement

Given the agronomic and environmental significance of feral crops, it is essential to consider these populations in environmental impact assessments of GM crops and the consideration of trait confinement (Van Acker et al. 2007a). Physical isolation of

cultivated crops from known feral populations is a simple means of limiting gene flow potential. This could be achieved by the implementation of strict rules or by prohibiting the cultivation of GM crops in the areas where there is a potential for outcrossing with feral plants and wild relatives.

To prevent pollen mediated gene flow, physical separation could be achieved by strict regulations, but there are still risks associated with human error (Marvier and Van Acker 2005). Genetic engineering solutions to gene flow may be more reliable in theory (Van Acker et al. 2007b). Mathematical models and empirical experimental evidence suggest that genetic solutions could be very effective in preventing the introgression of novel traits into feral populations, even if these feral populations are not reproductively isolated from cultivated crops (Lee and Natesan 2006). A wide range of genetic mitigation strategies have been proposed by Daniell (2002). Chloroplast engineering is a useful approach to target novel traits to the chloroplast genome (Khan and Maliga 1999; Kumar et al. 2004). This approach could potentially prevent pollen and seed from carrying novel traits. Al-Ahmad et al. (2006) proposed another strategy where a mitigation gene is designed to counteract any fitness advantage of a novel trait hybrid. To do so they used a tandem construct containing a selectivity unfit gene (Δ*gai*) that blocks the stimulation of growth and causes dwarfism in plants, thus affecting their ability to harvest sunlight and to reproduce. Another strategy is the use of tissue specific promoters (Potenza et al. 2004). Transgenic crops do not need to express the protein in all parts of the plants. Instead, they could be targeted to express only in some plant parts using tissue specific promoters, avoiding transgene expression in pollen and seed (Sunilkumar et al. 2006). This would help prevent the adventitious presence (AP) of novel traits in the environment. Currently, the problem with suggesting genetic solutions is that none have been tested for reliability, and none are commercially available (Van Acker et al. 2007b).

Given that a key trait of feral populations is persistence, limiting seed production of feral populations as well as seed migration to feral sites are important management tactics for preventing the establishment of feral crops in roadside verges (Claessen et al. 2005a). Rognli et al. (2000) suggested that a combination of isolation distance, population size and differential flowering time greatly limit gene flow from cultivated to feral

populations. These observations, however, are difficult to translate into practical management advice given that feral populations can be quite variable with respect to flowering timing, and limiting the pollen donor population defeats the crop production purpose. Because seed survival is a key characteristic for effective feral populations, GM crops should be tested for their seed dormancy potential. Gulden et al. (2003) noted a considerable variation in the dormancy propensity of oilseed rape cultivars and they recommended that breeders consider this trait as a means of reducing volunteer canola problems in western Canada.

In the absence of commercial genetic means of trait confinement and the selection of crop cultivars that are less susceptible to persistence in non-cropped areas via seed dormancy, it is important to recognize that feral populations can play a role in novel trait movement, and as such the management of trait movement must include the monitoring and active management of feral populations. It would also be prudent to enact greater regulatory diligence with respect to the commercialization of GM crops, especially those which contain novel traits which need to be contained. In these cases, the crop platform should be thoroughly scrutinized in regard to its ability to form effective feral populations within the intended region of cultivation.

3.0 Biology and Ecology of Feral Alfalfa and its Implications for Novel Trait Confinement

3.1 Abstract

Alfalfa (*Medicago sativa* L.) is an important forage crop worldwide. Apart from cultivated fields, alfalfa is also found along roadsides and in natural and semi-natural habitats. However, little information is available on the establishment capabilities of alfalfa in non-cultivated areas and the potential of these founding populations to become feral. Some crop species have sustained many wild characteristics during the domestication process and with several traits favoring weediness, alfalfa could be one among those that can become feral. There is great interest in the feral potential of alfalfa, particularly due to the concerns that feral plants could act as genetic bridges and facilitate novel trait movement at the landscape level. Alfalfa is the first perennial, insect-pollinated crop to be genetically modified and to be approved for unconfined release into the environment. This review investigates and compiles information in the literature that reveals the life history components that can influence ferality in alfalfa. Characteristics that can contribute to ferality in alfalfa include high genetic diversity, perenniality, quick regrowth potential, persistence, symbiotic nitrogen fixation, deep tap root system, drought and cold tolerance, and seed dormancy. With these traits, alfalfa is arguably equipped to invade and dominate unmanaged habitats. Feral alfalfa populations can and will act as bridges for long-distance gene flow and facilitate the adventitious presence (AP) of novel traits in the environment. As such, feral populations will become a potential barrier for achieving co-existence of transgenic and non-transgenic alfalfa fields. Implications of ferality, including gene flow and hybridization with compatible

wild relatives are also discussed in detail. This review serves as a resource for environmental risk assessment for the release of alfalfa containing novel traits.

3.2 Introduction

Investigation of wild forms of domesticated species has long been a topic of interest in plant evolutionary theory (Darwin 1883), taxonomy (Harlan and de Wet 1971), breeding (Jenczewski et al. 1999a) and recently, in gene flow and novel trait confinement (Ellstrand 2003). Genetically modified (GM) crops have been commercially grown worldwide for over a decade. However, the potential risks of transgene movement from GM to non-GM forms and associated consequences are still substantial and are hard to ignore (Gressel 2005a). The widespread cultivation of GM crops has raised concerns over the ability of novel traits to escape cultivation and to present adventitiously in the environment. Further, possibilities for gene flow from cultivated crops to the compatible wild and weedy relatives in the landscape are substantial (Ellstrand 2003; Gepts and Papa 2003). Such risks appear to be more prominent in plant species that possess life histories and morphological characteristics that favor gene flow and introgression. In most cases, synchronous flowering, outcrossing via wind and insect pollination and presence of self-incompatibility are key characteristics that favor gene flow among cultivated and compatible wild relatives (Papa and Gepts 2004).

There exist reports on the occurrence of gene flow from crop species to their wild and weedy relatives (Ellstrand 2003). Naturally occurring crop-wild gene flow was confirmed in several crops including potato (Scurrah et al. 2008), wheat (Loureiro et al. 2008), sunflower (Arias and Rieseberg 1994; Ureta et al. 2008), sugar beet (Arnaud et al. 2003), sorghum (Tesso et al. 2008), alfalfa (Jenczewski et al.1999a), rice (Chen et al. 2004), maize (Baltazar et al. 2005), oilseed rape (Chevre et al. 2003) and radish (Snow et al. 2001). This evidence suggests that this type of gene flow is indeed natural and the potential for this to occur between GM and non-GM forms is possible.

Similar to wild and weedy relatives, feral crop populations are now considered an important element in intra-specific gene flow from GM to non-GM forms (Gressel

2005a). Feral crop species are those from which individuals escape a managed area to survive, reproduce and establish self-perpetuating populations in either natural or semi-natural habitats (Bagavathiannan and Van Acker 2008a) (Chapter 2.0). For many crop species, domestication has rendered them completely dependent on humans such that they are no longer capable of independent population establishment and maintenance. However, some crop species are less domesticated and may have the potential to found feral populations (Baki et al. 2000; Doebley et al. 2006). Although escaped populations are common for many cultivated species, fewer species can self-sustain populations and be truly feral (Crawley et al. 1993; Pessel et al. 2001).

Occurrence of feral populations has been reported for some crop species including oilseed rape (Pessel et al. 2001; Claessen et al. 2005a,b; Garnier et al. 2006), radish (Snow and Campbell 2005; Hedge et al. 2006), rye (Burger and Ellstrand 2005; White et al. 2006), cotton (Ellstrand et al. 1999; USEPA 2001), sugar beet (Ellstrand 2003; Sukopp et al. 2005) and sunflower (Massinga et al. 2003; Berville et al. 2005a). Key feral traits include but are not limited to high levels of outcrossing, prolific seed production, seed dispersal, seed dormancy, discontinuous germination, rapid vegetative growth, tolerance to competition and tolerance to biotic as well as abiotic stresses.

Feral and cultivated forms of species are typically compatible with synchronized flowering periods and common pollinator insects. Feral populations could act as both sources and sinks for the movement of novel trait (s) at the landscape level. In nature, the intra-specific movement of traits among sub-populations occurs in the context of metapopulations (Crawley and Brown 1995) and for crop species, these metapopulations include subpopulations of cultivated crops, volunteers and feral plants (Van Acker 2007). Feral sub-populations established in semi-natural and natural habitats could act as genetic bridges allowing novel traits to move among crops and perhaps to wild compatible relatives. Further, they could act as repositories for engineered genes where the pollen source and pollen recipient are sexually compatible (Ellstrand 2006). A more detailed review on crop ferality and its implications for novel trait confinement is provided in chapter 2.0.

Alfalfa (*Medicago sativa* L.) is another cultivated crop that has the potential to form feral populations (SWSS 1998). Alfalfa populations are commonly observed in roadsides and other unmanaged habitats in alfalfa growing regions (Jenczewski et al. 1999a; Fitzpatrick et al. 2003; Kendrick et al. 2005; Bagavathiannan et al. 2006; Prosperi et al. 2006). Unlike other reported feral crops species, alfalfa is a perennial and a highly outcrossing species in which pollination is often facilitated by insects. The life history characteristics of alfalfa make for high gene flow and ferality potential, suggesting that novel trait confinement will be challenging in this species. Genetically modified herbicide resistant alfalfa was already approved in the United States (US) (APHIS 2005) and planted on over 200,000 acres. In Canada, it has been approved for food and feed use (CFIA 2005), but it has not yet been commercialized. A recent moratorium on herbicide resistant alfalfa in the US triggered interest in appropriate environmental impact assessment programs, including the role of feral alfalfa plants in novel trait movement (USDC 2007). A summary of the submissions/approvals of alfalfa containing novel traits worldwide is provided in Table 3.1. Alfalfa is also an effective platform for the production of industrial enzymes and biopharmaceuticals (Daniell et al. 2001; Bardor et al. 2006; Sparrow et al. 2007). In this respect, novel trait confinement would be critical yet likely very challenging given the life history characteristics of alfalfa.

In regard to environmental impact assessments and considerations of novel trait movement, many reports are available on the life history traits of successful weeds (Baker 1974; Baker 1991; Crawley et al. 1996; Sutherland 2004; Hamilton et al. 2005) and feral crops (Crawley et al. 1993; Hails et al. 2002; Claessen et al. 2005a, b; Gressel 2005a; Garnier and Lecomte 2006), but similar accounts do not exist for alfalfa. The objective of this review is to document the genetically heterogeneous, highly outcrossing nature of alfalfa, its propensity to form feral populations and to discuss the implications relevant to novel trait confinement.

Table 3.1 Summary of submissions/approvals of alfalfa plants containing novel traits for food, feed and environmental release

Country	Trait	Applicant	Year	Remarks	Reference
Australia / New Zealand	Glyphosate resistance (Events J101, J163)	Monsanto Australia Ltd.	2006	Approved for food and animal feed use and not intended for cultivation in either Australia or in New Zealand.	FSANZ (2006)
Philippines	Glyphosate resistance (Events J101, J163)	Monsanto Philippines Inc.	2006	Approved for direct use as food or feed or for processing. However, it is not intended for environmental release.	NBCP (2006)
Japan	Glyphosate resistance (Events J101, J163)	Monsanto Japan Ltd.	2005, 2006	Approved for food and feed and environmental release.	JBCH (2006)
South Korea	Glyphosate resistance (Events J101, J163)	Monsanto Korea Inc.	2007, 2008	Approved for food and feed use and not for environmental release	KBCH (2008)
Canada	Glyphosate resistance (Events J101, J163)	Monsanto Canada Inc.	2005	Approved for use as a food and livestock feed and unconfined release into the environment. However, the company didn't apply for variety registration.	CFIA (2005)
Mexico	Glyphosate resistance (Events J101, J163)	Monsanto Comercial, S.A. de C.V.	2005	Approved for food and feed use and not intended for environmental release	COFEPRIS (2005)
USA	Glyphosate resistance (Events J101, J163)	Monsanto Company / Forage Genetics International	2005	Approved for food and feed use and unconfined release but currently under regulated status for unconfined environmental release.	APHIS (2005)
Belgium	Alteration of lignin biosynthesis	Plant Genetic Systems NV	1994	Notified under EU directive 2001/18/EC (B/BE/94/V8). No further information available.	DFTEU (1994)
Spain	Virus resistance (alfalfa mosaic virus)	Semillas Pioneer SA	1994	Notified under EU directive 2001/18/EC (B/ES/94/04). No further information available.	DFTEU (1994)

3.3 Plant description

Alfalfa, a member of Fabaceae family, is also known as lucerne, medic, buffalo herb, Chilean clover, jatt, kaba yonca, mielga, mu su, sai pi li ka, yonja, feuille de luzerna, purple medick, luzerne, blaue luzerne, murasaki-umagoyashi, sinimailanen, blalusem and luzerna (Rehm 1994; Wiersema and Leon 1999). A short, bushy, deep tap-rooted perennial, it has a simple and unifoliate first foliar leaf and pinnately trifoliate leaves are alternately arranged on stems. The stem of alfalfa is erect and grows up to 1m in height rising from the crown. Alfalfa shoots are indeterminate and continue to produce both vegetative and reproductive organs. The inflorescence is an axillary raceme consisting of several florets. Alfalfa has a range of flower colors including yellow, white, purple, violet, blue or variegated (Barnes 1972). The fruit is a pod 5 to 9 mm in diameter and spirally coiled. The seed is small (1-2 mm long, 1-2 mm wide and 1 mm thick), kidney shaped and can number up to 220,000 per lb (Teuber and Brick 1988).

Alfalfa occurs both as a diploid and tetraploid species although tetraploid cultivars are more common (Brummer et al. 1991). The chromosome number of species in the genus *Medicago* is 2n = 16 (Lesins and Gillies 1972). Aneuploidy in *M. sativa* is rare, however, plants with a chromosome number of 2n = 4x = 31, 33 and 35 have been found (Bolton 1962). According to Small and Jomphe (1989), the genus *Medicago* was classified into 12 sections and eight subsections. The genus comprises of 83 species and 18 infraspecific taxa. The taxonomic nomenclature of Small and Jomphe (1989) treats alfalfa and alfalfa complex as infraspecific taxa.

M. sativa ssp. *sativa* (*M. sativa*), *M. sativa* ssp. *falcata* (*M. falcata*) and *M. sativa* ssp. x *varia* (*M. varia*) are recognized sub-species in the *M. sativa* complex (Frame et al. 1998). Other subspecies in the complex include subsp. *caerulea*, subsp. *glutinosa*, subsp. x *tunetana*, subsp. x *polychroa* and subsp. x *hemicycla* (Quiros and Bauchan 1988) (Fig. 3.1). All the members of the *M. sativa* complex have contributed germplasm to alfalfa. *M. sativa* is a high yielding yet less hardy species characterized by purple flowers, deep tap root, erect growth habit and coiled pods (Quiros and Bauchan 1988), whereas *M. falcata* is lower yielding but more winter-hardy with yellow flowers, fasciculate roots, a

prostrate growth habit and sickle-shaped pods (Julier et al. 1995). *M. sativa* and *M. falcata* both naturally occur as diploid and tetraploid populations (Clement 1962; Bingham 1975). *M. varia* is a hybrid between *M. falcata* and *M. sativa* ssp. *sativa* or ssp. *caerulea* (Small and Jomphe 1989; Frame et al. 1998).

The members of the *M. sativa* complex consist of both diploid and tetraploid forms and the natural occurrence of gene transfer between these forms is evident (Small and Jomphe 1989). For example, *M. sativa* ssp. *caerulea* is a diploid form (2n = 2x = 16) similar to ssp. *sativa* and is able to naturally hybridize with members of the *M. sativa* complex that are tetraploid (2n = 4x = 32). *M. varia* can be an example of such inter-ploidy hybridization (Small and Jomphe 1989). The occurrence of unreduced (2n) gametes often facilitates gene transfers between ploidy levels (Stanford et al. 1972; Vorsa and Bingham 1979; Veronesi et al. 1986). Bingham and Saunders (1974) showed the successful transfer of genes from higher to lower ploidy levels through haploidy. Similarly, Bingham (1968) demonstrated the gene transfer from diploid *M. sativa* and *M. falcata* and their hybrids to the tetraploid levels. They further observed that the inter-ploidy hybridization was equally efficient as that of the same ploidy hybridization and the hybrids were vigorous and fertile. It is evident that the members of *M. sativa* complex can occur sympatrically in the landscape and are capable of naturally hybridizing with each other both at diploid and tetraploid levels. This evidence has implications for the confinement of novel traits in regions where these populations occur in nature.

3.4 Origin and distribution

Within the scope of this study, 'alfalfa' refers to the cultivated forms of *M. sativa* ssp. *sativa*, *M. sativa* ssp. *falcata* and *M. sativa* ssp. *varia*. Wild (natural populations of ssp. *sativa* morphologically original to the cultivated form; Muller et al. 2005) and feral (cultivated alfalfa occurring outside of cultivated lands; Chapter 2.0) forms of alfalfa are mentioned as applicable.

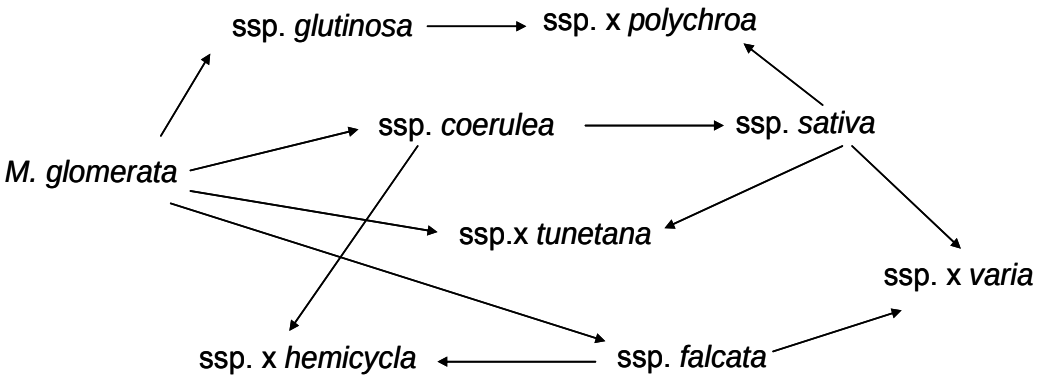

Fig. 3.1 Evolutionary pathway of members in *M. sativa* complex (adapted from Quiros and Bauchan 1988)

3.4.1 Centre of origin

Alfalfa likely originated in Near Eastern centre which includes Asia Minor, Transcaucasia, Iran and the highlands of Turkmenistan (Bolton 1962). However, there exist different schools of thought on the exact place of origin of alfalfa. Klinkowski (1933) considered Media, the north western part of modern Persia, as the place of origin of alfalfa. Sinskaya (1950), on the other hand, stated that alfalfa had two centers of origin namely the mountainous region of Transcaucasia and central Asia. Sinskaya also believes that Asia Minor and the areas adjoining the northwestern Iran could have been the center of origin of alfalfa. However, Iran is most often regarded as the place of origin of alfalfa (Bolton et al. 1972).

3.4.2 Geographical distribution

Alfalfa has a long history of cultivation and it was the first forage crop to be domesticated and recognized as a valuable crop plant (Chessmore 1979). There are references to alfalfa in Turkey from 3250 B.P. and in Babylonia from 2650 B.P. (Bolton et al. 1972). As early as 5950 B.P., maritime trade was well developed in the Mediterranean region, which could have contributed to the widespread use of alfalfa. During the pre-Christian period, the use of alfalfa further spread to Asia, Africa and Europe with advancements in trade and exchange of goods among these regions. It is only within the last 500 years that its use has extended far from its centre of origin (Leach 1978). The discovery and colonization of America by the Portuguese and Spaniards in the 15th century led to the introduction and spread of alfalfa throughout Latin and North America (Barnes et al. 1988). In the 18th century, alfalfa was taken to Australia and New Zealand. Alfalfa is now widely acclimatized in many regions including Australia, South Africa, New Zealand and South and North America.

The occurrence of alfalfa outside of cultivation is frequently observed in geographical regions where alfalfa is commonly cultivated (Small and Jomphe 1989; Muller et al. 2005). Wild alfalfa populations are often limited to the regions around its centre of domestication and are reported to occur from Near East to Central Asia (Small 1982) and

in Spain (Enguita 1986).

3.4.3 Distribution in Canada and the US

The primary global centre of alfalfa cultivation is North America with more than half of all current acreage. Alfalfa was brought to North America as early as 1736 (Stewart 1926), but it was only in the mid 1850s that alfalfa cultivation spread to the irrigated soils of the western regions and the Southern Great Plains of the US. The cultivated area of alfalfa in the US increased from a few acres in 1854 to 2 million, 10 million and 20 million acres in 1900, 1924 and 1950, respectively. Wisconsin, South Dakota, North Dakota, Minnesota, California, Idaho, Kansas, Iowa, Michigan, Montana and Nebraska are all important alfalfa growing states in the US (Barnes et al. 1988). With the introduction of winter-hardy types, it was possible to grow alfalfa in the northern states and in Canada. Alfalfa was first introduced into eastern Canada in 1871 with seed from Lorraine, France that was developed into the regional strain known as 'Canadian Variegated' (Melton et al. 1988). Alfalfa's use spread gradually throughout Ontario, Quebec and the Atlantic provinces. In Ontario, alfalfa production was concentrated near the Great Lakes. In the prairie provinces of western Canada, winter-hardiness is essential for persistence and because of the introduction of extremely winter-hardy types such as 'Grimm' and 'Baltic' from Minnesota in early 1900's, the use of alfalfa eventually spread across western Canada (Bolton 1962). The important alfalfa growing provinces of Canada include Alberta, Saskatchewan, Manitoba, Ontario and Quebec (i.e. where livestock are common).

Feral alfalfa populations are commonly observed in most of the alfalfa growing regions in North America. Boe et al. (2004) reported the occurrence of naturalized *M. falcata* populations on private and public rangelands in north western South Dakota. Results of the feral alfalfa surveys conducted in five states (California, Idaho, Pennsylvania, South Dakota and Wisconsin) in the US (Kendrick et al. 2005) and in three rural municipalities (Springfield, Hanover and MacDonald) in southern Manitoba, Canada (Bagavathiannan et al. 2008) (Chapter 6.0) confirm the widespread occurrence of feral alfalfa populations

in field shoulders, roadsides and other unmanaged habitats in alfalfa growing regions.

3.5 Traits favoring ferality potential in alfalfa

Plant breeding efforts in perennial forage crops including alfalfa have uniquely focused on selecting for traits that support persistence over time under conditions of mowing and grazing in a broad range of low input environments. It eventually facilitated the broad adaptation and wide global distribution of alfalfa. Many of the traits that facilitated broad geographical adaptation may also support ferality. There may be little difference between breeding for persistent forage varieties and breeding for ferality/weediness. Following is a description of key traits that support ferality in alfalfa.

3.5.1 Drought, cold and salt tolerance

The ability of alfalfa plants to become dormant when exposed to extreme cold or drought and to regenerate when conditions become favorable is an important adaptive trait (Chessmore 1979; Peterson et al. 1992). Because alfalfa can withstand drought, heat and cold, it is well-adapted to grow in many regions (Leach and Clements 1984; Michaud et al. 1988). The water use efficiency of alfalfa is greater than for most field crops (Putnam 2004) and it is more drought tolerant than most other temperate forage legumes (Peterson et al. 1992). Prosperi et al. (2006), for instance, observed the existence of natural alfalfa populations along roadsides in areas receiving less than 350 mm of annual rainfall (a rainfall level considered inadequate for the survival of many perennial herbaceous species). With a well-established deep root system, alfalfa can extract moisture from soil depths beyond depths possible for many other crops and this helps it to withstand drought conditions (Bolton et al. 1972). Though uncommon, alfalfa roots have been recorded at depths of up to 9 to 12 m (Bolton 1962). Alfalfa also produces lateral roots close to the soil surface (Bolton 1962) enabling the exploitation of resources at different depths in the soil profile.

Alfalfa possesses a high degree of winter-hardiness and the roots and crowns can survive temperatures as low as -20^{o}C (McKenzie et al. 1988). The winter-hardiness of alfalfa is

due to a series of biochemical, biophysical and morphological changes that occur within the plant during the fall hardening period (McKenzie et al. 1988). Cold tolerance begins in autumn when mean air temperatures are around 10^{o}C and the tolerance accelerates when temperatures fall below 5^{o}C (Tysdal 1933). Although a short photoperiod is essential for the initiation of cold tolerance, alfalfa plants continue to harden regardless of photoperiod (Hodgson 1964).

Apart from drought and winter-hardiness, alfalfa also has a high degree of tolerance for alkaline and high salt content soils (Sheaffer et al. 1988). Alfalfa can tolerate salt levels of up to 0.25% in soil, which is greater than that of most common grain crops (Geng 1989). Peng et al. (2008) reported that alfalfa is adaptive to salt stress and could also tolerate mixed salt-alkaline conditions to a certain extent. The work of Peng et al. (2008) revealed that the proline content in alfalfa increased with increasing salinity and alkalinity levels indicating that the physiological responses exhibited by alfalfa facilitated the adaptation to salt and alkaline stresses.

3.5.2 Competitiveness

With an efficient deep root system, profuse branching and leaf distribution, alfalfa is a competitive species (Bittman et al. 1991). Inclusion of alfalfa in crop rotations effectively suppresses weed populations in subsequent cereal crops (Entz et al. 1995; Ominski et al. 1999). Often in hay mixtures it is challenging to maintain a perennial grass population because alfalfa can be so competitive (Chamblee and Collins 1988). Alfalfa is more efficient at intercepting solar radiation compared to grasses (Brown and Blaser 1968). Competition for light is associated with rapid and early emergence and alfalfa seedlings have an ability to emerge at relatively low soil temperatures. The minimum temperature for the germination of alfalfa seed is 1^{o}C, whereas for timothy grass, for example, it is 3^{o}C (Wilsie 1952). Alfalfa is competitive for soil moisture not only because of its ability to set deep roots, but because it is also competitive for water at shallow soil depths (Chamblee 1972). Alfalfa is also very effective at scavenging nutrients with a cation-exchange capacity (CEC) nearly double that of perennial grasses common either in hay

mixtures or roadside verges (Drake et al. 1951). Entz et al. (2001) reported that alfalfa more efficiently extracts subsoil nitrogen compared to perennial grasses. In addition, alfalfa competes well for phosphorus since its root system extends deeper than the root systems of most grass species it is commonly grown with. Alfalfa cultivars with a high degree of parentage from *M. falcata* have better persistence and adaptability and this may enhance competitiveness, particularly in unmanaged habitats (Berdahl et al. 1989; Bittman and McCartney 1994).

3.5.3 Persistence

In comparison to most common field crops, alfalfa is highly persistent. Alfalfa stands were reported to be persistent after constant cropping for twenty-five years and sometimes even much longer than this (Coburn 1906). A germplasm survey revealed the persistence of alfalfa populations in South Dakota rangelands for even more than 50 to 75 years. These populations were comprised primarily of *M. falcata* and their origin could be traced back to introductions by N.E. Hanson in the early 1900s (Berdahl et al. 1986). Similarly, a germplasm pool of *M. falcata* collected at Palmer, Alaska persisted for over 50 years (Berdahl et al. 1986). These examples demonstrate the persistence potential of alfalfa but most of the available reports on the persistence of alfalfa in arable lands are limited to less than 10 years because commercial stands are typically terminated before they are 10 years old. The average persistence of alfalfa stands in the Canadian prairie region is 3 to 5 years in wet areas and 6 to 9 years in dry regions (Entz et al. 1995). In the northern US, Jewett et al. (1996) reported that among common perennial crops used in the Conservation Reserve Program (CRP), alfalfa was the most persistent legume with persistence ratings of almost 90%, 6 to 8 years after seeding. Similarly, Coruh and Tan (2008) showed that alfalfa persisted well for six years and produced high yields with low weed content. Brown et al. (2005) compared the persistence of alfalfa, chicory and red clover over a six year period and found that alfalfa had the greatest persistence with 94% (dryland) and 55% (irrigated) of the botanical composition of swards in the sixth year, compared to 61% dryland and 55% irrigated for chicory and 0% in both dryland and irrigated for red clover. There is also variation in persistence among alfalfa synthetics.

A high degree of parentage from *M. falcata* has often been cited as a factor contributing to high levels of persistence in alfalfa (Frame et al. 1998; Katepa-Mupondwa et al. 2002). Berdahl et al. (1989) reported that alfalfa cultivars with a high degree of *M. falcata* parentage had greater yields than *M. sativa* cultivars even seven years after they were inter-seeded into unimproved rangelands.

Alfalfa can occur in cultivated fields as a volunteer weed. A hard seed coat and seed dormancy may mean that alfalfa can form a relatively persistent seedbank (Bass et al. 1988). However, the size and dynamics of the alfalfa seed bank is not well understood. The impact of volunteer alfalfa may be greater than for volunteers of annual crops because adult alfalfa volunteers are perennial and they may persist to be problematic in more than one cropping season, if they are left uncontrolled. The longevity and persistence of volunteers depends to a great extent on agronomic practices. For example, crop management practices including intensive tillage are detrimental for the persistence of established volunteers, whereas volunteer alfalfa can be more common and persistent under reduced tillage conditions (Heller 2008). In Manitoba, Canada, volunteer alfalfa ranked as the 29th most abundant weed in field crops (Thomas et al. 1997) and Leeson et al. (2005) reported that alfalfa was the 37th most predominant weed of annual crops in western Canada during the 2000s.

3.5.4 Genetic diversity

For many crop species early domestication events included a population bottleneck resulting in a reduction in genetic diversity (Walker et al. 1998). To some extent, alfalfa is an exception in this regard (Muller et al. 2002). It is an inherently heterogeneous crop and possesses relatively high levels of genetic diversity within stands because it is an insect pollinated, highly outcrossing tetraploid that has little tolerance to inbreeding. Cross-compatibility and gene flow with cultivated populations (Jenczewski et al. 1999a) as well as the existence of self-incompatibility (Allard 1988) help to maintain high levels of genetic diversity within natural populations of this species as well. The existence and maintenance of this genetic diversity through cross-pollination is an important factor

contributing to the successful adaptation of alfalfa under a wide range of soil and climatic conditions (Bolton et al. 1972).

Julier et al. (2000) discovered that there is as much diversity within as among alfalfa cultivars. They also noted that within cultivar diversity is a valuable source of genetic variation in breeding programs. Alfalfa is an autotetraploid and there is evidence that polyploidy facilitates the adaptation of crops (Thompson and Lumaret 1992; Thompson et al. 1997). In addition, the level of heterozygosity at multi-allelic loci contributes to the vigor and yield of autopolyploid forage crops (McElroy (1991).

3.5.5 Perenniality and regrowth

Alfalfa is a long-lived perennial plant and its perennial nature is essential for its successful establishment in newly colonized areas. Alfalfa seedlings produce secondary stems from the axillary buds of trifoliate leaves and subsequently tertiary stems arise from the axils of leaves on the secondary stems. Any cutting or damage to the growing point after this stage will not kill seedlings, but facilitate regrowth. As such, mowing along roadsides is not detrimental to recruiting feral alfalfa seedlings since they will either escape because of minimum mower height (typically 15 to 20 cm above ground) or they will have already become hardy enough to withstand mowing and regrow. Alfalfa regrows quickly after mowing, often more quickly than common vegetation found in roadside habitats (Bagavathiannan and Van Acker 2007) (Fig.3.2). Regrowth from established plants produces shoots faster than seedling recruitment and alfalfa's regrowth ability is both a useful persistence and competitiveness trait (Pearson and Hunt 1972). The alfalfa crown enables regrowth and perennation. In warmer climates, crowns at or above ground level are characteristic while in colder climates, crowns develop partially below the soil surface as an adaptation to the cold (Bolton 1962).

3.5.6 Nodulation and symbiotic nitrogen fixation

Alfalfa fixes atmospheric nitrogen in association with *Rhizobium meliloti*, an alfalfa specific cross-inoculation group in the family Rhizobiaceae (Burton 1972). Alfalfa and

the symbiont coexist in a comparatively equilibrated state and efficiently fix atmospheric nitrogen (Burton 1972). Symbiotic N_2 fixation enhances the establishment and survival of alfalfa even in marginal lands with poor soil fertility. Vance et al. (1988) reported a contribution of about 200 kg of N_2 ha^{-1} yr^{-1} on average through symbiotic N_2 fixation in alfalfa. However, the amount of symbiotic N_2 fixed by alfalfa can range from 40 to 420 lb N_2 $acre^{-1}$ depending on biotype, bacterial strain interactions and management practices (Carter and Sheaffer 1983). Russelle et al. (1994) stated that symbiotic N_2 fixation is an adaptive process that declines with N uptake from other sources. The capacity to fix atmospheric nitrogen may allow alfalfa to become established and persist in nitrogen poor soils, contributing to its invasive and competitive potential in comparison to non-leguminous colonizing species.

3.5.7 Pollination biology

Outcrossing can increase species fitness, particularly in natural environments (Sakai et al. 1989; Rathcke and Real 1993) and it may enhance adaptability and range. Pollination in alfalfa is primarily insect-mediated since the presence of a thicker and stronger stigmatic cuticle prevents self-pollination before tripping (Viands et al. 1988). Tripping is naturally affected by mechanical pressure applied by pollinating insects and influenced by environmental factors (wind, rain, temperature). Pollen is stored in the flower from anthesis until tripping, which lasts normally for two weeks. This creates a long window during which tripping and pollination will be effective. If tripping does not occur during this period, the flowers rarely set seed (Bolton 1962). The interesting characteristic feature of alfalfa pollen is that it is usually sticky and readily adheres to most pollination insects (Barnes et al. 1972). Honey bees (*Apis mellifera*), leaf cutter bees (*Megachile rotundata*), alkali bees (*Nomia melander)* and bumble bees (*Bombus* spp.) are all effective pollinators for alfalfa (Rincker et al. 1988). They usually avoid already tripped flowers and they rarely revisit tripped flowers (Vansell and Todd 1946). Floral morphology and the tripping mechanism cause alfalfa to be predominately outcrossing (Viands et al. 1988).

Fig. 3.2 Regrowth potential of feral alfalfa populations in roadside habitats after road verge mowing

The alfalfa shoot is indeterminate and this is an important characteristic in relation to adaptation because it lengthens the pollination and reproductive maturity period. This, in turn, increases the chances of cross pollination and reproductive success, in areas where potential pollinators may be distant and pollination opportunities may be stochastic. Low flower abundance often evidenced in feral populations, may cause competition among pollinators, increasing pollinator movement between plants and effecting more cross-pollination and increased seed set (Strickler 1999). Seed set of alfalfa is greater when the number of flowers is lower and vice versa (Piper et al. 1914). If there are more flowers per plant, pollinators move more among the flowers on the same plant resulting in more self pollination (Strickler 1999). Self-incompatibility or self-sterility is commonly present in alfalfa (Viands et al. 1988) and self-fertilized plants usually demonstrate a substantial reduction in forage and seed yield (Rumbaugh et al. 1988).

3.5.8 Seed characteristics and seed bank survival

3.5.8.1 Hard seededness

Impermeable or hard seeds are common in alfalfa. Hard seeds have viable embryos but fail to imbibe water when placed in a moist environment due to the thickened outer walls of the palisade cells. The lens, which is the weakest point in the palisade layer, provides a point of entry for water during seed germination (Lutte 1928). Hard seediness prevents germination and facilitates persistence (Fick et al. 1988). Hard seededness may also be a mechanism to ensure sufficient moisture for successful recruitment following germination. Possession of hard seed coat is an important characteristic allowing alfalfa seed to remain dormant for years (Ballard 1973). Wilton et al. (1978) reported the successful germination of alfalfa seeds stored for 70 years. Even when alfalfa seeds are stored in continuous subfreezing temperatures for 20 years, the decline in germination ranged only between 3 and 13% (Rincker 1983). In Western Canada, average hard seed content ranged from 22 to 37% in pedigreed seed lots, while it ranged from 14 to 30% in non-pedigreed seeds (Fairey and Lefkovitch 1991).

Though alfalfa seeds may not be permeable to moisture when harvested, permeability increases over time. The rate of increase depends upon temperature, relative humidity and mechanical abrasions of the seed coat during harvest and post-harvest handling. Seed germination level is influenced by soil moisture, soil temperature, light and other microsite conditions (Meyer 1999). Further, there are linkages between seed size, dormancy and persistence for common weed species (reviewed by Van Acker 2009).

3.5.8.2 Factors affecting hard seededness

Percentage of hard seededness depends on genetic and/or environmental factors during and after seed maturation (Bass et al. 1988; Copeland and McDonald 1995). It is believed that temperature during and immediately after seed maturation in particular, plays a vital role in the percentage of hard seededness in legumes (Bass et al. 1988). Likewise, other factors including photoperiodic regime, soil moisture and relative humidity are known to affect the level of impermeability in legume seeds (Harrington 1949; Barton 1965; Clua and Gimenez 2003).

Acharya et al. (1998) observed the existence of genetic variability for hard seededness in alfalfa. Watson (1948) reported that alfalfa cultivars with greater proportional parentage from *M. falcata* had high levels of hard seededness. Alfalfa seed from lower altitues appear to have lower content of hard seeds when compared to the seed from higher altitudes (Roltson 1978). In the US, Bass et al. (1988) demonstrated that hard seededness in alfalfa varied across climatic regions with hard seed content ranging between 40 and 50% in cooler regions of the Pacific Northwest, while it was less than 20% in warmer regions of southern California. In addition, late harvested alfalfa appeared to have more hard seeds when compared to early harvested seeds (Dexter 1955). In *M. truncatula*, pods left on the soil surface tended to have more hard seeds 27 days later (about 97% of pods with hard seeds), when compared to the seeds buried 2cm below the soil surface (<17% of buried seeds with hard seeds) (Kitchner and Andrew 1971). The amount of impermeable seeds and the rate of softening may be a function of the level of moisture present in the seeds. The hard seed content increases with decreases in seed moisture

(Barton 1965). Similarly, low relative humidity can also increase the level of hard seededness (Harrington 1949). However, the level of soil fertility is less likely to play a major role in the development of hard seeds (Rolston 1978).

In the context of ferality, hard seed coat and dormancy mechanisms can contribute to discontinuous germination and seedbank persistence. These are important traits contributing to ferality potential in many crops (Hails et al. 2002; Garnier and Lecomte 2006) including alfalfa (Bagavathiannan and Van Acker 2008b) (Chapter 2.0). In addition, hard seededness in alfalfa facilitates its dispersal by animals (Russelle 2001). In this regard, alfalfa seeds may be scarified by the process of digestion, leading to germination after deposition into a nutrient rich environment (AIS 2008).

3.5.9 Useful chemical compounds

Reports indicate that chemical compounds such as phenolics, lignins, and saponins found in alfalfa can help provide some level of pest and disease resistance (Howarth 1988). Resistance to pests can facilitate the adaptability and range of a species. Phenolic compounds in alfalfa include coumarins, flavonoids and anthocyanins which provide essential pest and disease resistant properties (Howarth 1988). Lignin strengthens the most fragile polysaccharide constituents of the plant cell wall enhancing the fiber content and strength of alfalfa plants and improving pest resistance. Alfalfa herbage contains 5 to 14% lignin, levels greater than for a range of temperate grasses (Harkin 1973). Saponins are glycosides, which inhibit the growth of several potentially pathogenic fungi including *Sclerotium rolfsii*, *Pythium* spp., and *Rhizoctonia solani* in alfalfa (Leath et al. 1972). High saponin content in alfalfa increases resistance to the pea aphid *Acyrthosiphon pisum* (Pedersen et al. 1976). Alfalfa herbage has saponin levels of 2 to 3%. The concentration of saponin in alfalfa is lowest in spring, increases in the summer when the soil moisture levels are lowest and temperatures are highest, and then declines in the fall (Pedersen 1978), suggesting perhaps that plants grown under stressed conditions (conditions common in unmanaged habitats) may have greater saponin levels and associated pest resistance.

However, more recently, alfalfa breeders have focused on limiting many of these secondary metabolites for their anti-nutritional properties (Reed 1995). As such, phenolic compounds may play only a minor role in facilitating wide adaptation of escaped plants from modern alfalfa synthetics.

3.6 Evolution of adaptation

A species that easily adapts to the unmanaged environments presents a unique challenge in regard to confining the movement of novel traits across the landscape. Species that are genetically heterogeneous have the advantage of drawing on genetic diversity, allowing populations to continue to persist in new environments (Crawley et al. 1996). Frankel et al. (1995) proposed that genetic divergence is a result of the interaction of populations with abiotic, biotic, genetic and stochastic factors. Any selection pressure imposed on the escaped populations will lead to genetic divergence from the original cultivar(s), and lead to greater adaptiveness in new environments (Levin 1990). Because of the highly heterogeneous nature of alfalfa and its high level of outcrossing, selection pressures could lead to significant genetic shifts resulting in populations that are more adaptive to their surroundings than the original synthetic and this could contribute to ferality potential (Jenczewski et al. 1998).

Prosperi et al. (2006) tested the persistence of naturally occurring roadside alfalfa populations in Spain and found that natural populations were more adaptive and persistent with 71% of plants surviving after five years compared to 48% survival for plants from cultivated varieties common to the region. Reports indicate that natural selection pressures in wild alfalfa populations induce differences in morphological characteristics (Casellas 1962; Enguita 1986; Prosperi et al. 2006). A range of variabilities induced in wild alfalfa by natural selection were reported by Jenczewski et al. (1998; 1999b). Natural selection mechanisms prevented the maintenance of cultivated traits in natural populations of *M. sativa* (Jenczewski et al. 1999b). In most cases, natural selection favored prostrate, creeping growth habits and rhizome production (Jenczewski et al. 1999a), which are the traits associated with grazing and drought tolerance (Prosperi

et al. 2006). These reports are good examples of feral alfalfa populations adapting to new and unique environments.

Annicchiarico (2007) reported that alfalfa plants adapted to sandy loam soils produced more root biomass and larger leaflets compared to plants adapted to silty-clay loam soil which produced more shoots per plant and had greater autumn as well as winter growth. Populations with *M. falcata* parentage are generally more adapted to resource poor environments (Berdahl et al. 1989; Bittman and McCartney 1994). Because alfalfa cultivars selected for rangelands have greater levels of parentage from *M. falcata* and improved persistence (Heinrichs 1963), escaped plants from those cultivars would likely have a greater potential for establishing feral populations, as evident from the South Dakota example mentioned previously (Berdahl et al. 1986).

3.7. Factors hampering feral population establishment and spread

Although alfalfa is highly adapted to a wide range of soil and environmental conditions and alfalfa's biology aids its successful establishment in unmanaged habitats, the rate of feral population establishment and spread may be limited under some conditions.

3.7.1 Unfavorable soil and environmental conditions

During seedling establishment, alfalfa is sensitive to insufficient or excess water conditions. Grass seedlings establishing with alfalfa in hay or pasture mixtures tend to be deeper rooted and more robust with better survival under droughty conditions (Chamblee and Collins 1988), whereas shallow rooted alfalfa seedlings struggle for moisture (Gist and Mott 1957). Alfalfa plants cannot withstand prolonged waterlogging periods (Sheaffer et al. 1988). Successful seedling establishment is also affected by low light intensity. Under conditions of severe shading and inadequate light, only one axillary bud (usually the unifoliate) develops into a stem (Meyer 1999) and seedlings are weak.

Shading can also affect root development (Hall 1974), nodulation (Burton 1972) and the initiation of crown buds (Chamblee and Lovvorn 1953). Alfalfa seedlings are also

sensitive to fluctuations in soil temperature. Baldocchi et al. (1981) showed that the growth rate of alfalfa seedlings was reduced when temperatures were outside the range of 10 to 37^{o}C. Nitrogen fixation was reduced by approximately 50% when temperatures were increased from 16^{o}C to 30^{o}C (Barta 1978). Similarly, temperatures below 15^{o}C severely restrict and may completely inhibit symbiotic bacterial association with alfalfa and successful nodulation (McKenzie et al. 1988). Alfalfa does not tolerate acidic soils (pH below 6) (Sheaffer et al. 1988). Both alfalfa and its associated nitrogen fixing bacteria are poorly adapted to low pH soils and when soil pH levels are low, nitrogen fixation and alfalfa yields are reduced. In Spain and Portugal, Prosperi et al. (2006) noted that natural *M. sativa* populations were absent from any sites with acidic soils.

3.7.2 Auto-toxicity

The establishment of alfalfa following alfalfa is limited due to auto-toxicity, which is the inhibition of new alfalfa seedling establishment, caused by the allelopathic compounds produced by existing alfalfa stands (Seguin et al. 2002). Auto-toxicity is the most common cause of reseeding failure in commercial alfalfa stands (Miller 1996) and the reason why inter-seeding to increase alfalfa stand density is typically not successful (Jennings and Nelson 1998). Auto-toxicity may limit population growth for feral alfalfa stands if there is no seed dispersal. This will depend as well on the size of the allelopathic zone around a given alfalfa plant. Jennings and Nelson (2002) planted alfalfa seeds around mother alfalfa plants and found that the seedlings were weak and the yield was much reduced when seeding was done within 1m of mother plants. Widely dispersed alfalfa seeds could escape this auto-toxic limitation. Seed predation events may aid the dispersal of alfalfa seeds away from the auto-toxic zone. Jennings and Nelson (1998) found that auto-toxicity was influenced by soil texture and rainfall patterns which might in turn affect the activity of water-soluble chemicals, their concentration and movement in the soil. They noted that auto-toxic effects were greater in fine sandy loam versus silty clay loam soils.

3.8 Feral alfalfa and gene flow

Cultivated and feral alfalfa share ploidy levels, overlap in flowering periods, share pollinators and occur sympatrically in the landscape (Prosperi et al. 2006). As such, feral alfalfa populations can involve in trait movement via gene flow with cropped and volunteer alfalfa populations, with other subspecies in the *M. sativa* complex and also with wild relatives in the genus *Medicago* (Fig. 3.3). Because of cross-compatibility, feral alfalfa populations can act as sources and sinks for novel traits at the landscape level (Knispel et al. 2009). Therefore, feral alfalfa populations are an important but largely unrecognized consideration in gene flow and trait confinement in alfalfa (Putnam 2006).

3.8.1 Gene flow among escaped, feral and cultivated *M. sativa*

Jenczewski et al. (1999a) and Prosperi et al. (2006) confirmed gene flow from cultivated landraces to feral alfalfa populations occurring along roadsides in Spain. Because alfalfa is mostly pollinated by bees, long-distance gene flow is possible. In alfalfa, Teuber et al. (2004) found bee mediated gene flow in alfalfa at distances up to 4km. Likewise, St. Amand et al. (2000) confirmed the gene flow from hay and seed production fields at a distance of 1 km (although they tested the gene flow only up to this distance).

Under leaf cutter bee-mediated pollination, Fitzpatrick et al. (2003) showed that outcrossing rates at 152m and 274m were 1.7% and 0.3%, respectively, and they detected a single outcrossing occurrence (from 30,000 seeds tested) at a distance of 792m. St. Amand et al. (2000) investigated the transgene movement among widely dispersed, individual feral plants along roadsides and confirmed gene flow at a distance of 230m with an outcrossing frequency of 92.2%.

Considering the distance that pollen can move from cultivated fields, hybridization with feral populations is inevitable, considering that roadside feral populations are commonly observed within outcrossing distance to cultivated alfalfa fields (Bagavathiannan et al. 2008) (Chapter 6.0). This evidence suggests that it is highly probable that feral alfalfa plants growing in unmanaged habitats close to cultivated fields will act as conduits for long-distance transgene dispersal.

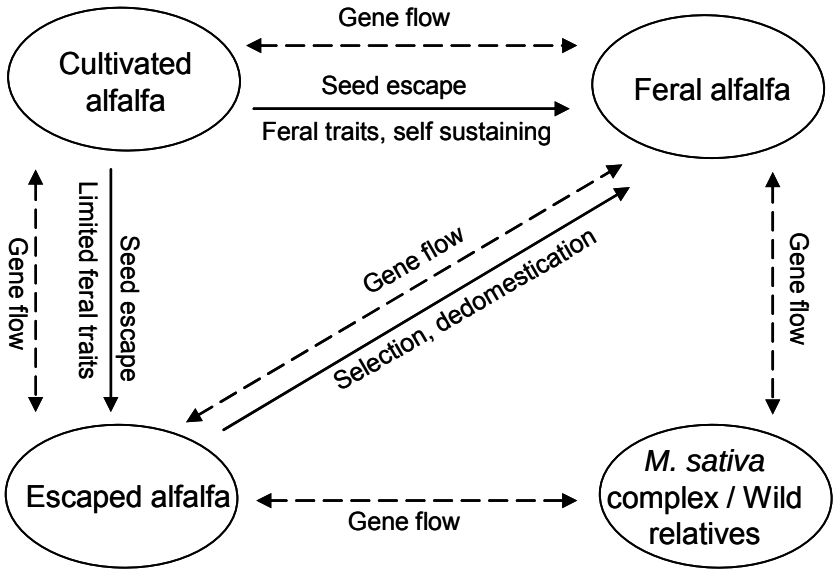

Fig. 3.3 Pathways of gene flow involving feral alfalfa populations. Feral alfalfa could potentially interact with cultivated alfalfa, escaped alfalfa and wild relatives both through forward and backward gene transfer. Consequently, they act as a genetic bridge or reservoir for the novel traits for further contamination of compatible species in the ecosystem.

3.8.2 Hybridization within the *M. sativa* complex

All members of the *M. sativa* complex are cross compatible and readily cross-pollinate (Lesins and Lesins 1979; McCoy and Bingham 1988). Inter sub-specific hybrids have very good chromosome pairing in F_1 and are typically fertile in F_1 and later generations (McCoy and Bingham 1988).

Small (1984) confirmed the natural occurrence of hybrid swarms and backcross hybrids between cultivated *M. sativa* and wild *M. falcata* at both diploid and tetraploid levels. In North America, *M. sativa, M. falcata* and *M. varia* are commonly observed (USDA 2008) both as cultivated and escaped populations. The widespread occurrence of intra-specific hybridization will favor the adventitious presence of novel traits in the environment. In addition, possible hybrids produced through outcrossing with *M. falcata* might show increased adaptation to resource poor natural environments (Berdahl et al. 1989; Bittman and McCartney 1994).

Introgression of genes from the members of *M. sativa* complex to their wild relatives is also possible particularly in regions where wild relatives are prevalent (Lesins and Lesins 1979; McCoy and Bingham 1988) (Refer Table 3.2). Feral alfalfa populations occurring in these regions might facilitate the movement of novel traits into wild relatives.

3.8.3 Inter-specific hybridization

Alfalfa has been shown to hybridize with compatible species and produce inter-specific hybrids (Table 3.2). Fertilization studies have shown the successful formation of zygotes and embryos for a number of inter-specific combinations (Sangduen et al. 1983; McCoy and Smith 1986). *Medicago* has 83 species and only a third of these are perennials including cultivated *M. sativa*. Production of hybrids between annuals and perennials is rare and there is no evidence for their occurrence in nature. However, Sangduen et al. (1982) reported the recovery of a hybrid between perennial *M. sativa* and annual *M. scutellata* using gibberellic acid (GA) treatment.

Cultivated alfalfa has been hybridized successfully with *M. cancellata, M. daghestanica,*

M. dzhawakhetica, M. glomerata, M. hybrida, M. marina, M. papillosa, M. pironae, M. prostrata, M. rhodopea, M. rupestris, M. saxatilis and *M. scutellata* (McCoy and Bingham 1988). In most of these cases, ovule and embryo culture methods favored the successful recovery of inter-specific hybrids (McCoy and Smith 1986).

The potential for gene flow and introgression in nature often depends on overlapping geography and congruence of ploidy level among the species involved in hybridization. For instance, natural hybridization between the members of *M. sativa* complex and *M. prostrata* and *M. glomerata* is possible due to congruent ploidy levels (2n = 2x = 16) and sympatric distribution (McCoy and Bingham 1988). Inter-ploidy gene flow could also be possible through the production of unreduced (2n) gametes and Stanford et al. (1972) argued that ploidy differences present no great barrier to gene flow in alfalfa.

In North America, *M. lupulina* (black medick) is the most common wild relative of cultivated alfalfa (Darbyshire et al. 2000). Turkington and Cavers (1979) reported the production of hybrids between black medick and cultivated alfalfa. However, the occurrence of hybrids between *M. sativa* and *M. lupulina* are unlikely in nature (Lesins and Lesins 1979). The occurrence of other wild relatives namely *M. hybrida* in Indiana and *M. scutellata* in Maryland has been documented (USDA 2008). Nevertheless, natural hybridization among these species is unlikely and that the risk of inter-specific hybridization of *M. sativa* with related wild species is very low in North America.

3.8.4 Seed mediated gene escape

Ferality potential in alfalfa also has implications for seed mediated gene escape. Seed escape from transgenic varieties followed by successful establishment in unmanaged habitats will directly result in the adventitious presence of transgenes in the environment. Seed escapes might occur during planting, harvesting and transport. Crawley and Brown (1995) documented the contribution of seed spill from the transport trucks to the establishment of feral oilseed rape (*Brassica napus*) populations in roadsides in the UK. In addition, seed predation by insects, rodents and birds may facilitate long distance seed dispersal out of cultivated fields.

Table 3.2 Evidence for successful inter-specific hybridization involving *M. sativa*[1]

Species	Plant description	Global distribution	Remarks	References
M. glomerata (2n = 2x =16)	Prostrate, stems covered with simple appressed hairs, yellow flowers and coiled pods with glandular hairs.	Southern Europe and northern Africa, particularly in Tunisia.	Hybrids readily obtained. However, high meiotic irregularities and low seed set observed in F_1 hybrids. Natural outcrossing between *M. sativa* and *M. glomerata* was also observed.	Lesins (1968); Lesins and Lesins (1979); Quiros and Bauchan (1988)
M. prostrata (2n = 2x = 16; 2n = 4x = 32)	Glandular stem hairs, Yellow flowers with tightly coiled pods. Plants occur in dry, rocky hillsides.	Eastern Austria and Italy, eastern Adriatic coast to Greece.	Easily hybridized both at diploid and tetraploid levels. However, *M. prostrata* as a female parent resulted in low seed set.	Lesins (1962); Sorensen et al. (1980)
M. cancellata (2n = 6x = 68)	Narrow leaflets, yellow flowers and coiled pods with reticulate veins. Grows in poor soils composed of sandstone.	Southeastern European Russia, north of Caucasus.	Successful hybridization with tetraploid and hexaploid *M. sativa*	Lesins (1961); Lesins and Lesins (1979); Yen and Murphy (1979); Smith et al. (1984)
M. rhodopea (2n = 2x = 16)	Semi-prostrate, profuse branching, yellow flowers and coiled pods with or without spines. Plants occur in calcareous rocky sites, in lower mountain zones.	Mountain ranges of Bulgaria, particularly in the Rhodope mountains.	Successful hybridization, especially when using ovule-embryo culture for recovering the hybrids. Chromosome pairing was excellent in F_1 hybrids.	Lesins (1972); McCoy and Smith (1986)

M. rupestris (2n = 2x = 16)	Wedge-shaped leaflets, yellow flowers and coiled pods with prominent veins. Plants occur in calcareous rocky sites, in lower mountain zones	Eastern Europe particularly in Ukraine (Crimean mountains)	Hybrids and backcross progenies were easily recovered using ovule-embryo culture. Chromosome pairing was excellent in F_1 hybrids.	McCoy (1985); McCoy and Smith (1986)
M. saxatilis (2n = 6x = 48)	Obovate leaves, yellow flowers and pods with glandular, articulated hairs and also with spines or corrugated edges. Plants occur in calcareous rocky sites in mid-mountain zones.	Eastern Europe particularly in Ukraine (Crimean mountains)	Hybrids were readily obtained both with tetraploid and hexaploid *M. sativa*. Good chromosome pairing was observed.	Yen and Murphy (1979); Smith et al. (1984)
M. daghestanica (2n = 2x = 16)	Prostrate, thin stems, obovate leaflets, purple flowers and coiled pods with short, conical spines. Plants occur in calcareous, weathered rock substrates.	Mid-mountain zone of Daghestan, Russia	Successful hybridization using ovule-embryo culture. Trispecies hybrids (*M.sativa* x *M. daghestanica-M.pironae*) were also produced using colchicine treatment	Lesins (1971)
M. pironae (2n = 2x = 16)	Semi-erect, wiry stems, yellow flowers and coiled spined pods with glandular, articulate hairs. Plants grow in sub-mountain rocky hillsides.	Eastern Alps particularly in northeast Italy (endemic to Friuli and Gorizia districts)	Hybrids were recovered using ovule-embryo culture. Trispecies hybrids (*M.sativa* x *M. daghestanica-M. pironae*) were also produced using colchicine treatment	Lesins (1971)

M. dzhawakhetica (2n = 4x = 32)	Semi-erect stems, obovate leaflets, yellow flowers and glabrous pods with no hairs. Grows in valleys of the mid mountain zones of about 1200 to 1500 above mean sea level.	Mountains of Transcaucasia	Triploid hybrids with two genomes of *M. dzhawakhetica* and one genome of *M. sativa* were obtained. The level of chromosome pairing in triploid hybrids was excellent. Unequal ploidy levels were essential.	McCoy and Smith (1984)
M. papillosa (2n = 2x = 16; 2n = 4x = 32)	Semi-erect stem, yellow flowers and pods covered with articulate, semitransparent, glandular hairs. Grows in calcareous rocky mountains and soils of volcanic origin.	Pontus mountains of north-eastern Anatolia to adjacent Caucasus mountains of Transcaucasia.	Triploid hybrids with two genomes of *M. papillosa* and one genome of *M. sativa* were recovered. Hybridization was possible only with uneven ploidy levels	Lesins (1961); Clement (1963); McCoy and Smith (1986)
M. marina (2n = 2x = 16)	Dense simple hairs on stems and pods, plants look grayish. Grows exclusively on seashores, in loose sand.	Mediterranean and Black Sea shores, Atlantic coast of Iberia and France	Successful when crossed with diploid *M. sativa*. Hybrids were recovered using ovule-embryo culture. However, the hybrids were extremely weak.	McCoy and Smith (1986)
M. hybrida (2n = 2x = 16)	Prostrate stems, vegetative parts covered with sparse hairs, yellow flowers, pods slightly curved and glabrous.	Southwestern Europe particularly in Corbier mountains and east Pyrenees in France, US (Indiana).	Successful when crossing the parents at diploid level. Hybrids were recovered using ovule-embryo culture	McCoy and Smith (1986); USDA (2008)

M. arborea (2n = 4x = 32)	Evergreen shrub growing up to 2m, flowers are hermaphrodite having both male and female organs. Grows in dry soils in rocky hillsides	Southern Europe, Asia Minor, Mediterranean basin.	Hybrids were recovered using protoplast fusion	(Nenz et al. 1996)
M. scutellata (Snail medick) (2n = 30)	Annual species. Plants prostrate to decumbent, densely covered with glandular hairs, yellow to orange yellow flowers, pods oval or cup-shaped.	Mediterranean region, US (Maryland)	Successful hybridization but unstable chromosome numbers in the hybrid. No progeny have been obtained.	(Sangduen et al. 1982); USDA (2008)

[1]Substantial information was obtained from Lesins and Lesins (1979) and McCoy and Bingham (1988). Distribution data in North America were obtained from USDA plants database (USDA 2008).

Alfalfa seeds are rich in protein and serve as a nutritious food resource for seed predators. Graham (1941) reported that about 27 species of birds and 46 mammals are associated with alfalfa. It is possible that the herbivores including deer (*Odocoileus virginianus*) may likely facilitate alfalfa seed dispersal from feral populations (Leach 1956; Kufeld 1973) and aid in GM trait movement.

3.9 Conclusions

Plant breeding efforts in alfalfa have been focused on developing cultivars that are adapted to a broad range of often low input environments and this facilitated the wide adaptation and distribution of alfalfa. Many of the characteristics of alfalfa that facilitated broad geographical adaptation also support ferality. Among these, high outcrossing rates and the genetic heterogeneity of alfalfa populations are very important. The combination of high levels of genetic diversity within populations and the ease of allele movement through outcrossing which is often facilitated by insect pollinators allows alfalfa to occupy a broad array of niches by providing a reservoir of genetic diversity and a means of gene flow that supports adaptation to new areas. High ferality potential makes gene flow even more probable in this species. Auto-toxicity may limit alfalfa seedling development and the growth rates of already established feral alfalfa populations. However, auto-toxicity would be less of an influence on seedling growth and development during initial stand establishment.

This literature review suggests that alfalfa may establish persistent populations in unmanaged habitats. Persistence of alfalfa in unmanaged habitats has implications for novel trait confinement in alfalfa. Trait confinement protocols and practices may need to consider the occurrence of feral populations in order to reduce the adventitious presence (AP) levels for specific traits.

4.0 Demography of Feral Alfalfa Populations Occurring in Roadside Habitats in Southern Manitoba, Canada

4.1 Abstract

Feral populations of cultivated crops can act as reservoirs for novel genetically modified (GM) traits in the environment. However, little information is available on the potential of cultivated crops to become feral. In this study, I investigated the ferality of roadside alfalfa populations occurring in roadside habitats in southern Manitoba, Canada. I studied the demography of roadside alfalfa populations including seedbank, seedling recruitment and fecundity and examined the impact of road verge mowing on key life stages of these populations. I also compared the growth and reproductive attributes of roadside and cultivated alfalfa populations. The results revealed that alfalfa is reproductively successful in roadside habitats and capable of establishing self-perpetuating populations. A substantial portion of the alfalfa seeds I extracted from seedbank samples were viable but not germinable, suggesting some degree of seedbank persistence in roadside habitats. Alfalfa seedlings recruited successfully, however, seedling mortality was high when seedlings were in close proximity to established alfalfa plants. Mowing dramatically reduced and even prevented the reproductive success of roadside alfalfa. Generally, growth and reproduction of roadside alfalfa was comparable to cultivated alfalfa except for total fecundity. Nevertheless, considering the long (>10 years) lifespan and profuse seed production, long-term persistence of roadside alfalfa seems reasonable in my study populations. In the context of novel trait confinement, my results suggest that feral alfalfa populations need to be managed if there is a desire/need to confine novel traits in alfalfa.

4. 2 Introduction

Ferality is observed in many crop species wherein "individuals of a cultivated crop escape a managed area, survive, reproduce successfully and establish a self-perpetuating population in either a natural or semi-natural habitat" (Bagavathiannan and Van Acker 2008a) (Chapter 2.0). Feral crops typically originate from seed escapes during transport, planting, harvesting operations or via seed predators (Crawley and Brown 1995; Gray and Raybould 1998; Yoshimura et al. 2006). Escape from cultivation is often facilitated by the presence of wild or weedy traits not lost during domestication (Doebley et al. 2006). Feral traits include, but are not limited to, high fecundity, seedbank persistence and successful generalized seedling recruitment (Arriola and Ellstrand 1996; Claessen et al. 2005a). As such, some crop species are more capable of surviving outside of managed cultivation than others (Gepts 2002).

Relatively little attention has been paid to feral crop populations or their significance in agricultural landscapes. The potential contribution of crop ferality to my understanding of the evolutionary ecology and dedomestication of cropped species has been largely neglected. However, with the introduction of genetically modified (GM) crop varieties, there was a realization that feral crops could be barriers for the co-existence of GM and non-GM crop cultivars since feral crops may serve as genetic bridges for gene flow (Gressel 2005a). In addition, feral crops may act as repositories for engineered genes (Ellstrand 2006; Knispel et al. 2008). Van Acker (2007) noted that feral crops may serve as a component of the metapopulation of cropped species in agricultural landscapes and thereby aid in novel trait movement among sexually compatible populations in the landscape. As such, the persistence and spread of engineered genes may be facilitated by feral crop populations in natural habitats (Wolfenbarger and Phifer 2000). Genetically engineered (GM) glyphosate-resistant alfalfa received authorization for environmental release in the US and Canada (APHIS 2005; CFIA 2005) and more information on the dynamics of roadside alfalfa populations would be valuable in designing efficient trait confinement protocols and co-existence strategies in this highly outcrossing species. In this respect, understanding the processes leading to the persistence of feral plants is important (Garnier and Lecomte 2006).

To date, the assessment of ferality potential and the role of feral populations in novel trait movement have typically been based on literature analysis and not based on field-based experiments. To understand the feral capacity of escaped populations, it is necessary to conduct investigations on the demography, including fecundity, seedbank, seedling emergence and the survival of different life stages (Claessen et al. 2005a; Garnier and Lecomte 2006). High fecundity favors the persistence of many plant species particularly annuals in competitive environments (Abhilasha and Joshi 2009). Ronce and Olivieri (1997) have shown that perennials with low reproductive effort have a competitive advantage over other plants. Occurrence of seed dormancy and the formation of a persistent seedbank are often considered to be important for the long-term persistence of plant populations (van Klinken et al. 2008). In particular, the existence of physical dormancy (hard seed coat) can serve as a bet-hedging strategy spreading the risk of recruitment failure over years (Evans et al. 2007). In addition to seedbank persistence, excellent seedling recruitment ability is a fundamental life-history trait that greatly influences population growth rate and population fitness (Xavier et al. 2003). Seed dispersal may be helpful in population spread (Bass et al. 2006) but for some species broad niche adaptation may be more important than dispersal (Caspersen and Saprunoff 2005).

Alfalfa is a perennial legume crop adapted to a wide range of soil and climatic conditions (Bolton 1972). In addition to cultivated fields, alfalfa could also be found in natural and semi-natural habitats in regions where alfalfa is commonly grown (Jenczewski et al.1999a; Kendrick et al. 2005). The ecology and biology of alfalfa including its deep tap root system, ability to fix nitrogen, perenniality, and drought and cold tolerance may favor successful establishment in unmanaged habitats (reviewed in Bagavathiannan and Van Acker 2009) (Chapter 3.0). Alfalfa is a prolific seed producer and its seeds have a hard coat that contributes to dormancy and persistence (Bass et al. 1988; Fick et al. 1988). Self-seeding and recruitment has been observed in alfalfa (Dubbs 1971; Rumbaugh 1982) but the seedlings that recruit in already existing stands do not perform well due to auto-allelopathy and competition from other alfalfa plants (Rice 1984; Jennings and Nelson 2002). Long-term persistence of alfalfa populations in pastures and

rangelands has been reported by several authors (Kilcher and Heinrichs 1965; Pearse 1965; Rumbaugh and Pedersen 1979). However, there have been no studies on the life history stages of feral alfalfa populations in natural or semi-natural habitats.

The objective of this study was to characterize the demography of roadside alfalfa populations occurring in roadside habitats in southern Manitoba, Canada. In particular, I addressed the following questions: a) Are alfalfa plants reproductively successful in roadside habitats? b) How large is the seedbank and what is the level of seedling recruitment? c) What is the survival of different life stages in roadside habitats? and d) What is the impact of occasional mowing of road verges on the dynamics of feral alfalfa populations growing in roadside habitats?

4.3 Materials and Methods

4.3.1 Study area and site selection

Roadside alfalfa populations were studied from 2006 to 2009 in three rural municipalities (RM) in southern Manitoba, including Hanover (49° 28' N; 96° 50' W, area = 718 km^2), MacDonald (49° 40' N; 97° 30' W, area = 1059 km^2) and Springfield (49° 55' N; 96° 45' W, area = 1106 km^2). The regions are characterized by a continental climate consisting of cold winters and warm summers. The average seasonal temperature ranges from -13^oC to 26^oC with about 115 frost free days per annum. The average annual precipitation is 520 mm of which about 23% is received as snowfall (MCP 2008). Alfalfa is one of the important crops in the RMs of Hanover and Springfield while it is grown less frequently in MacDonald. In 2008, alfalfa was planted in 81 and 53 farms respectively in Hanover (1727 ha; 4% of total cultivated area) and Springfield (2917 ha; 11% of total cultivated area), while it was cultivated on only 34 farms in MacDonald (976 ha; <1% of total cultivated area) (MMPP 2009).

In each RM, four roadside alfalfa populations were chosen randomly for detailed examination over four years (see Table 4.1 for details on site characteristics).

Table 4.1 Characteristics of the roadside sites studied in southern Manitoba, Canada.

Population	Road type	Length of the population (m)	Ditch width (m)		Steepness‡	Adjacent environment†
			Total	Inner verge*		
Hanover 1	Gravel	225	8	5	2	1,5
Hanover 2	Paved	290	11	5	3	3,4
Hanover 3	Provincial highway	210	12.5	8	4	1,5
Hanover 4	Provincial highway	162	22	16	3	2,5
MacDonald 1	Gravel	222	9	4	4	2
MacDonald 2	Gravel	225	7	3	3	2
MacDonald 3	Gravel	238	6	3.5	2	2
MacDonald 4	Paved	131	18	8	3	2,5
Springfield 1	Gravel	207	11	5	2	2
Springfield 2	Gravel	58	5	5	5	1,6
Springfield 3	Gravel	132	4.5	4	1	2
Springfield 4	Gravel	110	14	9	5	1,2

*Width from the road shoulder to the deepest point of the ditch

‡Measured in a scale of 1 to 5 with 5 being very steep

†Type of landuse in the adjacent environment (1-alfalfa field; 2-cultivated crop other than alfalfa; 3-pasture/grazing; 4-woods; 5-yard/residential; 6-wasteland)

Each roadside research site was about 100 to 150 m long and 5 to 15 m wide, depending on the size of the population. At each roadside population, 30 alfalfa plants were selected randomly across the length and width of the site without any preference on size, age and location within the study site. In total, 360 plants were studied across the three RMs. The 30 plants were assigned to a mowing treatment (mowed or not-mowed) only after the mowing treatment was implemented. Mowing is usually carried out by RMs as part of the roadside stewardship program twice per year: once between early June and early July and again between late August and mid September at a cutting height of about 20cm. Generally, only the area immediately adjacent to the road shoulder (about 3.5m wide) is mowed (Fig. 4.1). The study plants were cut during the first mowing by the RMs. However, they were harvested manually prior to the second mowing. Herbicide application (2,4-D) for controlling noxious weeds in roadside habitats can occur in this region but none of my observation sites were sprayed with herbicide at any time during the study.

4.3.2 Data collection

4.3.2.1 Roadside seedbank determination

Three soil cores (10cm diameter and 7cm deep) were taken within 30cm around each study plant (30 plants per study site) during early May in 2006 and 2007. Cores from each plant were pooled and the number of germinable seeds in the seedbank was estimated by a grow out procedure in a greenhouse. The seedbank samples were subjected to freeze-thaw cycle twice to break seed dormancy (Nightingale and Baker 1995). In 2007, soil cores from five plants per population (n = 15) were selected randomly and washed out on a fine mesh sieve to find seeds that failed to germinate. Following elutriation, viability of the seeds was tested using a tetrazolium test (ISTA 1999). Seed coats were pierced and seeds were imbibed in 1% (w/v) tetrazolium solution at room temperature (~24^{o}C) for 24 hours before viability determination. Seed viability was confirmed based on the presence of colored tissues following the tetrazolium test.

Fig. 4.1 Pattern of road verge mowing in rural Manitoba, Canada

To estimate over-winter seed survival, artificial seedbanks were established in roadside sites in the fall of 2006 and 2007 by placing seeds in nylon mesh bags (5cm x 5cm) (Kalisz 1991). Pooled samples from alfalfa seeds harvested from roadside feral populations in the respective years were used in the seed bags. Five hundred alfalfa seeds were placed into each nylon bag with no additional soil and the bags were buried 2 cm deep in the roadside sites in early November. Three seed bags were buried in each of the four roadside sites per municipality in all the three municipalities selected in this study (n = 36). The bags were retrieved in early May of the following year and winter survival of alfalfa seeds was estimated by germination in petri dishes. After five days, ungerminated seeds were scarified using sand paper and subjected to a second germination test in petri dishes. Scarification induced germination in all remaining seeds.

4.3.2.2 Seedling recruitment in roadside habitats

The number of seedlings (identified by the presence of cotyledons) that recruited around the study plants was determined during early May prior to the collection of seedbank samples in 2006 and 2007. Seedling recruitment was counted in four quadrats (25cm x 25cm) in the immediate vicinity of each study plant. The seedlings recruited in May 2007 were marked and their survival was studied during May (winter survival) and August (summer survival) of each year until August 2009.

4.3.2.3 Growth, reproduction and survival of adult plants

Data on growth parameters (plant height, number of shoots plant^{-1}) and reproductive attributes (number of racemes plant^{-1}, number of flowers raceme^{-1}, number of pod clusters plant^{-1} and number of pods cluster^{-1}) were recorded, as available, from the study plants at monthly intervals from May to August. These observations were carried out on 30 alfalfa plants selected priorly without any preference on growth type, size and location, distributed within the study site (~150m long and ~10m wide) in each roadside population.

Plant height (cm) was measured on the central shoot of the study plant. Reproductive attributes such as the number of racemes and number of pod clusters were counted on the central shoot and then calculated for total number of shoots in each study plant. Observations on the number of flowers raceme^{-1} and number of pods cluster^{-1} were carried out on five random samples derived within the plant and the values were averaged for each plant. Study plants were manually harvested during late August in 2006 and 2007, dried on a hot air bed for a week and whole plant dry biomass was estimated. The plants were then threshed using a mechanical thresher and the mature plump seeds were separated from light seeds using a pneumatic seed blower. In my study population, the reproductive success of a plant was determined based on the production of at least a single fully matured seed at the time of harvest. In addition, the study plants were tagged and their survival was recorded in mid May (winter survival) and late August (summer survival) each year from 2006 to 2009.

4.3.2.4 Soil characteristics

Four random soil cores (10cm dia x 7cm deep) were collected from the area within each roadside study site in May 2006. Forty eight soil cores were collected from 12 roadside sites selected in this study (four sites per municipality). The soil samples collected from each site were pooled, air-dried and analyzed for nitrogen (N), phosphorus (P), potassium (K), organic matter (OM), sulphur (S) and zinc (Zn) content and electrical conductivity (EC). Soil samples were also collected from an adjacent cultivated field to each roadside study site (four soil cores per field) and soil nutrient status (N, P, K, OM, S, Zn and EC) was estimated for each cultivated field from the pooled sample. The soil samples were analyzed by Agvise Laboratories Inc., USA (www.agviselabs.com).

4.3.2.5 Comparison of roadside and cultivated alfalfa

Observations on the growth (plant height, number of shoots plant^{-1}) and reproductive attributes (number of racemes plant^{-1}, number of flowers raceme^{-1}, number of pod clusters plant^{-1} and number of pods cluster^{-1}) were also carried out on alfalfa plants in seed production fields located near to my roadside study sites in 2007. Ten plants per

field were randomly selected from one field in MacDonald RM and four in Hanover RM. Alfalfa varieties in these fields included Quest, Stealth and Haygrazer. Data were collected as per the procedures followed in the roadside study populations (refer 4.3.2.3).

4.3.3 Data analysis

All data were analyzed using the mixed procedure analysis (Littell et al. 1996) in SAS (Statistical Analysis Software version 9.1) (SAS Institute 2003). Prior to analysis of variance (ANOVA), outliers were removed based on the studentized residual values using Lund's test (Lund 1975) and normality of the residuals was confirmed using the Kolmogorov-Smirnov test. Least square means were calculated and mean separation was performed using Fisher's protected Least Significant Difference (LSD) at $\alpha = 0.05$, using the PDMIX800 macro in SAS (Saxton 1998). Dependent variables (growth and reproductive attributes, fecundity, soil seedbank levels and seedling recruitment) were examined using a two-way ANOVA with municipality (3 levels), mowing (2 levels) and their interaction as fixed effects and the populations within each municipality as random effects. Over-winter survival of the seeds buried in the soil was analyzed using a factorial ANOVA model where year and municipality were considered fixed effects and populations within a municipality were considered random. Differences in soil nutrient status as well as the growth and reproduction of roadside and cultivated alfalfa populations were compared using one-way ANOVA, considering municipalities as fixed effects and the populations within each municipality as random. In this regard, growth and reproductive data from alfalfa plants collected in seed production fields (number of plants (n) = 50) were compared with roadside alfalfa plants that were not-mowed (n = 116).

All the response variables from this study were analyzed by year (2006, 2007) and municipality (Hanover, MacDonald and Springfield) due to high-level interactions. In each year and municipality, the growth and reproduction of mowed (n: Hanover-45; MacDonald-112 and Springfield-87) and not-mowed (n: Hanover-75; MacDonald-8 and Springfield-33) plants were compared. Data on the survival of newly recruited alfalfa seedlings and adult alfalfa plants were collected at regular intervals. The survivorship of

the seedlings and adult alfalfa plants was analyzed using a repeated measures ANOVA using PROC MIXED of SAS (Littell et al. 1998; Marshall et al. 2008).

A Pearson correlation co-efficient matrix of soil variables (nitrogen, phosphorus, potassium, organic matter, sulphur, zinc and electrical conductivity) and plant dry weight and fecundity was computed using PROC CORR of SAS. To limit Type 1 errors, Bonferroni's family wise error rate adjustment was applied to the significances of the correlation output.. In this analysis, plant dry weight and fecundity data of only the not-mowed adult alfalfa plants (n = 116) were used since these variables were affected by mowing.

4.4 Results

4.4.1 Adult growth, reproduction and survival

For the adult plants, the August data reflected the growth and reproductive attributes over the entire season, and so only the August data are presented. In general, mowing had little impact on growth (plant height, shoots plant^{-1}) and reproductive attributes (racemes plant^{-1}, flowers raceme^{-1}, pod clusters plant^{-1}, pods cluster^{-1}) of roadside alfalfa populations at the end of the season (Table 4.2). No significant differences were detected for total dry biomass production between mowed and not-mowed plants among the municipalities and years (Fig. 4.2). However, variations within municipalities and populations were substantial (Table 4.2; fig. 4.2).

I observed reproductive success in roadside alfalfa (Fig.4.3). Despite similar growth and reproductive attributes between mowed and not-mowed alfalfa plants, fecundity was affected by mowing (Table 4.2; fig. 4.2). In addition, these parameters varied among different years of observation, indicating the significance of temporal effects in determining the growth and reproduction of alfalfa in roadside habitats. Over the term of my study and among all populations, 29% of the mowed and 92% of the not-mowed plants were reproductively successful (Fig. 4.4).

Table 4.2 Mean growth and reproductive attributes of roadside alfalfa populations for mowed and not-mowed plants across three rural municipalities in southern Manitoba, Canada.ǂ‡

Municipality	Plant height (cm)		Number of shoots plant^{-1}		Number of racemes plant^{-1}		Number of flowers raceme^{-1}		Number of pod clusters plant^{-1}		Number of pods cluster^{-1}	
	Mowed†	Not-mowed	Mowed	Not-mowed	Mowed	Not-mowed	Mowed	Not-mowed	Mowed	Not mowed	Mowed	Not mowed
Hanover	76.3 (7.2)	90.2 (9.6)	24.7 (4.5)	15.2 ab* (3.1)	33.1 (14.5)	32.4 (12.6)	8.6 (1.9)	8.1 (0.2)	43.2 (15.2)	76.9 (20.8)	4.7 (0.9)	6.1 (0.2)
MacDonald	64.2 (3.8)	75.7 (14.2)	18.5 (2.5)	9.1 b (0.6)	28.4 (7.2)	20.1 (13.6)	8.8 (0.7)	8.5 (0.5)	18.5 (3.7)	44.3 (44.3)	4.3 (0.2)	2.9 (2.9)
Springfield	62.0 (6.5)	88.5 * (5.7)	16.6 (1.6)	21.4 a (3.2)	21.4 (6.8)	29.9 (6.4)	8.7 (1.1)	8.6 (1.0)	16.5 (4.3)	105.9 (34.4)	4.7 (0.5)	6.4 * (0.8)

ǂData obtained during late-August 2007

‡Sample size (n)-Hanover (mowed-45; not-mowed-75); MacDonald (mowed-112; not-mowed-8); Springfield (mowed-87; not-mowed-33)

†Mowing was carried out between early June and early-July

Values in parenthesis indicate standard errors

Values within each column (within each treatment variable) followed by different letters are significantly different, as determined using Fisher's protected LSD ($\alpha = 0.05$)

*Within each row (municipality), denotes significant differences between mowed and not-mowed treatments for each variable, determined using Fisher's protected LSD ($\alpha = 0.05$)

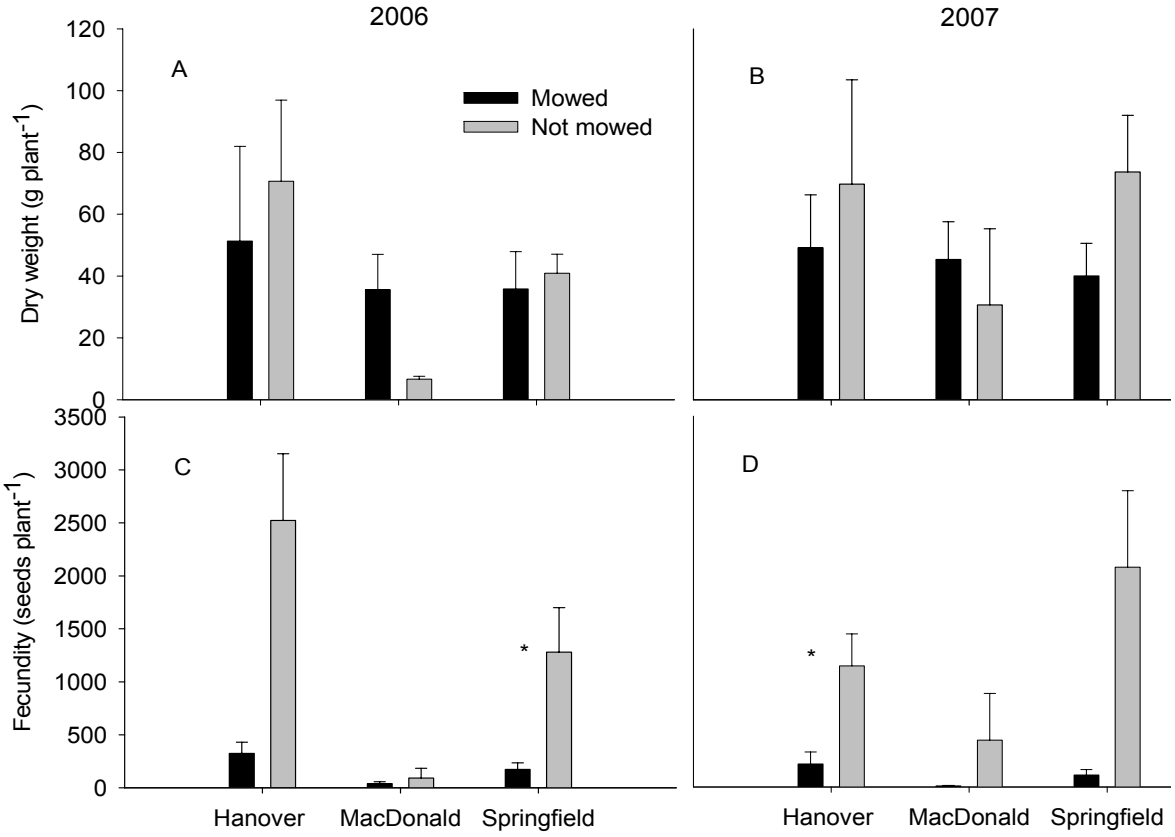

Fig. 4.2 Mean plant dry weight (A, B) and fecundity (C, D) of roadside alfalfa plants under mowed and not-mowed conditions across selected municipalities (Hanover, MacDonald and Springfield) in southern Manitoba, Canada, in 2006 and 2007. *denontes significant difference between treatment means, determined using Fisher's protected LSD ($\alpha = 0.05$). Bars above columns represent standard errors of the means.

Fig. 4.3 Reproductive success in roadside alfalfa populations in southern Manitoba, Canada

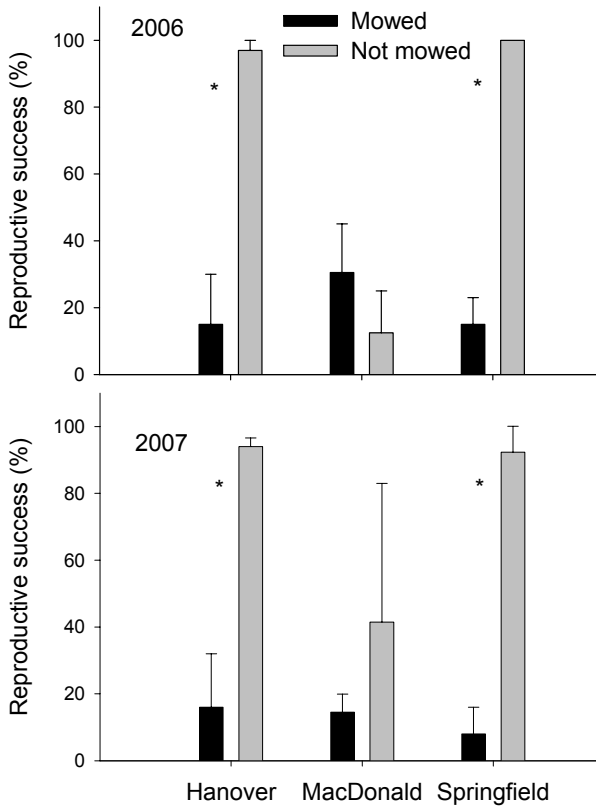

Fig. 4.4 Mean reproductive success of mowed and not-mowed alfalfa plants in roadside habitats in selected rural municipalities (Hanover, MacDonald and Springfield) in southern Manitoba, Canada in 2006 and 2007. *denontes significant difference between treatment means, determined using Fisher's protected LSD ($\alpha = 0.05$). Bars above columns represent standard errors of the means.

Average fecundity ranged from 10 to 325 seeds plant^{-1} for mowed plants and from 92 to 2523 seed plant^{-1} for plants that were not-mowed (Fig. 4.2). In general, the reproductive success and fecundity of roadside alfalfa did not follow a constant pattern among municipalities or between years.

I observed high levels of adult winter survival in roadside alfalfa populations. During the course of study (from May 2006 to May 2009), average adult mortality ranged between 13 and 22% across municipalities (Fig. 4.5). Generally, winter mortality was greater than summer mortality. There were significant differences in the level of mortality among municipalities. Adult mortality was significantly greater in MacDonald in May and August 2009 ($P \leq 0.05$) but was comparable among the locations at other times of the year (Fig. 4.5). There was no significant difference in proportional adult mortality between mowed and not-mowed plants within each population.

4.4.2 Soil seedbank

I found substantial alfalfa seed in the seedbank in roadside habitats. Seedbank levels represented only a fraction of the fecundity observed in adult plants. Total seedbank levels varied greatly between mowed and not-mowed areas and among municipalities (Fig. 4.6). Average seedbank densities ranged from 15 to 187 seeds m^{-2} in the mowed area and from 59 to 208 seeds m^{-2} in areas not-mowed (Fig. 4.6). Elutriation and tetrazolium testing revealed the presence of about 38% more seeds in the roadside alfalfa seedbank that were viable but ungerminable despite being subjected to repeated freeze-thaw cycles. Overall among all populations, there was a 66% increase in seedbank levels in 2007 versus 2006 and the increase was greatest in mowed areas where it increased from 16 seeds m^{-2} in 2006 to 116 m^{-2} in 2007.

Seed mortality over winter contributed little to reductions in soil seedbank levels. On average, the level of seed mortality over winter ranged from 21 to 24% in 2006 and from 14 to 16% in 2007 (Fig. 4.7). Differences in seed mortality over winter between years were significant ($P \leq 0.05$) but the differences among the municipalities within each year were not significant, indicating that year had a greater effect than site on alfalfa seed winter survival.

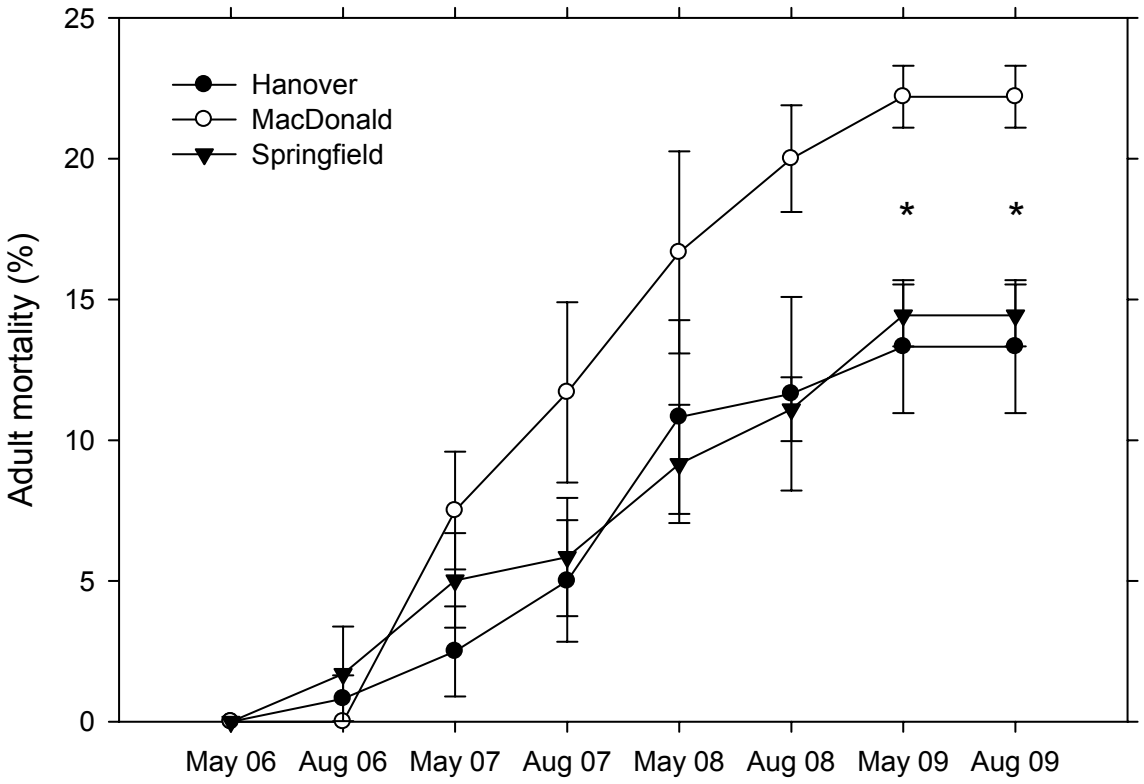

Fig. 4.5 Mean mortality rate of adult alfalfa plants in roadside habitats in three rural municipalities (Hanover, MacDonald and Springfield) in southern Manitoba, Canada, as revealed by repeated measures ANOVA. *Denotes significant differences in mortality rates among the municipalities, determined using Fisher's protected LSD ($\alpha = 0.05$). Bars above and below the data points represent standard errors of the means.

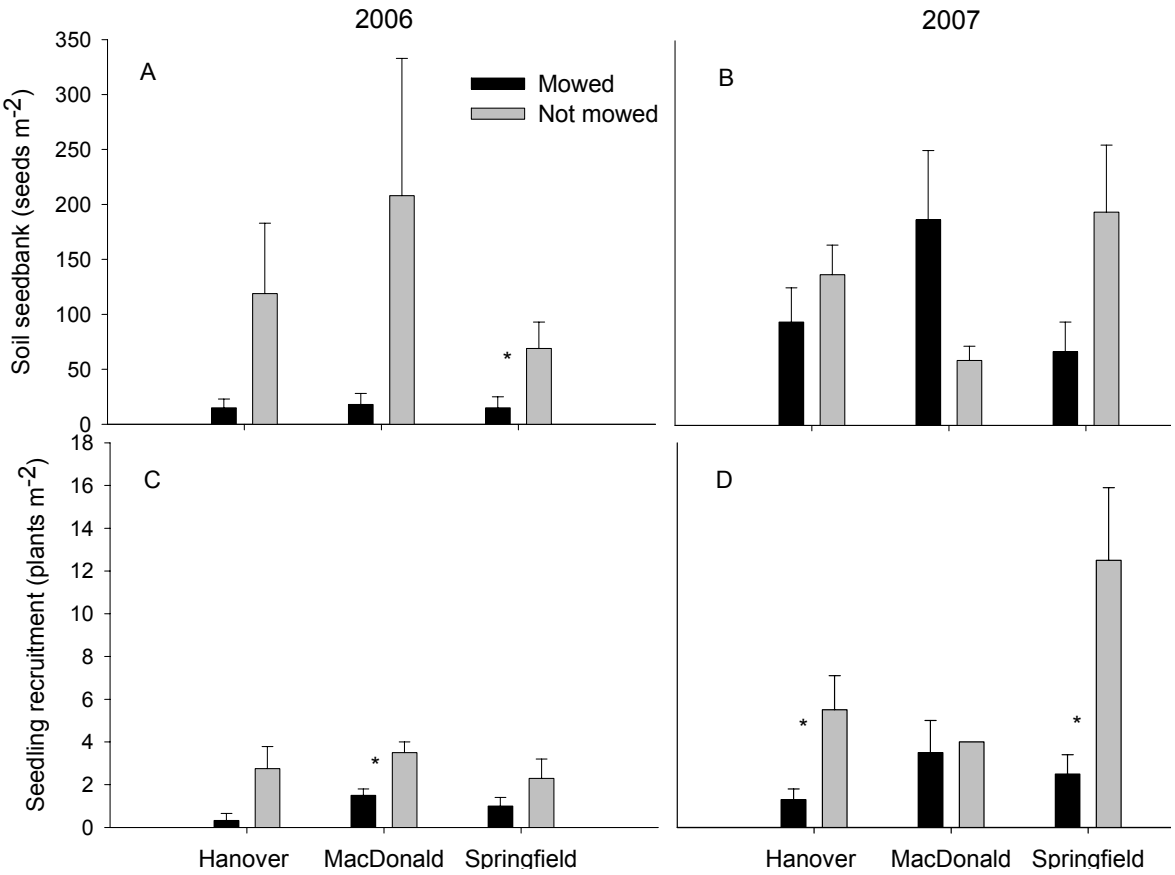

Fig. 4.6 Soil seedbank (A, B) and seedling recruitment (C, D) in roadside alfalfa populations under mowed and not-mowed conditions across different municipalities (Hanover, MacDonald and Springfield) in southern Manitoba, Canada, in 2006 and 2007. *denontes significant difference between treatment means, determined using Fisher's protected LSD ($\alpha = 0.05$). Bars above columns represent standard errors of the means.

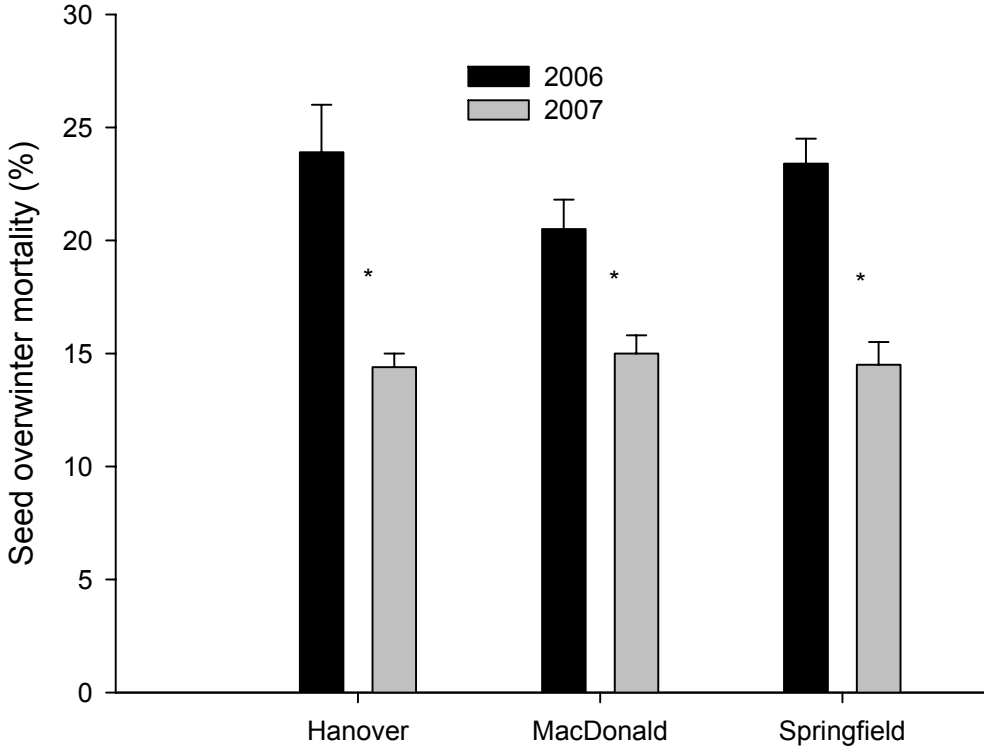

Fig. 4.7 Winter mortality of alfalfa seeds in roadside habitats in selected rural municipalities (Hanover, MacDonald and Springfield) in southern Manitoba, Canada, as revealed by seed bag experiments (2006 and 2007). *denotes significant difference between treatment means, determined using Fisher's protected LSD ($\alpha = 0.05$). Bars above columns represent standard errors of the means.

4.4.3 Seedling recruitment

Alfalfa seedlings recruited successfully in roadside habitats (Fig. 4.8). On average, seasonal seedling recruitment represented only about 3% of the germinable seedbank. There was a relationship between adult seed production levels, seedbank size and seedling recruitment and mowing affected the number of seedlings recruited because it reduced fecundity (Fig. 4.6). The effect of mowing on total seedling recruitment was significant in MacDonald in 2006 ($P \leq 0.01$) and in Hanover and Springfield in 2007 ($P \leq 0.05$) (Fig. 4.6). Within year no significant differences were detected for total seedling recruitment among the municipalities but variation within municipality was substantial. Significant year effects on seedling recruitment were detected in Springfield ($P \leq 0.05$) where the recruitment was more than 350% greater in 2007 than in 2006 (Fig. 4.6). Year effects were not significant in the other municipalities even though increases in recruitment between years were 120% and 50%, respectively, in Hanover and MacDonald.

There was a general increase in seedling mortality over the course of my study and two years after recruitment only 16% of the seedlings survived on average (Fig. 4.9). Seedling mortality rates were greatest between August 2007 and May 2008, suggesting that winter had a greater impact on the survival of the newly recruited seedlings versus the winter of 2008-2009. There was significantly greater seedling mortality in MacDonald in July 2007 ($P \leq 0.05$) but all other repeated measures results were not significant (Fig. 4.9).

4.4.4 Comparison of roadside and cultivated alfalfa

Roadside and cultivated alfalfa significantly differed in shoot production and fecundity (Table 4.3). While the average number of shoots produced plant^{-1} was significantly greater in roadside populations, fecundity was significantly greater in cultivated alfalfa (138% greater) than roadside alfalfa (Table 4.3). Roadside and cultivated alfalfa were comparable for other attributes including dry biomass production, despite significant differences in soil nutrient status between these two environments (Table 4.4). Correlation analysis indicated that dry biomass production and fecundity of roadside alfalfa showed a negative relationship with soil N, P, K and EC (Table 4.5).

Fig. 4.8 Recruitment of alfalfa seedlings from roadside seedbanks in southern Manitoba, Canada

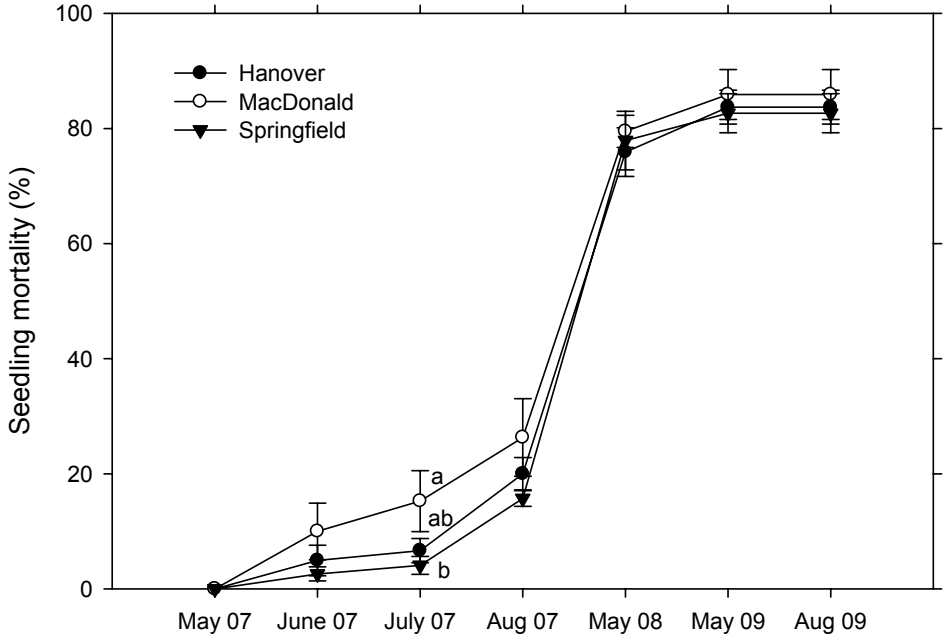

Fig. 4.9 Alfalfa seedling mortality in roadside habitats in southern Manitoba, Canada, as revealed by repeated measures ANOVA. Seedling mortality rates were significantly different among the municipalities in July 2007 ($P \leq 0.05$). Treatment differences are denoted by the letters a, b; Bars above and below the data points represent standard error of mean.

Table 4.3 Comparison of the growth and reproductive attributes for roadside and cultivated alfalfa populations in southern Manitoba, Canada*

Parameter	Roadside population (n = 116)	Cultivated population (n = 50)
Plant height (cm)	86.8 (2.4)	97.2 (3.0)
Number of shoots plant^{-1}	16.6 (2.2) **a**	11.9 (1.3) **b**
Number of racemes plant^{-1}	28.9 (5.7)	30.0 (7.1)
Number of flowers raceme^{-1}	8.5 (0.4)	8.1 (1.4)
Number of pod clusters plant^{-1}	82.0 (17.8)	101.0 (10.8)
Number of pods cluster^{-1}	6.0 (0.3)	7.1 (0.5)
Dry weight (g plant^{-1})	63.3 (15.5)	65.1 (3.2)
Fecundity (seeds plant^{-1})	1370 (360) **b**	3274 (283) **a**

*Data obtained in August 2007

Values in parenthesis indicate standard errors

Values within each row not followed by the same letter are significantly different, as determined using Fisher's protected LSD ($\alpha = 0.05$)

Table 4.4 Soil nutrient status for roadside habitats and cultivated fields in rural municipalities in southern Manitoba, Canada.

Compound	Roadside soils			Cultivated field
	Hanover	MacDonald	Springfield	
N (kg ha^{-1})	17.0 (8.9) **bc**	30.3 (4.3) **ab**	6.5 (1.7) **c**	35.3 (5.8) **a**
P (ppm)	6.5 (1.2) **b**	18.3 (3.3) **b**	11.5 (2.6) **b**	42.3 (8.6) **a**
K (ppm)	224.3 (72.4) **b**	308.5 (74.1) **b**	258.5 (40.3) **b**	504.7 (44.8) **a**
OM (%)	4.1 (0.6) **b**	3.7 (0.4) **b**	3.2 (0.5) **b**	6.2 (0.5) **a**
S (kg ha^{-1})	23.5 (5.5)	37.0 (6.1)	21.0 (1.9)	32.2 (9.2)
Zn (ppm)	1.26 (0.13)	1.17 (0.13)	1.15 (0.12)	1.25 (0.10)
EC (mmho cm^{-1})	0.35 (0.10) **b**	0.48 (0.09) **ab**	0.34 (0.04) **b**	0.71 (0.06) **a**

N-nitrogen; P-phosphorus; K-potassium; OM – organic matter; S-sulphur; Zn-zinc; EC-electrical conductivity

Values in parenthesis indicate standard errors

Values within each row not followed by the same letter are significantly different, as determined using Fisher's protected LSD ($\alpha = 0.05$)

Table 4.5 Correlation coefficient matrix for soil nutrient status, dry matter production and reproductive output of alfalfa in roadside habitats in southern Manitoba, Canada.

	N	P	K	OM	S	Zn	EC	DW	FE
N	1								
P	-0.25	1							
K	0.85 *	-0.1	1						
OM	0.81 *	-0.44 *	0.72 *	1					
S	-0.09	0.34 *	-0.33 *	0.08	1				
Zn	0.53 *	-0.31 *	0.43 *	0.49 *	-0.11	1			
EC	0.95 *	-0.31 *	0.95 *	0.84 *	-0.23	0.48 *	1		
DW	-0.21	-0.20	-0.22	-0.06	-0.15	-0.08	-0.2	1	
FE	-0.24	-0.25	-0.32 *	-0.1	-0.08	-0.08	-0.27	0.62 *	1

N-nitrogen (kg ha^{-1}); P-phosphorus (ppm); K-potassium (ppm); OM – organic matter (%); S-sulphur (kg ha^{-1}); Zn-zinc (ppm); EC-electrical conductivity (mmho cm^{-1}); DW-plant dry weight (g); FE- fecundity (seeds $plant^{-1}$).

*The effects are significant at $P \leq 0.005$ (determined using Bonferroni's family wise error correction).

4.5 Discussion

The demography of roadside alfalfa indicates that alfalfa is capable of establishing self-perpetuating feral populations in roadside habitats with key elements including persistent seedbanks, successful seedling recruitment and adult reproductive success. Feral alfalfa populations adapt to roadside habitats. Roadside habitats are typically characterized by poor nutrient conditions and a correlation analysis showed that alfalfa grows well despite the limited nutrient levels in these habitats. This corresponds to the fact that alfalfa fixes its own nitrogen and develops a deep tap root system that is very efficient at extracting nutrients from depths not available to other typical roadside vegetation (Bolton et al.1972; Horton and Hart 1998). My study may suggest the importance of root traits on the adaptability and competitive ability of alfalfa in low-nutrient environments. This corroborates the findings of Funk and Vitousek (2007) and Drenovsky et al. (2008).

Mowing affected the reproductive success and fecundity of roadside alfalfa plants because the length of the period between mowings (first and second round) was often insufficient for plants to reach full reproductive maturity. In the rural municipalities where my study took place, the pattern and timing of mowing varies in space and time (Moffat B. Springfield municipality manager, Personal communication) and the potential effect on the reproductive success of roadside alfalfa populations can vary tremendously. The fate of low seed production in mowed plants and their importance to the population dynamics is not clear. In this respect, the plants that are not-mowed may form a more dynamic population.

Low fecundity in roadside alfalfa (in not-mowed plants at the time of harvesting) relative to cultivated alfalfa may be attributed to the delayed maturity of roadside populations (over the cultivated fields I observed) as a result of competition with surrounding vegetation (personal observation). In this regard, studies have shown that the maturity of alfalfa is delayed under competition for light, moisture and nutrients (McCordick et al. 2008; McGraw et al. 2008). In addition, the number of seeds pod^{-1} could have been less in the feral populations, which I did not measure. In alfalfa, the number of seeds produced pod^{-1} can be affected by a combination of variables including type of pollination (cross vs self), growing conditions and genotype as has been shown by

Pedersen and Nye (1962) and Strickler (1999). Furthermore, lygus bugs (*Lygus* spp.) have been known to reduce the number of seeds produced per pod (Soroka 1991; MAFRI 2009). Nevertheless, the levels of fecundity I found in roadside alfalfa populations appear to be sufficient to perpetuate these populations.

The difference between seed production levels and the much lower seed densities I found in the roadside seedbanks did not appear to be due to a loss of viability from over-wintering. It is likely that seed predation levels could be very high in roadside habitats, especially for high quality protein rich alfalfa seeds in low density feral alfalfa populations. However, the results from the seed over-wintering study may not be fully representative of possible scenarios for seed mortality over-winter given that I used only one burial depth. Seed placed on the surface may have been subjected to much harsher conditions with a greater effect on viability. Nevertheless, the seed bag study was novel in these conditions and it did provide novel baseline data on seed survival. A substantial portion of the seeds I retrieved from my roadside seedbank samples had a very hard seed coat and were viable but not germinable. This suggests that alfalfa can form a persistent seedbank in roadside habitats. Alfalfa seed dormancy has been demonstrated elsewhere (Wilton et al. 1978; Rincker 1983) but not for seed extracted from roadside seedbanks. Occurrence of seed dormancy is a key attribute in feral populations because it may aid their persistence in unmanaged habitats (Gressel 2005b).

High levels of winter mortality in seedlings recruited immediately around established alfalfa plants may have reflected by auto-allelopathy (Jennings and Nelson 1991). The effects of allelopathy and competition appear to be additive (Rice 1984; Jennings and Nelson 2002). Alfalfa seedlings are sensitive to competition (Wilson and Burgener 2009) and severe shading can affect the development of roots (Hall 1974) and the initiation of crown buds in seedlings (Chamblee and Lovvorn 1953) (Fig. 4.10 vs. fig. 4.8). Crown buds are essential for the winter survival of alfalfa seedlings (Cunningham and Volenec 1998). Nevertheless, I observed a considerable number of alfalfa seedlings to survive for two years and evidence from the literature suggests that seedlings that survive for two years will most likely continue to survive (Rumbaugh 1982). I noticed some alfalfa seedling recruitment in the areas away from established alfalfa plants.

Fig. 4.10 Development of weak seedlings as a result of auto-allelopathy/competition when recruitment occurs in proximity to the established alfalfa plants

These seedlings were more vigorous than the seedlings that recruited around the established alfalfa plants (personal observation). Dispersal of alfalfa seed away from established feral mother plants may be an important factor influencing the dynamics, spread and persistence of roadside alfalfa populations.

High fecundity may considerably decrease the extinction risk of many species (Kery et al. 2000). I noted great variation in the fecundity of roadside feral populations. Nevertheless, the long life span (>10 years) (Berdahl et al. 1989) of alfalfa and the existence of seed dormancy can have a positive impact on the persistence of roadside populations irrespective of reproductive output and dispersal capacity (Honnay and Bossuyt 2005; Bossuyt and Honnay 2006). The long life span in perennial herbaceous plants buffers temporal fluctuations in population size and thereby increases population stability (Garcia et al. 2008). I also observed high levels of winter survival in adult alfalfa plants in roadside habitats. As such, the levels of fecundity, seedling recruitment and survival I detected in roadside alfalfa populations may be sufficient for long-term persistence of these populations.

Persistence of alfalfa populations in roadside habitats has implications for novel trait confinement because feral populations may act as sources and sinks for novel traits. I present the evidence that roadside alfalfa populations are capable of establishing self-sustaining feral populations. If there is a necessity to confine novel traits in alfalfa, then the location and management of roadside feral populations need to be considered in confinement protocols and practices. Timely mowing can prevent the reproductive success of feral populations but the results from my study suggest that it would not likely be sufficient to drive these populations to extinction in the short-term. Population dynamic models can be helpful in predicting the long-term dynamics of feral populations and the findings from this experiment can provide baseline information for these models.

5.0 Establishment of Alfalfa Under Different Dispersal Times and Disturbance Treatments in a Semi-Natural Habitat

5.1 Abstract

Alfalfa (*Medicago sativa* L.) is an important forage crop that is widely adapted to different geographical regions of the world. Because alfalfa cultivars are typically selected for their persistence in competitive environments, they are often capable of establishing and surviving in unmanaged habitats such as the roadsides. However, little is known about the degree of establishment particularly when subjected to disturbances. The overall objective was to estimate the establishment of alfalfa in a grass sward subjected to different disturbances. The study was set-up in a split plot design with two main plots (spring and fall seed dispersal) and five sub plots (mowing, sward scarring, herbicide spray, tilled seedbed and undisturbed control). Alfalfa recruitment success ranged between 0.5% and 9.7% across treatments and the level of recruitment was influenced by the sward cover. Plant density in fall-dispersed plots was 82% lower than in spring-dispersed plots. Sward scarring reduced the density of alfalfa <50% of the initial density. However, low plant density was compensated over time by increased numbers of shoots and racemes plant^{-1} and thereby increased seed output. Herbicide application effectively controlled all emerged alfalfa plants but some dispersed seeds remained dormant forming a seedbank. Although mowing did not kill alfalfa plants, mowed plants did not produce seeds. My study shows that alfalfa is readily capable of establishing in roadside habitats and rapidly recovering from moderate level disturbances. Mowing may be useful in restricting the reproductive success and thereby population growth.

5.2 Introduction

Agriculture started about 10,000 years ago with crop domestication, a process aimed at selecting preferable traits for human use from wild plants (Harlan 1992; Doebley et al. 2006). Domestication was followed by continuous crop evolution, a process facilitated by plant breeding programs and novel production practices (Frary and Douanlar 2003). Both domestication and crop evolution favored the differentiation of modern cultivars from their wild progenitors for specific traits (Hawkes 1983) and such traits collectively make up the "domestication syndrome" (Harlan 1992). This process resulted in some crops being incapable of surviving outside of managed cultivation (Doebley et al. 2006). However, some crops, forages for example, are only partially domesticated and vary in the make up of their domestication syndrome when compared to more domesticated crops including typical grains (Gepts 2002).

Alfalfa (*Medicago sativa* L.) is a common and important forage crop domesticated as early as 5000 BC in the Near East (Muller et al. 2005). Because alfalfa cultivars are often selected for their persistence and ability to withstand inter-specific competition in grass mixtures, many of the traits that support the adaptation of alfalfa as a cultivated crop may also favor their escape and establishment in natural and semi-natural habitats. In particular, high genetic diversity, perenniality, fast regrowth potential, symbiotic nitrogen fixation, deep tap roots, and drought and cold tolerance are traits of alfalfa that may contribute to persistence (reviewed in Bagavathiannan and Van Acker 2009) (Chapter 3.0). Alfalfa has also been shown to be very competitive, particularly with grasses, and often out-competes perennial grasses in mixtures (Chamblee and Collins 1988; Kilcher 2006). In addition, alfalfa has a hard seed coat and alfalfa seeds can persist in the soil for several years (Bass et al. 1988). With these traits, alfalfa is inherently equipped to escape and persist in unmanaged habitats.

Alfalfa is the first perennial, highly outcrossing, insect pollinated crop species genetically modified for the production of novel agronomic (CFIA 2005) and industrial (Sparrow et al. 2007) traits. Alfalfa populations are common in roadsides, field edges and abandoned lands in alfalfa growing regions (Kendrick et al. 2005; Prosperi et al. 2006; Bagavathiannan et al. 2008) (Chapter 6.0). My works indicate that alfalfa is capable of

establishing self-sustaining feral populations in unmanaged habitats (Bagavathiannan et al. 2009) (Chapter 4.0). In addition, alfalfa populations can displace native vegetation and reduce species diversity in natural and semi-natural habitats (SWSS 1998). Feral populations can serve as reservoirs for novel traits (Bagavathiannan and Van Acker 2008b) (Chapter 6.0). As such, the ability of alfalfa to establish in unmanaged habitats has implications for novel trait confinement (CAST 2008).

Seed escape in road verges may occur during specific periods in spring and fall, facilitated by seeding and harvesting operations, respectively. The Northern Great Plains of North America, and in particular western Canada is characterized by extreme weather conditions with cold winters and hot summers and it is not clear whether the time of seed dispersal has any effect on the establishment of alfalfa. In addition, road verges are often subjected to disturbances caused by mowing, herbicide spray, and traffic. The impact of such disturbances on the establishment and persistence of alfalfa is not well documented. No study to-date has investigated alfalfa establishment outside of cultivated fields in natural or semi-natural habitats. The overall objective of this study was to quantify establishment of alfalfa in a grass sward as influenced by time of seed dispersal and disturbance regime. The following specific questions were addressed. (1) What is the level of establishment of alfalfa in a grass sward? (2) What management approaches are likely to restrict alfalfa population growth? (3) What is the effect of time of seed dispersal on alfalfa population establishment? (4) How is establishment affected by disturbances?

5.3 Materials and methods

5.3.1 Study site

The experiments were conducted between May 2006 and September 2008 at 'The Point' a field research facility of the University of Manitoba located on campus in Winnipeg, Canada (50°38'N 96°19'W). The climate is temperate with extreme winters and warm summers (Fig. 5.1). The study was conducted in a grass sward similar to roadside vegetation cover. The site was also suitable with respect to safety and accessibility.

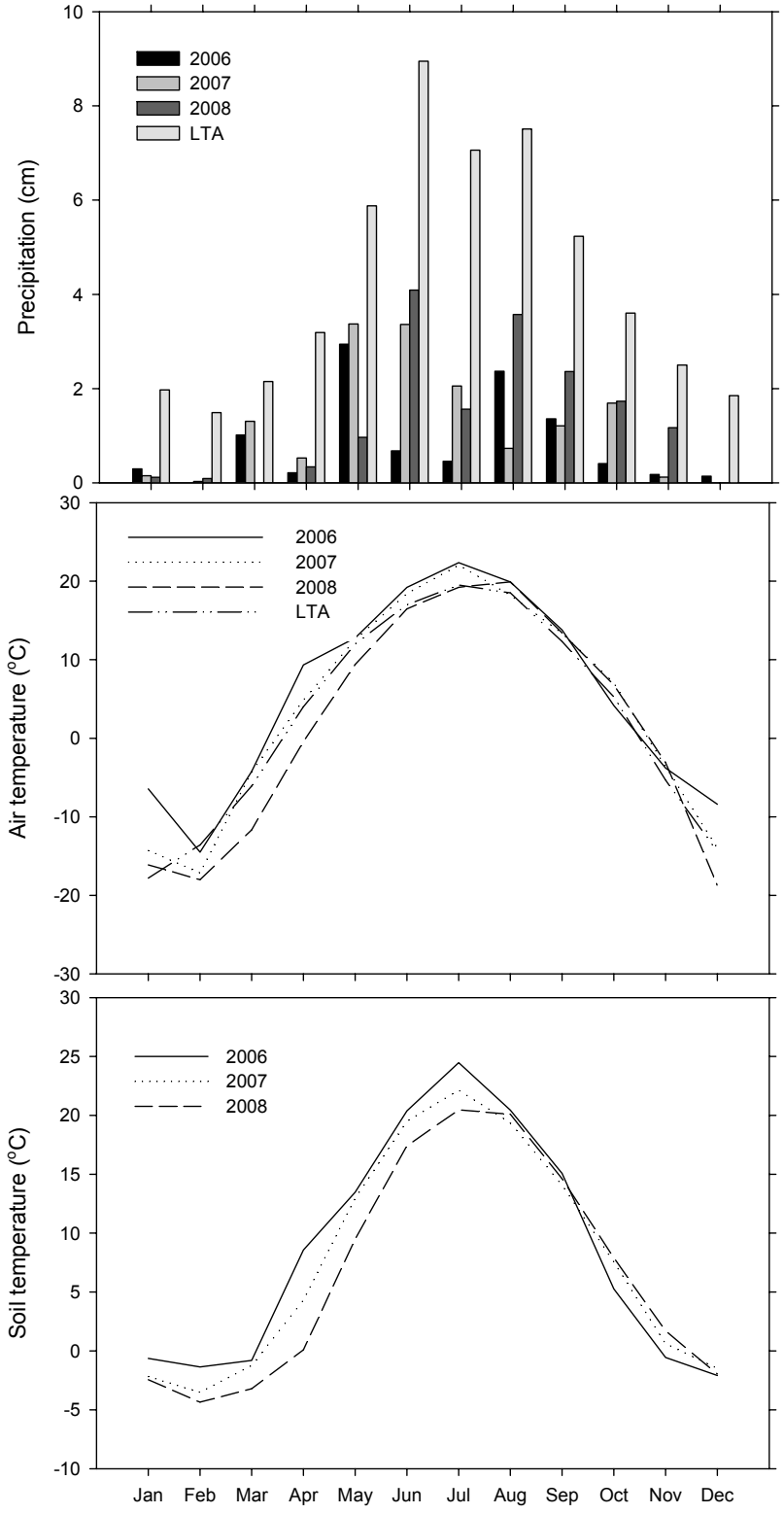

Fig. 5.1 Weather conditions of the experimental site during the three years of study. LTA - Long term average (30 years)

The plant community at the site was dominated by *Festuca rubra* L. (red fescue), *Festuca arundinacea* L. (tall fescue), *Poa pratensis* L. (Kentucky blue grass), *Elymus repens* L. (quack grass), *Trifolium repens* L. (white clover), *Taraxacum officinale* L. (dandelion), *Cirsium arvense* (L) Scop. (Canada thistle), *Sonchus oleraceus* L. (sowthistle) and *Tragopogon dubius* Scop. (goat's-beard). The ground cover of the sward ranged between 80% and 95%. Prior to the experiment, the sward was subjected to regular mowing, a regime that resembled roadside mowing practices in the region.

5.3.2 Experimental design

I studied the establishment of alfalfa in a two factor, multi-year field experiment. The experiment consisted of a factorial combination of time of seed dispersal and disturbance regimes in a split-plot design with four replicates. Time of seed dispersal was assigned to the main plots and the disturbance regimes were assigned to subplots, each subplot being 2m x 3m (Fig. 5.2). To capture the spatial and temporal variation in population establishment, the study was established (repeated) in two consecutive years. Experiment I was initiated in 2006 and alfalfa populations were studied for three years (until 2008), while experiment II was established in an adjacent sward area in 2007 and followed for only two years (2007 and 2008). Initial ground vegetation cover was between 80 and 90%, and 90% to 95% in experiments I and II, respectively. These swards were chosen randomly with no bias towards the per cent vegetation cover.

5.3.3 Treatment details

The main plot treatments consisted of spring (early May) and fall (early September) seed dispersal. These two times represent the time of potential seed spill or dispersal that may occur at roadside habitats as a result of commercial alfalfa seeding and harvesting operations. The five sub-plot treatments included mowing, sward scarring (tilling using a tine cultivator), herbicide application, bare seedbed (well-tilled) and undisturbed control. Alfalfa seeds (variety: AC Caribou) were manually dispersed at a rate of 1500 seeds m^{-2} in each sub-plot before implementation of treatments. One exception to this was the well-tilled seedbed where the seeds were dispersed after tilling was carried out.

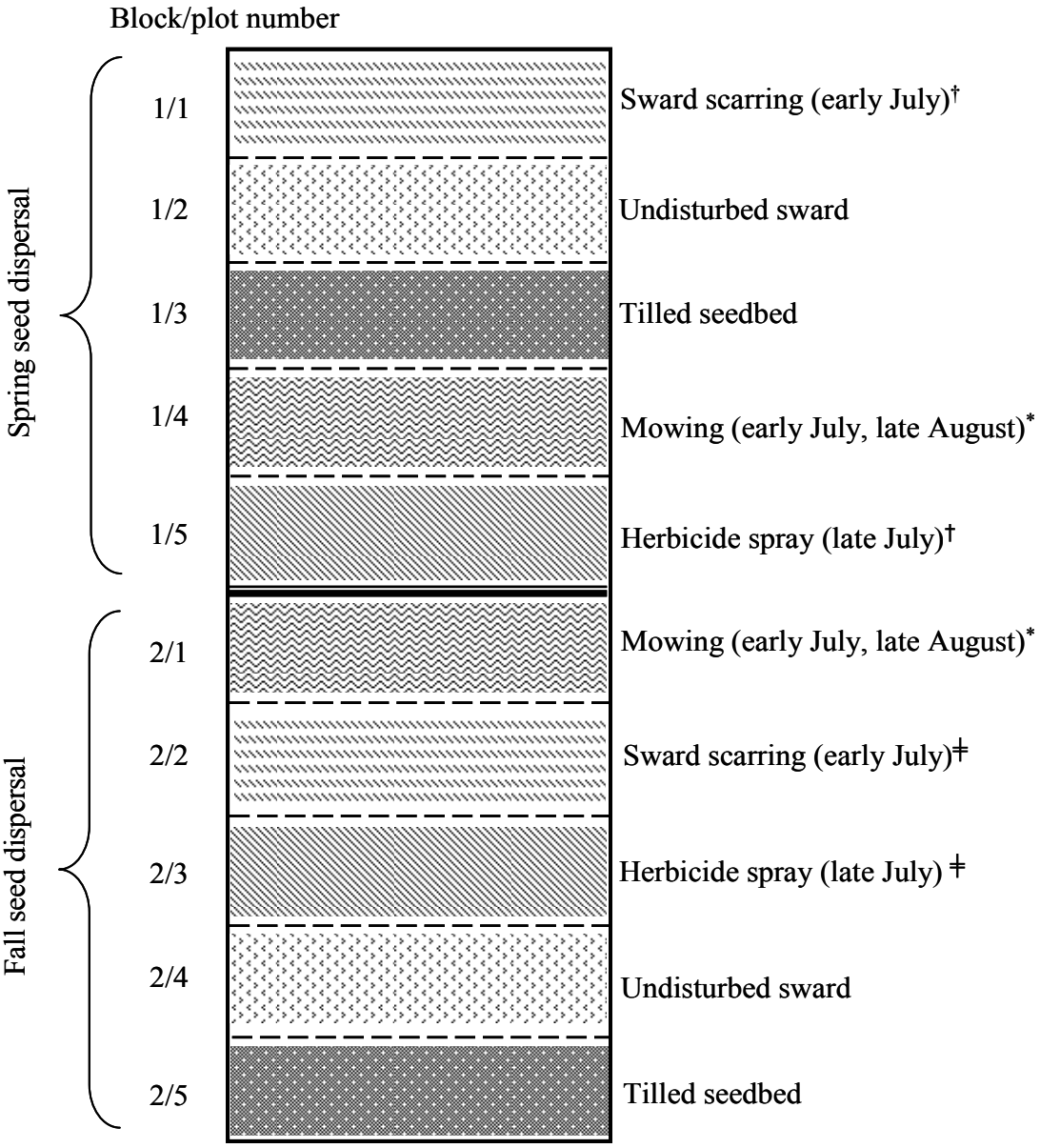

Fig. 5.2 Map of the study site showing the spatial arrangement of experimental units

† Treatment imposed only once in the year of establishment

ǂ Treatment imposed only once in the year following the year of establishment

* Treatment imposed twice every year

In the mowing treatment, plants were mowed at a height of 20cm twice each year; once in the first week of July and again in late August. These timings resemble typical mowing timings for rural municipalities in the region. A herbicide mix consisting of 2,4-D (2,4-dichlorophenoxy acetic acid) and dicamba is often sprayed along roadsides anytime from early July to early August to control thistles and other noxious broad leaved vegetation. Therefore, the commercial products 2,4-D amine and Banvel II were mixed and sprayed at a rate of 1.5 kg a.e.ha^{-1} and 384 g a.i. ha^{-1}, respectively (a rate used by rural municipalities in the region) (Ammeter, M., MacDonald municipality weed supervisor, personal communication). In my study, the herbicide was applied using a bicycle sprayer at a pressure of 310 kpa using a flat fan nozzle. Herbicide was applied only once in late July (in the year of establishment for spring dispersed plots and in the following summer for fall dispersed plots). Sward scarring was carried out using a tine cultivator to represent random disturbances caused by agricultural implements pulled across the sward, snow plow, road grater or roadside traffic. The soil was inverted up to a depth of 15cm and about 50% of the plant cover was disturbed in treated plots. Sward scarring was carried out only once in early July of the year of establishment for spring dispersed plots and the following summer for fall dispersed plots. The well-tilled seedbed treatment represented a cultivated field and was accomplished using a roto-tiller on the sward prior to alfalfa seed dispersal). Treatments also included an untreated control. Treatments were identical in experiments I and II.

5.3.4 Data collection

Plots were observed once a month between May and August each year from 2006 to 2008. Observations in the spring seeded plots (early May seed dispersal) commenced in the same year, whereas in the fall seeded plots (early September seed dispersal), measurements were collected beginning the following May.

Plant height, number of shoots $plant^{-1}$ and racemes and pod clusters $plant^{-1}$ were measured on five randomly selected alfalfa plants within each sub-plot, while observation on the number of recruited plants m^{-2}, plant dry biomass m^{-2} and fecundity (i.e seed output) m^{-2} were obtained from four randomly placed quadrats (25 x 25 cm) within each

sub-plot. Neither the study plants nor the quadrats were permanently marked and thus the samples were drawn randomly from each sub-plot at each time of observation.

Plant height (cm) was measured on the central shoot of each study plant. Number of flower racemes and pod clusters were counted on the central shoot of each study plant and then multiplied by the total number of shoots for each plant. Total number of alfalfa plants recruited in each of the four quadrats was counted and then plant population m^{-2} was calculated for each sub-plot.

The plants within each of the four quadrats per sub-plot were harvested during late August, dried on a hot-air bed until equilibrium and the whole plant dry biomass m^{-2} (g) was determined. The plants were threshed using a mechanical thresher and mature seeds were separated using a pneumatic seed blower. From this, total seed output (fecundity m^{-2}) was determined. Fecundity $plant^{-1}$ was also calculated based on the number of plants harvested from each quatrat. In addition, weight of thousand mature seeds (g) harvested from each sub-plot was also determined.

5.3.5 Data analysis

Data were analyzed using a Mixed Model analysis (PROC MIXED) (Littell et al. 1996) using the Statistical Analysis Software (SAS) version 9.1 (SAS Institute, 2003). Dependent variables were examined using a two-way analysis of variance (ANOVA). The year of observation, time of seed dispersal, disturbance treatments and their respective interactions were considered as fixed effects while replication and interaction of the time of seed dispersal x replication (main plot error) were regarded as random effects. Prior to ANOVA, outliers were removed based on the studentized residual values (Lund 1975) and normality of the residuals was confirmed using the Kolmogorov-Smirnov test. Variables with non-normal distribution were transformed using either a log transformation or square root transformation to conform to the assumptions of ANOVA. The specific transformation used in each case is indicated in the respective tables and figures. Mean separation was carried out using Fisher's protected Least Significant Difference (LSD) at $\alpha = 0.05$, using the PDMIX800 macro in SAS (Saxton 1998). For transformed data, the calculated means were back-transformed and presented. Pearson

correlation co-efficient matrix of the variables plant height (cm), shoots plant^{-1}, racemes plant^{-1}, pods plant^{-1}, fecundity plant^{-1}, thousand seed weight, plant density m^{-2}, dry biomass m^{-2} and fecundity m^{-2} was computed using PROC CORR of SAS. Bonferroni's family wise correction was applied to the correlation output, while determining the significance of the effects. A linear regression analysis was also carried out on the survivorship data to estimate the rate of change in survivorship over time. Data within years of observation and times of seed dispersal were analyzed separately due to significant higher-order interactions.

5.4 Results

Because the data obtained during August reflected the plant density, growth and reproductive attributes of alfala over the entire season, only the August data is presented.

5.4.1 Plant density

Alfalfa successfully recruited and established in the grass sward (Fig. 5.3). The recruitment success in undisturbed swards ranged between 0.5% and 9.7% (of the total number of seeds dispersed) across the times of dispersal over both experiments (Tables 5.1 and 5.2). Average density (plants m^{-2}) of alfalfa in undisturbed treatments in experiment II was 51% less than in experiment I. Fall dispersal resulted in lower establishment than spring dispersal. In the year of establishment and averaged over experiments, fall dispersal establishment levels were only 18% of spring dispersal establishment levels. Plant densities were affected substantially by disturbance treatments (Tables 5.1 and 5.2). In spring seeded plots, substantially greater densities were recorded in the well-tilled seed bed with over two-fold greater density when compared to undisturbed plots. Fall dispersal in the well-tilled beds resulted in lower establishment than spring dispersal (fall dispersal establishment being only 5.5% on average of spring dispersal establishment in this regard). Mowing did not substantially affect plant density. Sward scarring resulted in reduced establishment compared to undisturbed plots. Herbicide (2,4-D) applications effectively controlled alfalfa populations.

Fig. 5.3 Establishment success of alfalfa dispersed in a dense grass sward

Table 5.1 Average values of growth and reproductive variables of alfalfa as affected by seed dispersal and disturbance (experiment I).

Treatment	Plant density (m^{-2})	Plant height (cm)	Shoots $plant^{-1}$	Racemes $plant^{-1}$	Pods $plant^{-1}$	Dry biomass (gm^{-2})	Fecun-dity (gm^{-2})	TSW (g)
Spring seed dispersal								
Year I (2006)								
Control	146 b[a,b]	19.5 b	1.5 b	32.7	28.3 a	408 b	4.9 b[c]	1.54
Herbicide	na	na	na	na	na	na	na	na
Mowing	147 b	21.7 b	1.6 b	33.1	na	306 b	na	na
Scarring	70 c	20.5 b	1.7 ab	33.9	23.5 b	253 b	2.7 c	1.47
Seedbed	319 a	31.2 a	2.3 a	31.0	26.3 ab	1410 a	20.1 a	1.50
Year II (2007)								
Control	117 b	73.1 ab	4.4 bc	91.9 b	518.7 b	738 b	46.3 b	1.25
Herbicide	4 d	56.1 cd	2.8 c	86.3 b	329.4 bc	46 d	0.2 c	1.69
Mowing	131 b	49.2 d	5.7 b	104.4 b	86.6 c	529 c	na	na
Scarring	54 c	64.6 bc	4.3 bc	113.1 b	470.5 b	737 b	51.5 b	1.33
Seedbed	187 a	84.3 a	8.8 a	228.8 a	990.4 a	2091 a	69.1 a	1.31
Year III (2008)								
Control	62 b	111.7 ab	13.1 ab	574.4 ab	482.5 a	2258 b	150.2 a	1.65 b
Herbicide	6 d	96.1 b	10.1 abc	432.7 bc	331.0 ab	237 d	15.3 b	2.10 a
Mowing	61 b	43.9 c	6.4 bc	20.0 d	57.4 b	821 c	na	na
Scarring	30 c	125.6 a	17.4 a	717.9 a	609.2 a	2490 b	164.3 a	1.70 b
Seedbed	103 a	97.0 b	4.5 c	250.9 cd	369.0 ab	3143 a	212.5 a	1.66 b
Fall seed dispersal								
Year I (2006)								
Control	41 a	34.9 bc[c]	2.4 c[c]	93.9 a	96.9 b	136 b	4.1b	1.37
Herbicide	na	na	na	na	na	na	na	na
Mowing	45 a	29.9 c	2.7 c	17.5 bc	18.1 c	120 b	na	na
Scarring	30 ab	48.9 ab	3.8 b	56.0 ab	50.3 bc	96 b	2.2bc	1.38
Seedbed	14 bc	54.1 a	7.4 a	83.2 a	222.6 a	381 a	34.53a	1.48
Year II (2007)								
Control	27 b	86.5 ab	7.1 bc	310.6 bc	395.6 bc	1432 a	121.95 b	1.95
Herbicide	2 c	15.9 c	1.3 d	47.6 c	118.4 bc	32 c	2.73 c	2.17

Mowing	49 a	48.5 bc	5.2 cd	16.7 c	47.5 c	610 b	na	na
Scarring	27 b	98.6 ab	12.8 b	442.9 ab	465.1 ab	1786 a	161.95 a	1.95
Seedbed	10 c	114.4 a	24.8 a	781.7 a	779.5 a	1677 a	109.05 b	1.77
Effects [d]								
Year I (2006)								
Treatment	***	***	***	***	***	***	***	NS
Time	***	**	NS	*	**	***	***	NS
Treat*Time	***	NS	NS	NS	*	***	***	NS
Year II (2007)								
Treatment	***	***	***	***	***	***	***	**
Time	*	*	NS	NS	NS	*	NS	*
Treat*Time	*	*	***	**	NS	***	***	NS

TSW-Thousand Seed Weight; na-value not available

[a]Values within each treatment group not followed by the same letter are significantly different, as determined by Fisher's protected LSD ($\alpha = 0.05$)

[b]data log transformed; back transformed means are presented

[c]data square root transformed; back transformed means are presented

[d]Significance of the effects is given at three levels. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; NS - not significant

Table 5.2 Average values of growth and reproductive variables of alfalfa as affected by seed dispersal and disturbance (experiment II).

Treatment	Plant density (m^{-2})	Plant height (cm)	Shoots $plant^{-1}$	Racemes $plant^{-1}$	Pods $plant^{-1}$	Dry biomass (gm^{-2})	Fecun-dity (gm^{-2})	TSW (g)
Spring dispersal								
Year I								
Control	84 bc[a]	21.9 b	1.6 b	4.1 b[c]	11.3 b	96 b	0.02 b[c]	1.39
Herbicide	na	na	na	na	na	na	na	na
Mowing	100 b	22.2 b	1.8 b	na	na	69 b	na	na
Scarring	53 bc	20.5 b	1.5 b	4.8 b	15.8 b	71 b	0.01 b	1.45
Seedbed	395 a	32.6 a	3.1 a	73.1 a	55.5 a	973 a	169.2 a	1.31
Year II								
Control	31 bc	52.2 bc	3.3 b	117.1 a	63.9 abc	195 b	48.3 b	1.47
Herbicide	na	na	na	na	na	na	na	na
Mowing	42 b	40.1 c	3.6 b	23.3 b	31.1 bc	132 bc	na	na
Scarring	28 bc	64.4 ab	4.3 ab	158.1 a	113.1 a	241 b	96.6 b	1.47
Seedbed	203 a	84.7 a	5.3 a	179.2 a	82.3 ab	1357 a	1419 a	1.30
Fall dispersal								
Control	7 b[b]	24.6 b	1.5 bc	8.9 b	0.0 b	39.2 b[b]	0.20 b[c]	1.58
Herbicide	na	na	na	na	na	na	na	na
Mowing	6 b	23.9 b	1.4 bc	22.0 ab	na	19.5 c	na	na
Scarring	4 b	26.6 b	1.9 b	26.1 ab	8.7 b	29.9 bc	0.13 b	1.48
Seedbed	26 a	62.3 a	5.7 a	65.5 a	37.8 a	290.0 a	8.38 a	1.38
Effects[d]								
Treatment	***	***	***	***	**	***	***	NS
Time	*	**	NS	NS	**	***	**	NS
Treat*Time	***	NS	*	NS	*	***	***	NS

TSW-Thousand Seed Weight; na-value not available

[a]Values within each treatment group not followed by the same letter are significantly different, as determined by Fisher's protected LSD ($\alpha = 0.05$)

[b]data log transformed; back transformed means are presented

[c]data square root transformed; back transformed means are presented

[d]Significance of the effects is given at three levels. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; NS - not significant

There was a general decline in alfalfa density over time (Fig. 5.4) with more significant declines in treatments with greater initial plant densities. Among the treatments, tilled seed bed showed a 68% reduction from initial plant densities over the three year period and this was followed by mowed and undisturbed plots with a respective decline of 59% and 56%. In contrast, there was a considerable increase in plant density over years in herbicide treated plots in experiment I (Table 5.1). This was not observed in experiment II (Table 5.2).

5.4.2 Vegetative growth

Time of seed dispersal and disturbance regime had a direct impact on the vegetative growth attributes (plant height and number of shoots plant^{-1}) of individual alfalfa plants through their impact on plant density. In experiment I, fall seed dispersal resulted in 79% greater plant height and 60% greater number of shoots plant^{-1} versus spring seed dispersal in undisturbed plots in the year of establishment (Table 5.1). This difference was less important in experiment II (Table 5.2).

Disturbance treatments did not greatly affect plant height or number of shoots plant^{-1} in the year of establishment but had a significant impact in the following years. Sward scarring (both spring and fall dispersal) and well-tilled (fall dispersal) treatments produced plants with greater height and more shoots plant^{-1} when compared to undisturbed plots in the year (s) following establishment (Tables 5.1 and 5.2).

5.4.3 Dry biomass production

In this study, dry biomass accumulation for alfalfa ranged on average between 39 and 408g m^{-2} in the year of establishment. Dry biomass production was significantly influenced by the time of seed dispersal and disturbance treatments through an impact on plant density. In general, dry biomass was positively correlated with plant density (r = 0.33, $P \leq 0.001$) (Table 5.3).

Consequently, in the year of establishment, dry biomass production level in experiment II was only about 25% of the dry biomass production level in experiment I. Plants in the fall

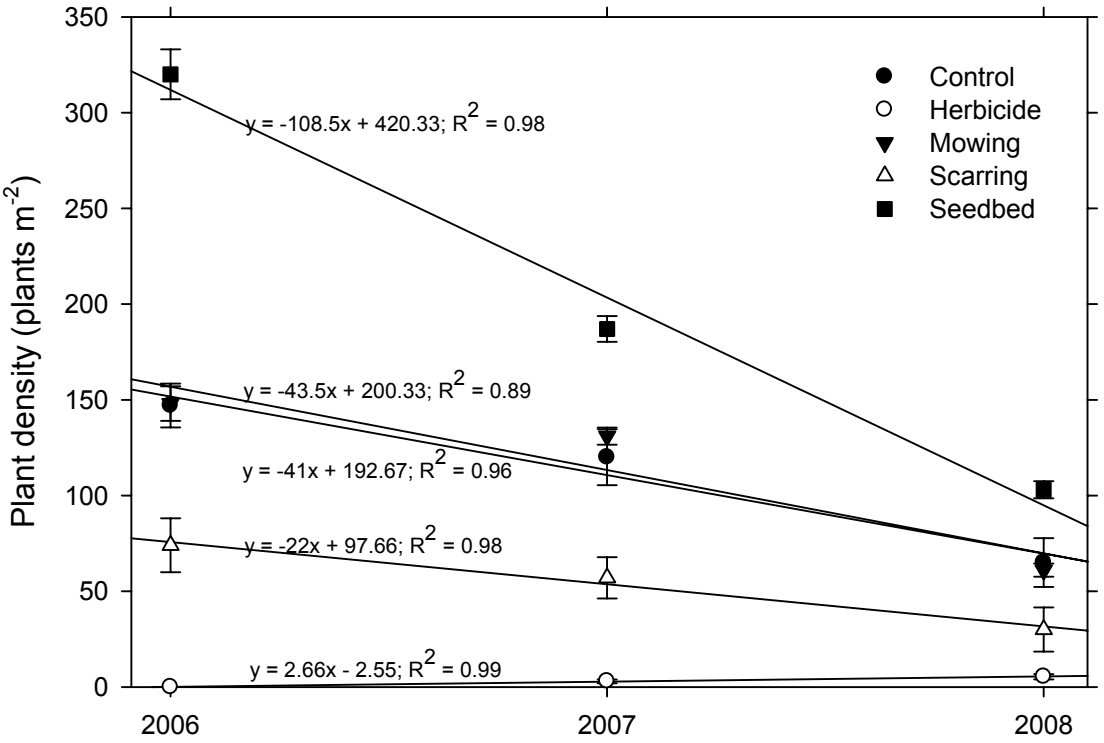

Fig. 5.4 Survivorship of the recruited alfalfa seedlings under different disturbance treatments over three years (2006-2008), presented as the change in plant density over time (observations from spring seed dispersal in experiment I). Bars above and below the data points represent standard errors of the means.

Table 5.3 Correlation coefficient matrix for growth and reproductive variables of alfalfa seeded in a grass sward

	Plant height (cm)	Shoots plant^{-1}	Racemes plant^{-1}	Pods plant^{-1}	Seeds plant^{-1}	1000 seed wt (g)	Plant density (m^{-2})	Dry biomass (gm^{-2})	Seed output (gm^{-2})
Plant height (cm)	1								
Shoots plant^{-1}	0.78*	1							
Racemes plant^{-1}	0.78*	0.71*	1						
Pods plant^{-1}	0.71*	0.62*	0.74*	1					
Seeds plant^{-1}	0.67*	0.82*	0.74*	0.58*	1				
1000-seed wt (g)	0.33	0.31	0.36*	0.13	0.43*	1			
Plant density (m^{-2})	0.05	-0.07	-0.07	0.01	-0.19	-0.32	1		
Dry biomass (gm^{-2})	0.74*	0.57*	0.62*	0.61*	0.54*	0.17	0.33*	1	
Seed output (gm^{-2})	0.75*	0.60*	0.67*	0.62*	0.68*	0.34	0.03	0.90*	1

*The effects are significant at $P \leq 0.005$ (determined using Bonferroni's family wise error correction)

dispersed treatment produced less than 50% dry biomass than that produced by plants in spring dispersed treatments. Among the disturbance treatments, dry biomass production was greatest in the well-tilled treatments (spring dispersed) (1410 gm^{-2}) and lowest in the herbicide treatments (0 gm^{-2}) in the establishment year (Tables 5.1 and 5.2).

The effect of low plant density on dry biomass production was compensated over time with increases in plant height and number of shoots $plant^{-1}$ of individual plants (Tables 5.1 and 5.2). In general, plant dry biomass production increased over time in all plots. The increase was most remarkable in sward scarring and undisturbed treatments with a more than two-fold increase between the second and third year (Table 5.1).

5.4.4 Reproductive success

In my experiments some of the alfalfa plants matured and produced seed in the establishment year. Number of reproductive units (racemes $plant^{-1}$and pods $plant^{-1}$) and fecundity $plant^{-1}$ and fecundity m^{-2} were significantly lower in experiment II vs. experiment I (Tables 5.1 and 5.2; fig. 5.5).

Fall seeded plots produced considerably greater numbers of reproductive units and fecundity (at the time of observation during the following spring) than spring seeded plots (observed in the same year) (Tables 5.1 and 5.2). Among different treatments, fecundity $plant^{-1}$ and fecundity m^{-2} were highest in the tilled treatment in the year of establishment. Mowed plants did not produce mature seeds yet later recruiting plants in the herbicide treated plots were reproductively successful.

Thousand seed weights were significantly greater in herbicide treated plots when compared to other treatments (Table 5.1). Correlation analysis revealed that the number of seeds $plant^{-1}$ was positively correlated with plant height (r = 0.67) and number of shoots $plant^{-1}$ (r = 0.82) (Table 5.3) but was negatively correlated to plant density m^{-2} (r = -0.19).

There was a substantial increase in reproductive output in both the sward scarring and undisturbed treatments in the years following the establishment year. In particular, from

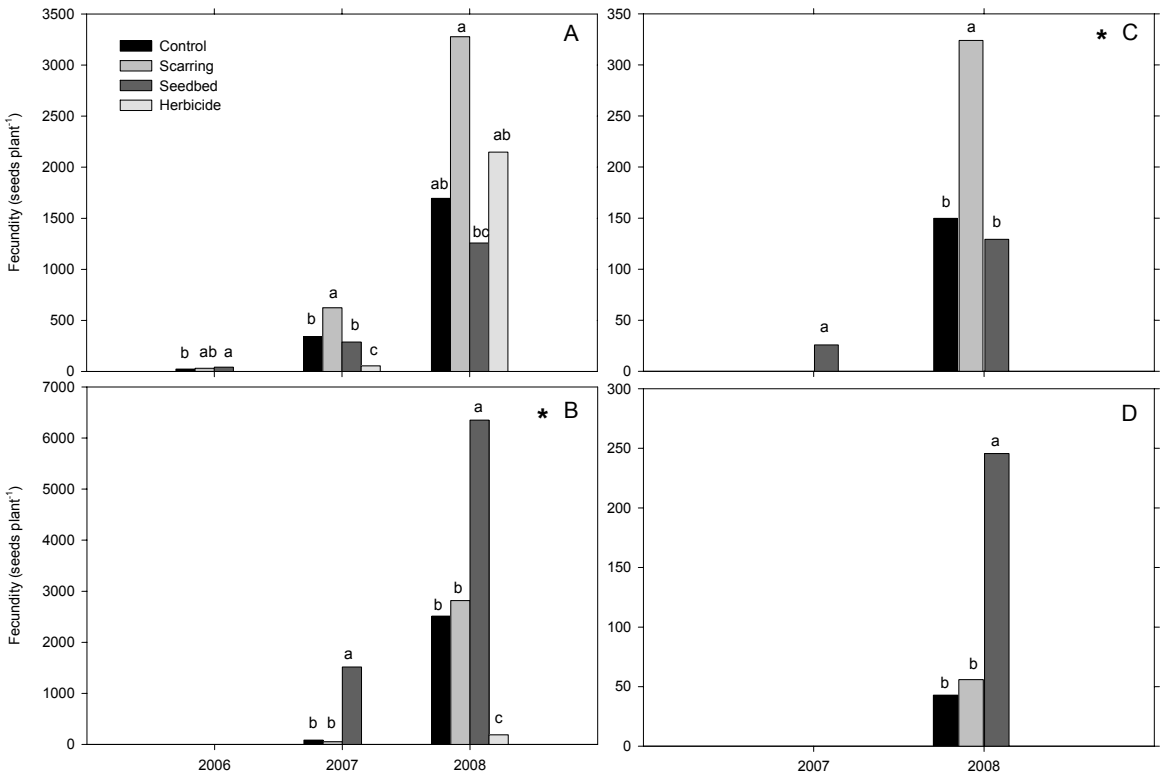

Fig. 5.5 Fecundity (seeds plant^{-1}) under different treatments: A) experiment I – spring seed dispersal; B) experiment I – fall seed dispersal; C) experiment II – spring seed dispersal; and D) experiment II – fall seed dispersal. Values within each treatment group not followed by the same letter are significantly different, as determined using Fisher's protected LSD ($\alpha = 0.05$); *data square root transformed; back transformed means are presented.

the second to the third year after establishment, seeds plant^{-1} increased by 425% and 397%, respectively, in the scarred and undisturbed treatments (Fig. 5.5).

5.5 Discussion

My findings confirm that alfalfa readily establishes in competitive environments. The extent of recruitment and establishment seemed to be affected by the level of vegetation cover of the sward as was evident from the differences in plant densities between the experiments I and II. Reduced ground cover allows for greater levels of incoming irradiance which can increase the soil temperature and encourage recruitment (Ghermandi and Bran 2004; Jorgelina et al. 2009). It is also known that alfalfa seeds have a hard seed coat which is a dormancy mechanism. Reports indicate that fluctuations in temperature and soil moisture can facilitate seedling recruitment (Midgley 1926; Rincker 1954; Ellis and Palmer 1973). Gaps in vegetation cover can promote a much more ephemeral microclimate and one which may encourage seed coat softening.

High levels of seedling recruitment and establishment in the well-tilled seedbeds could have been due to low inter-specific competition. Alfalfa seedling recruitment is affected by inter-specific competition during establishment (Hall et al. 1995; Hoy et al. 2002) and seedling vigor is related to the level of competition (Stout et al.1992). Indeed, severe shading causes alfalfa seedlings to be weak with limited crown bud initiation. These types of seedlings may never make it to adult stage (Chamblee and Lovvorn 1953).

High levels of winter mortality caused substantial reductions in plant densities in the fall dispersed treatments. In alfalfa, winter survival and spring regrowth is governed largely by sugar reserve accumulation in the fall (Dhont et al. 2003) and timing of fall seeding, in the case of my experiments, fall recruitment can be critical in this regard. Because the recruitment in my experiments was ad hoc and timing of recruitment was not managed as it would be in a commercial field, a significant proportion of recruited seedlings may have exhibited delayed emergence. In addition, seedling winter survival can be mitigated by microclimate. The grass sward can act as a snow trap and protect over-wintering seedlings (Leep and Jeranyama 2001). This may explain why the tilled treatment (fall

seeded) had the lowest plant densities over other treatments in experiment I. The opposite occurred in experiment II, which may have been due to differences in the time of seedling recruitment. This result is reflected in significant interaction among time of dispersal, treatment and year. These results thus highlight the importance of both microsite and macro climatic conditions to alfalfa seedling recruitment and winter survival.

The decline in plant density of the established stands over time was likely due to competition. Similar reduction in plant density in cultivated alfalfa stands was reported by several researchers (e.g. Stout 1998; Hall et al. 2004; Teixeira et al. 2007). The exception of the gradual increase in plant density in few of the herbicide treated plots was due to the delayed recruitment of dormant alfalfa seeds. Delayed recruitment in alfalfa due to the presence of hard seed was reported by Hall et al. (1993). In alfalfa, delay in recruitment is often facilitated by the presence of hard seed coat (Ballard 1973). Impermeable seeds may act as potential reserves and assist stand re-establishment following unfavorable conditions (Dexter 1955; Rolston 1978). Seed dormancy in alfalfa can last for several decades (Wilton et al. 1978; Rincker 1983), suggesting that slow recruitment of alfalfa seedlings may assist the re-establishment of alfalfa populations in roadside habitats even after disturbances such as herbicide spray. However, the degree to which this can occur may depend on the availability of suitable microsite for seedling recruitment and establishment.

The alfalfa plants in this study compensated for low plant densities by increases in numbers of shoots, racemes and pods. Reports indicate that this type of yield component compensation is typical in alfalfa (e.g. Volenec et al. 1987; Simko 1992; Hall 1993; Askarian et al. 1995). In particular, the compensation was most notable for the fall dispersal treatments. It is likely that spaced alfalfa plants have the opportunity to exploit their full yield potential when compared to the plants under high density, as demonstrated by Riday and Brummer (2004). Further, I observed little relationship between plant density and dry biomass production and yield of alfalfa after the establishment year. Similar findings were also observed by Askarian et al. (1995), Hall et al. (2004) and Teixeira et al. (2007), indicating that even low densities of roadside alfalfa can produce significant dry biomass and seed output.

Mowing restricted seed production due to the reduction in the length of growing period necessary for the production of mature seeds. In the herbicide treated plots, later recruiting alfalfa plants grew in a less competitive environment (both inter- and intra-specific), effectively utilizing available resources from the surrounding microsite. This may be the reason for greater thousand seed weights observed in herbicide treated plots when compared to other plots.

The results of this study show that alfalfa is capable of recruiting and establishing in competitive environments. Establishment of alfalfa in semi-natural habitats such as a grass sward may be facilitated by disturbances to the established vegetation in which it is establishing. However, that is not an absolute requirement and I documented successful establishment even in undisturbed grass swards. Low levels of establishment can still result in significant alfalfa stands in terms of plant biomass and seed production. The results of my study also suggest that density is not necessarily the primary determinant of seed production potential by feral alfalfa stands because alfalfa has a tremendous ability to compensate in terms of yield components. Some disturbances can limit feral alfalfa populations and I showed that timely mowing could completely prevent alfalfa seed production and 2,4-D application can control the established plants. The results of this study provide a novel baseline information on the establishment of alfalfa in unmanaged environments such as the roadsides. The findings will be useful in modeling the dynamics of feral alfalfa populations in unmanaged habitats and also for the risk assessment of alfalfa containing novel traits.

6.0 Occurrence of Feral Alfalfa Populations Along Roadside Habitats in Southern Manitoba, Canada and Their Role in Intra-specific Novel Trait Movement

6.1 Abstract

Feral populations of cultivated plants can facilitate transgene movement across the landscape and act as potential barriers for achieving co-existence between genetically modified (GM) and conventional crops. Alfalfa is a highly outcrossing perennial species often escapes cultivation and grow in unmanaged habitats as self-sustaining feral populations. Genetically modified glyphosate resistant (GR) alfalfa was approved for unconfined release in Canada but there remains little information available on the extent of the occurrence of feral alfalfa populations and their potential role in transgene movement in Canada. The main objectives of this study were a) to document the occurrence of feral alfalfa populations, and b) to estimate the levels of outcrossing facilitated by feral populations. A roadside survey revealed widespread occurrence of feral alfalfa populations with a frequency ranging from 0.2 to 1.7 populations km^{-1}. In 68% of the cases, the nearest feral alfalfa population was located within 250m of cultivated fields, a distance enough for outcrossing. Cultivated and feral alfalfa populations exhibit flowering synchrony and are cross compatible. In this study, estimated outcrossing levels involving feral alfalfa populations ranged between 62% and 85%. The results of this study show that feral alfalfa plants are prevalent in alfalfa producing regions in western Canada and that they can serve as genetic bridges for the movement of transgenes at landscape level. Achieving the sustainable co-existence of GM and conventional alfalfa in current production systems will require the management of feral alfalfa populations.

6.2 Introduction

Alfalfa (*Medicago sativa* L.) is regarded worldwide as a high protein source for livestock and is the most important forage crop in North America. With the expansion of alfalfa seed production in Manitoba and Saskatchewan, Canada is now one of the major exporters of alfalfa seed in the world (Wong 2008). In addition, alfalfa is also a major component of Canada's processed forage industry. In 2006, alfalfa was seeded both as a sole crop and in mixtures on over 88,000 farms across Canada with a total area of 12.5 million acres (Statistics Canada 2006). In Manitoba, in 2008, alfalfa was grown on 7,499 farms representing a total area of 664,851 acres and a total production of about one million metric tones (both alfalfa and alfalfa/grass mixtures) making Manitoba one of the important alfalfa producing regions in Canada (MMPP 2009).

Widespread cultivation of alfalfa has facilitated its escape and establishment in unmanaged natural and semi-natural habitats including roadsides. My work in southern Manitoba has revealed that roadside aflafla populations occuring in this region are capable of establishing self-perpetuating feral populations (see chapter 4.0). Alfalfa's deep tap root system, cold and drought tolerance, symbiotic nitrogen fixation and perenniality contribute to its successful establishment and persistence in competitive environments (reviewed in Bagavathiannan and Van Acker 2009) (Chapter 3.0). Occurrence of feral populations has implications for the release of GM crops because they can act as sources and sinks of transgenes and potentially aid in gene flow at the landscape level (Mueller 2004). In nature, intra-specific gene flow among sub-populations occurs in the context of a metapopulation (Crawley and Brown 1995). In agricultural landscapes, and for crop species, these metapopulations include subpopulations of cultivated crops, in-field volunteers and feral plants that occur in roadsides and other unmanaged habitats (Van Acker 2007).

In Canada, GM glyphosate resistant (GR) alfalfa was approved for unconfined release but authorization for commercial planting has not yet been granted (CFIA 2005). In the US, GR alfalfa was approved for commercial planting in 2005 (APHIS 2005) and was initially planted on over 200,000 acres. However, GR alfalfa is currently under regulated status, after a US court issued a permanent moratorium on further sales and cultivation of

GM-GR alfalfa (Fox 2007). This moratorium points to the need for thorough environmental impact assessments including the role feral alfalfa populations might play in transgene movement. In addition, alfalfa is currently used for the production of biopharmaceuticals (Sparrow et al. 2007) and the presence of feral populations may pose challenges for trait confinement once released under field conditions.

Previous studies have estimated the gene flow and outcrossing among different alfalfa populations using white flower color (Pedersen 1967, 1968, 1974; Kehr 1973; Pedersen and Barnes 1973) or herbicide-resistance (St. Amand et al. 2000; Fitzpatrick et al. 2003; Teuber et al. 2004) as scoreable markers. White flower color in alfalfa is a simple yet powerful phenotypic tool for determining outcrossing levels. The 'c' gene is a basic color factor in alfalfa that in the homozygous recessive condition produces white-flowered plants that are devoid of anthocyanin pigmentation in flowers, seeds, stems, leaves, and roots (Barnes 1972). White-flower color could be used as a scorable marker for estimating gene flow in alfafa (Kehr 1973; Pedersen and Barnes 1973). Therefore, in my study, white-flowering alfalfa clones were used as female parents and colored hypocotyls or colored flowers have been used for positive scoring for outcrossing.

The existence of alfalfa populations in unmanaged habitats have been noted in Europe (Jenczewski et al. 1999a) and the US (Kendrick et al. 2005). However, the extent of their occurrence in the agricultural landscapes on the Canadian prairies and the outcrossing levels involving feral alfalfa populations have not been studied. The main objectives of this study were a) to determine the extent of occurrence of feral alfalfa populations in roadside habitats in a key alfalfa growing region in western Canada (southern Manitoba), b) to estimate levels of outcrossing possible between feral and cultivated alfalfa, and c) to discuss the results in the context of environmental risk assessment of GM alfalfa.

6.3 Materials and Methods

6.3.1 Roadside survey

6.3.1.1 Location

A survey of feral alfalfa populations was conducted in each of three rural municipalities located in southern Manitoba: Springfield (49° 55' N; 96° 45' W), Hanover (49° 28' N; 96° 50' W) and MacDonald (49° 40' N; 97° 30' W) with total agricultural land areas of 1059 km^2, 718 km^2 and 1106 km^2, respectively (Fig. 6.1). Average winter and summer temperatures for this region are -13^oC and 26^oC, respectively and on average, precipitation in the region is 407 mm of rainfall and 112 mm of snow fall. In total 115 frost free days per year were recorded in this region in 2007 (MCP 2008).

6.3.1.2 Survey design and method

The survey methodology was a modified version of the stratified semi-random survey methodology used by Kendrick et al. (2005). The survey was started in early August 2006 and was completed before roadside mowing started in late August. At the time of the survey, alfalfa populations were at anthesis allowing for easy detection. The survey route was determined without any prior knowledge of land use patterns or the existence of feral alfalfa populations. The route was chosen to represent as much of each municipality as possible. Within each municipality, 30 observation sites were identified. Fifteen of these were random sites, chosen without any prior knowledge of the survey area (i.e. pre-determined sites) and these sites were all at least 5 km apart. Due to the possibility that feral alfalfa plants may be absent at these sites, another 15 sites were chosen (i.e. directed sites) each located between two subsequent pre-determined sites on the survey route. Directed sites did have feral alfalfa except in situations where feral populations were completely absent between two pre-determined sites.

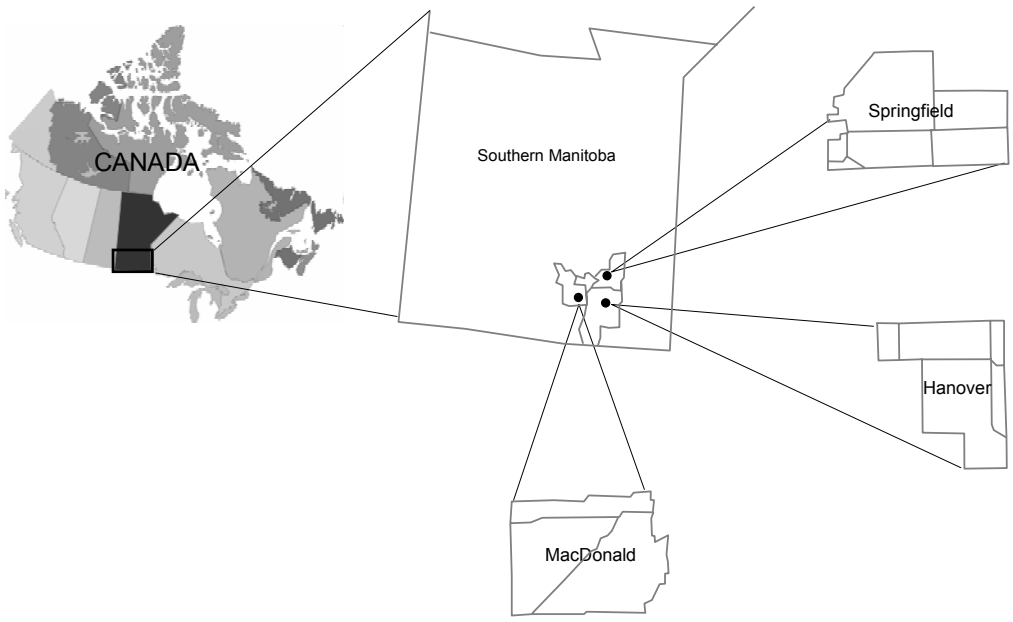

Fig. 6.1 Locations of rural municipalities included in the survey in southern Manitoba, Canada

At the observation sites, the road-side was divided into three zones arbitrarily; a mowed zone, a flood prone zone and the field side. The mowed zone was about 3 m wide (from the road shoulder). In this region of Manitoba, roadside mowing is typically done twice per year (early June to early July and late August to mid September). The flood prone zone was the area between the mowed zone to approximately 3 m from the field edge and it included the deepest point of the road-side ditch. This zone is subject to regular flooding and waterlogging. The field side zone was the area from the flood prone zone to the field edge. This zone was less subject to mowing and flooding but the occurrence of feral plants in this zone may be associated with the cultivation history of alfalfa in the adjacent field. Global positioning system (GPS) co-ordinates for each observation site were recorded.

6.3.1.3 Data collection

Observation sites: Data pertaining to the population size (number of patches population^{-1}) and size of each patch (number of individuals patch^{-1}) were documented at observation sites (~500 m^2 per site). Alfalfa plants that occurred within 1 m distance to each other were defined as a single patch and each patch consisted of one to many individual plants. The distribution of feral plants in different zones (mowed, floodprone and field side) within a ditch was noted. In addition, details on the land use pattern 1 km before and after the observation sites were also collected. In particular, the type of surrounding land use including residential areas, crop cultivation (alfalfa and other field crops) and lands allotted to range, pasture and woods were documented. Other observations include roadside mowing (yes/no), ditch vegetation cover (measured in a scale of 1 to 5 with 5 being more dense), ditch width (scale: 1 - <5 m, 2 - 5 to10 m, 3 - >10 m) and road surface type (paved, gravel or dirt).

Data collected along the survey route: Data pertaining to the number of feral populations km^{-1}, existence of cultivated alfalfa fields, type of production (hay/seed) and distance as well as flowering synchrony between cultivated and feral alfalfa populations were documented along the survey route (between the observation sites). Flowering in hay production fields was confirmed if there was at least one fully opened flower on at least

10% of the branches. The feral populations were defined such that two adjacent populations were separated by at least 100m. The extent of roadside mowing also was noted along the survey route (scored as mowed or not-mowed for each mile road).

6.3.2 Outcrossing experiment

A white-flowering alfalfa plant was collected in late summer 2006 from a seed production field, clonally multiplied in the green house and tested for white flower color prior to its use as the female pollen recipient population in the study. The clones were planted at the roadside experimental sites in early May 2007. The experiment consisted of four scenarios (treatments) and three planting sites per scenario (replicates) in a completely randomized design.

The treatments were designed to test the levels of gene flow in the following scenarios: a) among the individuals of feral alfalfa populations, b) from hay production fields to feral populations, c) from seed production fields to feral populations, and d) from feral to cultivated populations. In each treatment, 10 clones of white-flowering female plants were randomly planted in the roadside ditch with 5 m between plants. Any surrounding road-side alfalfa plants were controlled for a minimum distance of 500 m such that the pollen was obtained only from the male population under investigation. One exception to this was the test of gene flow within the individuals of feral populations where the pollen source was left uncontrolled. Leaf-cutter bees (*Megachile rotundata*) were released in the seed production fields and in all other treatments the seed set was based on natural pollinators (Fig. 6.2). Further descriptions of the treatments are given in table 6.1.

The clones were harvested in late August 2007 and the seeds were bulked. In order to detect the gene flow between alfalfa populations even at low levels, the following formula was used to determine the required sample size:

$$N = \ln(1-P)/\ln(1-p)$$ (Alibert et al. 2005)

Where, P-probability of detection of one individual in least frequent class; p-probability of the least frequent class. The theoretical sample sizes were as follows:

P	p			
	10%	5%	1%	0.1%
95%	28	58	298	2994
99%	42	90	458	4606
99.9%	66	180	916	6904

On this basis, 458 seeds were screened in each replication allowing for a 1% detection level (p = 0.01) with 99% probability (P = 0.99). The seeds were planted in small trays in a greenhouse and 458 healthy seedlings from each replication were grown until flowering. Selfed progeny produce white flower color and lack purple pigmentation in the roots. Positive scoring for outcrossing was carried out based on the occurrence of pigmented petals (other than white) and confirmed on the basis of pigmentation in the crown region of the roots.

6.3.3 Data analysis

All data were analyzed using the Statistical Analysis Software (SAS) version 9.1 (SAS Institute 2003). The significance of different factors investigated in the survey was tested by fitting poisson regression models to the data. Poisson regression is a generalized linear model, which is appropriate when the dependant variable is a count data and the model assumes that the variable has a poisson distribution. Poisson regression analysis was performed using the GENMOD procedure of SAS. Analysis of variance (ANOVA) was performed to reveal significant differences among treatments, following a Mixed Procedure analysis (PROC MIXED) (Littell et al. 1996), using SAS. Locations were considered fixed effects while observation sites were considered random effects. Gene flow data were analyzed following a Generalized Linear Model procedure (PROC GLM) of SAS. Prior to ANOVA in all the analyses, outliers were removed based on the studentized residual values (Lund 1975) and normality of the residuals was confirmed using the Kolmogorov-Smirnov test. Mean separation was performed using Fisher's

Fig. 6.2 Experimental set-up to determine the level of outcrossing from alfalfa seed production fields to an adjacent pollen recipient plant

Table 6.1 Treatments for an outcrossing experiment involving feral alfalfa populations in roadside habitats in southern Manitoba, Canada

Treatment	Experimental set-up	Density (plants m^{-2}) ǂ	Distance (m)‡
Gene flow within feral populations	Clones were space planted within a feral population in the ditch	25 - 50	5 m
Gene flow from hay fields to feral populations	Clones were space planted in the ditch adjacent to a hay field*	150 - 200	10 to 15 m
Gene flow from seed fields to feral populations	Clones were space planted in the ditch adjacent to a seed field*Ғ	125 - 175	10 to 15 m
Gene flow from feral alfalfa to field alfalfa	Clones were space planted in the field shoulder adjacent to a feral population in the ditch†	25 - 50	10 to 15 m

* Feral plants occurring in the surrounding area within a minimum distance of 500 m were not allowed to flower

Ғ Alfalfa leaf cutter bees were released for seed production

† Fields cultivated with crops other than alfalfa

ǂAverage density of pollen donor population

‡Average distance between pollen donor and pollen receptor plants

protected Least Significant Difference (LSD) at P≤0.05, and letter groupings were carried out using the PDMIX800 macro in SAS (Saxton 1998).

6.4 Results

Feral alfalfa populations were widespread in roadside habitats but varied in frequency (populations km^{-1}) and size among the municipalities. In MacDonald, the frequency was only about 20% of the frequency in Hanover and Springfield (Table 6.2). Frequency corresponded to the extent of alfalfa cultivation within a given municipality (Table 6.3). Average populations in Springfield were significantly larger in size versus the other two municipalities. In general, however, individual patches were small (about 3.5 individuals $patch^{-1}$) and there were no significant differences in patch size among municipalities.

Population size did not significantly vary according to the occurrence, type of production system and distance of alfalfa fields in the surrounding area (Table 6.4). Factorial ANOVA detected significant differences for landuse pattern with lower population size in residential areas when compared to other land uses (cultivated and pasture/range/woods). Further, population size was greater in mowed ditches compared to the not-mowed ditches (P<0.0009, Table 6.4).

Roadsides were mowed by the municipalities but the extent of mowing varied greatly among locations. On average, 87% of the ditches along the survey route were mowed during the first round of roadside management. In MacDonald, however, only 50% of the roadsides were mowed (Fig. 6.3). In addition, population size had a positive relationship with the ditch width (P<0.0003) and negative relationship with the ditch vegetation cover (P<0.0001). The road type however did not have any significant influence on the size of feral populations (Table 6.4).

The occurrence of feral alfalfa plants varied according to position within the roadside ditches. Almost 75% of feral alfalfa plants were found within the portion of the ditch that would be regularly mowed. Many fewer feral plants were found in the flood prone zone and the field shoulder (Fig. 6.4). One exception was for populations in the MacDonald

Table 6.2 Prevalence of feral alfalfa populations in roadside habitats in southern Manitoba, Canada

Parameter	Hanover		MacDonald		Springfield	
	Mean	SE	Mean	SE	Mean	SE
Number of populations km^{-1*}	1.68 **a**	0.16	0.21 **b**	0.04	1.32 **a**	0.15
Number of patches $population^{-1\dagger}$	11.52 **b**	1.11	12.57 **b**	1.33	17.54 **a**	1.66
Number of individuals $patch^{-1\dagger}$	3.04	0.38	4.06	0.67	3.68	0.37

*Data collected along the survey route

†Data collected in the detailed observation sites

Values within each row followed by different letters are significantly different, as determined using Fisher's protected LSD ($\alpha = 0.05$)

Table 6.3 Alfalfa production data (both seed and hay) of the rural municipalities included in the survey[†]

Particulars	Hanover	MacDonald	Springfield
Number of farms involved in alfalfa production	51	27	40
Production area (acres)	3,807	1849	2,320
Total production (tones)	7,656	2150	4,511

[†]Production data for 2006

Source: MMPP (2009)

Table 6.4 Poisson regression models for the influence of different factors on the size of roadside alfalfa populations[†]

Factor	Estimate	Chi-square	*P*
Loc	0.2062	26.85	<0.0001
Adjalf	-0.1275	0.59	0.4417
Distalf	-0.1420	3.23	0.0723
Prodsys	0.1525	1.12	0.2896
Mow	0.2967	10.92	0.0009
Roadsur	0.0212	0.14	0.7121
Landuse	0.2422	24.64	<0.0001
Ditchwid	0.2411	12.80	0.0003
Ditchveg	-0.3331	48.33	<0.0001

[†]Data collected in the detailed observation sites

Loc locations, *Adjalf* presence of adjacent alfalfa field, *Distalf* distance to the nearby alfalfa field (scale: 1 - <0.5 km, 2 - 0.5 to1.5 km, 3 - >1.5 km), *Prodsys* alfalfa production system (hay/seed), *Mow* mowing of roadside vegetation, *Roadsur* road surface (paved, gravel and dirt), *Landuse* type of landuse pattern in the immediate vicinity (residential, cultivated fields and pasture/range/woods), *Ditchwid* width of the ditch (scale: 1 - <5 m, 2 - 5 to10 m, 3 - > 10m), *Ditchveg* ditch vegetation cover (1 - sparse to 5 - dense)

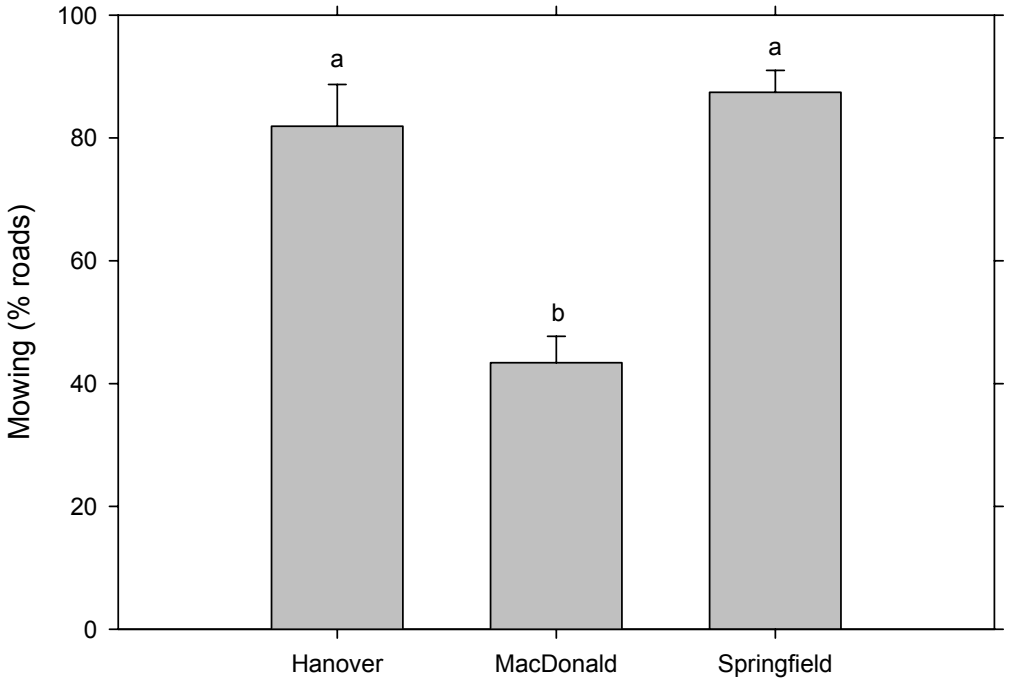

Fig. 6.3 Degree of roadside mowing along the survey route in each rural municipality selected in this study (Hanover, MacDonald and Springfield). Columns topped by different letters are significantly different, as determined using Fisher's protected LSD ($\alpha = 0.05$). Bars above columns represent standard errors of the means.

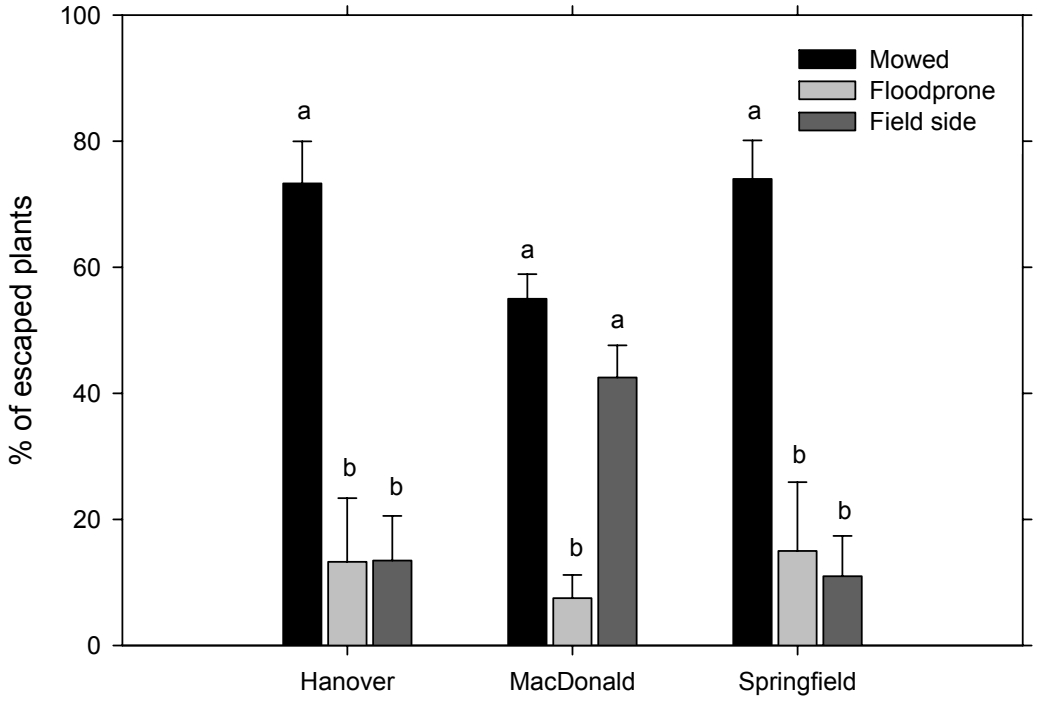

Fig. 6.4 Distribution of feral alfalfa plants in different zones within the roadside habitats in three rural municipalities (Hanover, MacDonald and Springfield) in southern Manitoba, Canada. 'Mowed' zone represents the area adjacent to road shoulder that is regularly mowed. 'Floodprone' zone is the shallow region of the ditch, which is often subjected to flooding and 'field side' represents the area adjacent to the field shoulder, which is located on the opposite side of the ditch. Columns within municipality not topped by the same letter are significantly different, as determined using Fisher's protected LSD ($\alpha = 0.05$). Bars above columns represent standard errors of the means.

municipality where occurrence of feral plants in the field shoulders was comparable to occurrence in the mowed zone.

Feral alfalfa populations were often observed close to cultivated alfalfa fields. In 68% of cases, feral populations were located 250 m or less from a cultivated field (Fig. 6.5). I found flowering synchrony between feral and cultivated alfalfa populations but it varied depending on whether the alfalfa field was a hay or seed field. Flowering synchrony between feral and cultivated alfalfa was 100% for seed production fields. However, less than 10% of the alfalfa fields along the survey route were seed fields. For hay fields, flowering was observed in almost 33% of fields at the time of the survey.

The gene flow study showed that feral alfalfa populations can aid intra-specific trait movement (Fig. 6.6). Estimated levels of outcrossing varied between 62% and 85% within a distance of 15m. Lower levels of outcrossing (62%) were observed when the pollen recipients were planted adjacent to seed production fields (which use leaf cutter bees for pollination) when compared to other treatments. High level of outcrossing (>80%) were also observed from hay fields to the adjacent feral population when considerable flowering was observed in the hay field.

6.5 Discussion

I observed widespread occurrence of feral alfalfa populations along the roadsides I surveyed, indicating that alfalfa can readily establish in roadside habitats. In particular, greater occurrence of feral populations was noted in regions with widespread alfalfa cultivation. Similar observations were made by Kendrick et al. (2005). Substantially greater frequencies of feral populations in areas with widespread alfalfa cultivation suggest that farming and related activities may play an important role in the occurrence of feral alfalfa populations along roadsides in rural municipalities.

There was no indication, however, that the size of individual populations was related to the extent of alfalfa cultivation in the region. Population size rather was related to other proximate factors including mowing, ditch width, density of the ditch vegetation and surrounding landuse. Mowing increases the amount of light penetrated through the

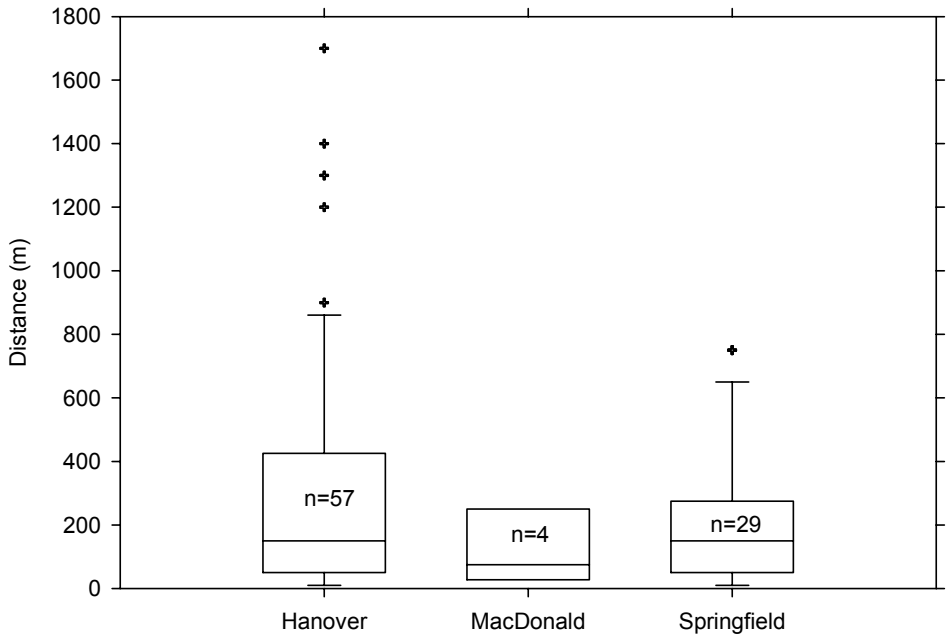

Fig. 6.5 Average distance of feral alfalfa populations to the alfalfa production fields in southern Manitoba, Canada. The center line of the boxes represents sample median. Lower and upper hinges are the estimates respectively of the first and third quartiles representing the lower and upper halves of the samples respectively. Data points above the bars denote outliers.

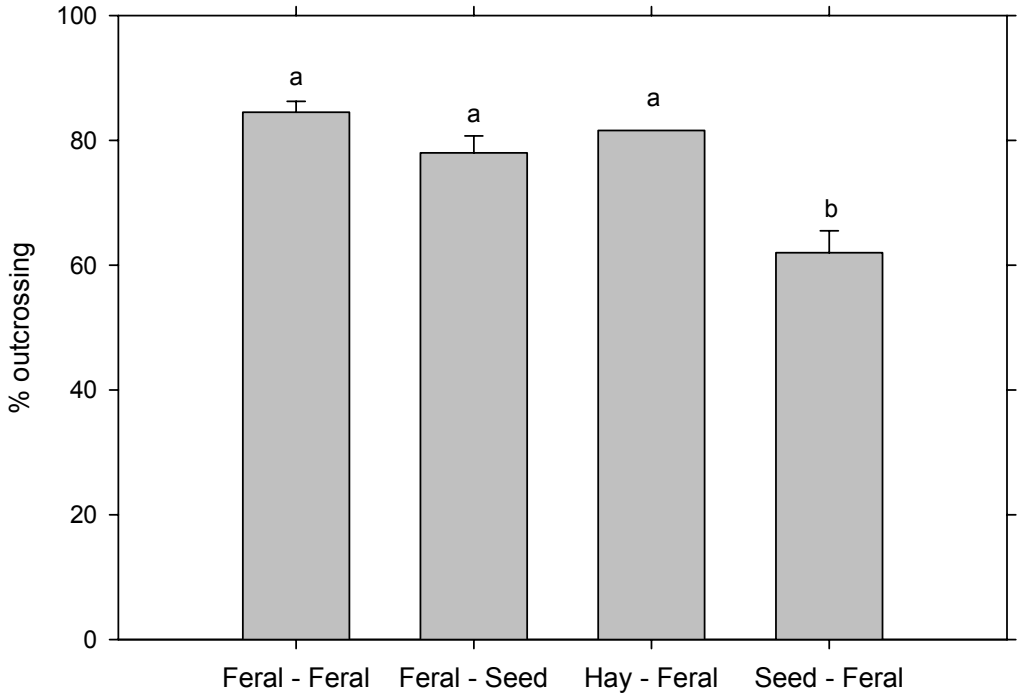

Fig. 6.6 Differences in outcrossing levels in feral alfalfa estimated by white-flowering clones. Columns not topped by the same letter are significantly different, as determined using Fisher's protected LSD ($\alpha = 0.05$). Bars above columns represent standard errors of the means.

canopies and thereby alters the micro environmental conditions. This could have increased successful seedling establishment (Bissels et al. 2006), survival and size (Satterthwaite et al. 2007; Williams et al. 2007) of the feral alfalfa populations in roadside habitats. Conversely, competition for resources including light and nutrients could have affected the seedling establishment and population size in not-mowed and also in very densely populated ditches (Chamblee and Lovvorn 1953; Meyer 1999). Comparatively smaller populations in residential areas could be attributed to potentially low external seed input and intensive management of the roadsides including frequent herbicide application when compared to farming areas. It was not clear why some patches were very small but auto-allelopathy can limit alfalfa patch growth (Jennings and Nelson 2002).

The lack of any significant relationship between population size and the occurrence of alfalfa fields indicates that feral populations are not necessarily affected by (or dependent upon) adjacent alfalfa cultivation. Existence of feral populations even in places where there were no current alfalfa fields in the surrounding area suggests that feral alfalfa populations persist over time. It is possible that some feral populations were founded by seed spills during transport as observed in the UK (Crawley and Brown 1995), Canada (Yoshimura et al. 2006), Japan (Nishizawa et al. 2009) and South Korea (Park et al. 2010) or intentional road-side planting. Because alfalfa seeds are small, they can easily be lost during transport and end up in nearby unmanaged habitats. Van Deynze et al. (2008) and Putnam (2006) suggested that feral alfalfa populations found in the US may have originated through unintentional escapes from cultivated fields or in some cases through intentional plantings. In western Canada, some roadsides were seeded to alfalfa during road construction (most of these 40 to 50 years ago) because of the excellent erosion control properties of alfalfa (Reid B., Springfield municipality agriculture representative, personal communication) and the populations originating from these roadside plantings may be persistent for decades. Several studies have reported the long-term persistence of alfalfa populations in pastures and rangelands (Kilcher and Heinrichs 1965; Pearse 1965; Rumbaugh and Pedersen 1979).

The distinct pattern of distribution of feral populations across the roadside ditches shows the influence of site characteristics on the presence and perhaps persistence of feral alfalfa. Alfalfa seeds lost during transport would most likely establish near the road shoulder and although this zone is typically mowed by rural municipalities every summer, the mowing frequency, timing, route and coverage varies among the municipalities and among years (Moffat B., Springfield municipality, personal communication). Inconsistent mowing allows populations to recover. However, feral plants occurring in the ditches that were not-mowed continue to grow and produce mature seeds (Chapter 4.0). Alfalfa plants establishing in the flood-prone zone may experience greater levels of mortality given that alfalfa does not tolerate prolonged waterlogging (Sheaffer et al. 1988). In the municipalities I surveyed, the field shoulder was not typically mowed or flooded. The presence of feral alfalfa plants along the field shoulder may be a function of the field history. And even infrequent seed invasion may be sufficient for population establishment given that alfalfa plants occurring in this zone are disturbed less and may persist for longer periods.

The successful movement of transgenes between cultivated and feral alfalfa populations depends on the following criteria: proximity of feral plants to the cultivated field, flowering synchrony, and the abundance of pollinator insects. In my study, a number of feral populations were observed within 250 m from cultivated fields, a distance sufficient for outcrossing at a level of 0.28% and 1.5% under leaf cutter bee (Fitzpatrick et al. 2003) and honey bee (Teuber et al. 2004) mediated pollination, respectively. Cultivated and feral populations exhibited flowering synchrony and the indeterminate growth habit of alfalfa extends the period of flowering synchrony (Bolton 1962). In Canada, leaf cutter bees are widely used for pollination in alfalfa seed production fields (Richards 1991) and they also occur as solitary insects in the wild (Bohart 1957). In addition, natural populations of honey bees (Bohart 1957), bumble bees (Holm 1966; Osborne et al. 2008) and alkali bees (Stephen 1959) were also reported to cause tripping and pollination in alfalfa. Thus, close proximity of feral populations to cultivated fields, synchronized flowering and the existence of natural pollinators likely favored gene flow in alfalfa in this study.

The outcrossing experiment revealed significant levels of gene flow between feral and cultivated populations. Significantly lower level of outcrossing between white-flowering clones and purple flowering seed production fields when compared to other treatments could possibly be due to the preferential pollination by leaf-cutter bees with respect to flower color (Kehr 1973; Steiner et al. 1992). Using white flower color as an outcrossing marker may thus result in a systematic underestimation of gene flow (Knapp and Teuber 1993). None the less, observed outcrossing levels were similar to those reported by Pedersen (1967), and Kehr (1973) who observed outcrossing levels between 50 and 55% in white-flowered alfalfa plants growing in a population of purple flowered alfalfa plants (with leaf-cutter bee pollination).

I did observe substantial outcrossing from hay fields to the adjacent feral population, particularly when a harvest delay resulted in considerable flowering in the hay field. Gene flow from hay fields to nearby seed production fields was also observed in a study conducted by Forage Genetics International (FGI 2008) using the glyphosate resistant marker, but they found only low outcrossing levels (0.21% at 45m, <0.05% at 175m) even when the hay fields were allowed to bloom at a level of 20% and honey bees were used for pollination. In their study, low outcrossing rates may have been caused by the dilution effects due to differences in the flower density/pollen load between the source and recipient population. Using glyphosate resistant marker and leaf-cutter bee pollination, St. Amand et al. (2000) detected significant outcrossing frequencies away from hay fields for distances upto 1000m (~10% outcrossing at this distance).

Although hay fields are typically harvested before flowering, it is not uncommon to notice flowering in uncut field corners and along field edges (Fig. 6.7). My own observations indicate that poor weather conditions can cause harvest delays, sometimes until after significant flowering has already occurred. Gene flow from feral populations to hay fields may be less of a concern because hay fields are rarely allowed to set seed.

I observed very high levels of outcrossing among individuals within feral populations. This might aid the persistence of transgenes in the environment. St. Amand et al. (2000) estimated an outcrossing rate of 92% in widely dispersed feral alfalfa plants (with gap distances of up to 230 m). The outcrossing levels noted in my study were comparable to

Fig. 6.7 Flowering in alfalfa hay fields: delayed harvesting and uncut field corners

their study. Outcrossing from feral populations to the white-flowered clones planted in the field shows that it is possible for genes to move back from feral populations to cultivated fields. Back and forth gene flow from cultivated to feral populations allows feral populations to act as genetic bridges for transgene movement. Likewise, Knispel et al. (2008) documented the accumulation of transgenes in feral roadside *Brassica napus* populations in southern Manitoba. Any bridging capacity, however, is mitigated via the dilution effect caused by relatively low pollen loads from feral plants to the cultivated fields (Van Deynze et al. 2008).

My results show that roadside alfalfa populations are common in southern Manitoba and that transgene movement to and from these populations and cultivated alfalfa fields occur under typical southern Manitoba growing conditions. If there is a need to confine traits in cultivated alfalfa fields then the strict implementation of best management practices, including the active management of feral alfalfa populations will be required to reduce the adventitious presence (AP) levels for specific traits. Seed production in feral alfalfa populations need to be prevented, if cutting is delayed and substantial flowering is possible in adjacent hay fields. However, the extent to which feral populations need to be managed will depend on the threshold levels of GM-AP allowed in non-GM crops. Realistic and workable threshold levels should be established for markets sensitive to the presence of novel traits. Landscape level gene flow models may be helpful in determining threshold levels and my findings will be useful in such models.

7.0 Genetic Diversity of Feral Alfalfa (*Medicago sativa* L.) Populations Occurring in Manitoba, Canada and Comparison With Alfalfa Cultivars: An Analysis Using SSR Markers and Phenotypic Traits

7.1 Abstract

Feral populations of cultivated crops may act as reservoirs for novel traits and aid in trait movement across the landscape. Knowledge on the genetic diversity of feral populations may provide new insights into their origin and evolution and may help in the design of efficient novel trait confinement protocols. In this study, the genetic diversity of 12 feral alfalfa (*Medicago sativa* L.) populations originating from southern Manitoba (Canada) and 10 alfalfa cultivars and a *M. falcata* germplasm were investigated using eight SSR markers (i.e. microsatellites) and 14 phenotypic traits. I found that the genetic diversity observed in feral populations was similar to the diversity detected among the 10 cultivars. Analysis of molecular variance (AMOVA) revealed that there was great genetic variation within (99.8%) rather than between different feral populations. Cluster analysis (UPGMA) revealed no differentiation between feral populations and cultivars for neutral loci. High levels of population differentiation for phenotypic traits (and not for neutral markers) suggest the occurrence of heterogeneous selection for adaptive traits. The phenotypic traits I studied did not distinctly separate feral populations from cultivars but there was evidence of natural selection pressure in feral populations for adaptive traits including winter survivability, rhizome production and prostrate growth habit. My results have implications for the risk assessment of alfalfa containing novel genetically modified (GM) traits and also for the conservation of plant genetic resources.

7.2 Introduction

Feral crops are those that escape cultivation and establish self-perpetuating populations in unmanaged habitats (Bagavathiannan and Van Acker 2008a) (Chapter 2.0). Crop ferality is an important phenomenon in the evolution of domesticated crops (Gressel 2005a). Feral forms of cultivated crops constitute a part of the metapopulations of cultivated crops (Van Acker 2007) and they can facilitate gene flow and novel trait movement (Knispel et al. 2008). Understanding the genetic diversity of feral populations can provide new insights into their origin (Burger et al. 2006) evolution (Campbell and Snow 2009), gene flow (Jenczewski et al. 1999a), colonization, invasiveness and extinction (Sakai et al. 2001). Knowledge on the genetic structure of feral populations has applications in the risk assessment of novel genetically modified (GM) crops (Raybould et al.1998).

Feral forms of cultivated crops are typically colonizing competitive habitats and the selection pressure is substantial in these environments (Sakai et al. 2001). Environmental stresses may increase the opportunity for selection and thereby enhance the evolutionary potential and differentiation among plant populations (Hoffmann and Hercus 2000; Stanton et al. 2000). Further, abiotic environmental stresses shape life histories of plant populations (Parsons 1990) and the traits selected during domestication are likely to be selected against in unmanaged habitats (Gressel 2005b). Genetic diversity is viewed as an important aspect to understand the dynamics of plant populations (Maron et al. 2004) and reduced genetic diversity may limit the ability of the populations to evolve (Gutierrez-Ozuna et al. 2009) and persist (Jump et al. 2008) in nature. Gene flow and heterogeneous selection (i.e. selection at specific loci) may play crucial roles in maintaining high levels of genetic variation found in natural populations (Jenczewski et al. 1999a; Yeaman and Jarvis 2006). Conversely, genetic bottlenecks resulting from a small number of founding individuals, limited gene flow, predominant self-fertilization and genetic drift may lead to reduced diversity and genetic disequilibrium (Glover and Barrett 1987; Husband and Barrett 1991).

Neutral genetic variation (i.e. variation at non-adaptive loci) reveals the demographic and genetic history of populations (Flajoulot et al. 2005; reviewed in Holderegger et al. 2006). As such, neutral markers have applications in studying gene flow and genetic

diversity (Bruschi et al. 2003), although they have limited use in investigating species fitness and evolutionary potential of populations (Conner and Hartl 2004). Phenotypic assessment can provide a direct and easy estimation of selection and evolution in plant populations (i.e. variation at non-neutral loci). However, intra-specific phenotypic differences can be incorrectly interpreted as evidence of different biotypes (Fritz et al. 2005). Further, variation for phenotypic traits does not necessarily reflect the genetic diversity of populations (Chiari et al. 2009). As such, investigations using both molecular markers and phenotypic traits can reveal a great deal of information about populations.

Alfalfa (*Medicago sativa* L.) is a perennial autotetraploid, allogamous species with 2n = 4x = 32. To study the genetic diversity of alfalfa, randomly amplified polymorphic DNA (RAPD) (Jenczewski et al. 1999b), amplified fragment length polymorphism (AFLP) (Greene et al. 2008) and Simple Sequence Repeat (SSR) (Flajoulot et al. 2005) markers have been used. Among these, SSR markers are efficient because they are highly polymorphic, co-dominant and are abundant in the genome (Tautz 1989). SSR markers have been first developed in *M. sativa* by Diwan et al. (1997). Other SSRs originating from *M. truncatula* have been used also to amplify and reveal polymorphism in alfalfa (Julier et al. 2003; Sledge et al. 2005). Since then a growing number of studies have used SSR markers for estimating the genetic diversity in alfalfa (e.g. Flajoulot et al. 2005). Wright's *F*-Statistics (Wright 1951) is the most commonly used measure to describe the amount of genetic variation found among populations. The availability of tools for analyzing co-dominant markers in autotetraploids (Thrall and Young 2000; Hardy and Vekemans 2002) has advanced the use of molecular markers in alfalfa. In addition, several studies have utilized phenotypic traits as a valuable tool for understanding the genetic diversity of different alfalfa populations (e.g. Crochemore et al. 1998).

In North America, alfalfa is an important forage crop and feral alfalfa populations are commonly observed in roadside habitats in alfalfa growing regions (Kendrick et al. 2005). Feral alfalfa populations may facilitate novel trait movement and as such thorough investigations on the nature of these populations is warranted. Studies have been carried out to assess differences among natural and cultivated alfalfa populations (Jenczewski et al. 1998, 1999a,b; Muller et al. 2003; Greene et al. 2008). However, no study has yet

established the genetic diversity of feral alfalfa populations occurring in roadside habitats in regions where alfalfa is cultivated in neighboring fields

The aim of this investigation was to estimate the genetic diversity of feral and cultivated alfalfa populations in Manitoba, Canada using SSR markers and phenotypic traits. The study was intended to provide answers, in part, to a number of important questions including: a) What is the likely origin of feral alfalfa populations? b) What is the degree of genetic diversity in feral compared to cultivated alfalfa populations? c) Is there any indication of gene flow among feral and cultivated populations? and d) Is there any evidence of natural selection in feral alfalfa populations?

7.3 Materials and methods

7.3.1 Molecular characterization

7.3.1.1 Plant material and DNA extraction

Twelve feral alfalfa populations originating from roadside habitats in three municipalities [Hanover (49° 28' N; 96° 50' W), MacDonald (49° 40' N; 97° 30' W) and Springfield (49° 55' N; 96° 45' W)] in southern Manitoba were included in the study (i.e. four feral population in each municipality). Adjacent feral populations were at least 4 km apart from each other. In addition, 10 alfalfa cultivars [Viking (VIKI), Ranger (RANG), Provence (PROV), Rangelander (RLAND), Grimm (GRIM), Vernal (VERN), ACLongview (ACLO), Beaver (BEAV), Algonquin (ALGO), Haygrazer (HAYG)] and a *M. falcata* (FALC) germplasm were chosen to represent a wide range of genetic backgrounds and years of release (Table 7.1). Among these cultivars, RANG, PROV and FALC are considered outgroups since they are not widely cultivated in my study region.

In each feral population, leaf tissue samples were collected from 30 randomly selected individuals and these were stored at -80^{o}C prior to DNA extraction. The DNA from feral populations was extracted following the method described by Mahuku (2004), which uses TES [Tris-Hcl, ethylene-diamine-tetra acetic acid (EDTA) and sodium dodecyl sulfate

Table 7.1 Characteristics of the alfalfa populations investigated in this study

S.No	Population	Year Available	Root type	Use	Flower type	Other remarks
1	Feral alfalfa	-	-	-	Variegated	Collected from the roadside habitats in the rural municipalities of Springfield, Hanover and MacDonald* (4 sub-populations at each location)
2	Viking	-	Taproot	Hay	Variegated	Medium winter tolerance
3	Ranger	1942	Taproot	Hay/pasture	Variegated	High winter tolerance
4	Provence	1950s	Taproot	Hay	Purple	French landrace adapted to Mediterranean climate, low winter tolerance
5	Rangelander	1978	Creeping	Hay/pasture	Variegated	Medium winter tolerance
6	Grimm	1903	Taproot	Hay	Variegated	High winter tolerance
7	Vernal	1953	Taproot	Hay	Variegated	High winter tolerance
8	AC Longview	1999	Taproot	Hay	Purple	Medium winter tolerance
9	Beaver	1961	Taproot	Hay/pasture	Variegated	Medium winter tolerance
10	Algonquin	1972	Taproot	Hay/pasture	Variegated	Medium winter tolerance
11	Haygrazer	2002	Branch	Hay/pasture	Variegated	Medium winter tolerance
12	*M. falcata*	-	Creeping	Pasture	Yellow	High winter tolerance

*Municipalities belong to southern Manitoba, Canada

(SDS)] extraction buffer. In this method, the extracted DNA was precipitated using isopropanol and stored at -20^{o}C in eppendorf tubes in the form of pelletes. The cultivars were grown in a greenhouse at INRA, Lusignan, France, and young leaf samples were collected from 30 individuals in each population. The DNA from alfalfa cultivars was extracted following the method described by Cheung et al. (1993), using the CTAB (hexa-decyl-trimethyl-ammonium bromide) extraction buffer. The DNA was precipitated using isopropanol and stored in TE buffer (10mM Tris, 1mM EDTA). A total of 690 individuals were studied from 23 populations.

7.3.1.2 SSR analysis

SSR analysis was carried out using eight primer pairs originating from *M. truncatula*, selected based on their position on the genetic linkage map (Julier et al. 2003; see Table 7.2 for details of the primers). Magali and Gabès, parents of a mapping population with already available dose information for each allele were used as positive controls in the study. PCR amplification, gel electrophoresis and scoring were carried out as per Flajoulot et al. (2005), allowing scoring of allele doses. The PCR products were separated using a 6.5% polyacrylamide gel in the automated DNA sequencer LI-COR IR^2 (LI-COR Inc.) and the gel images were scored using the GENE PROFILER software (Scanalytics Inc.)

7.3.1.3 Analysis of SSR data

To estimate the level of genetic diversity present in the populations from SSR data, I used the software AUTOTET (Thrall and Young 2000). For each population and for each SSR locus, the following genetic diversity indices were calculated using AUTOTET:

i) *A* (Allelic richness) = the sum of all unique alleles detected per locus

ii) *Ai* (Allelic richness within individuals) = average number of alleles per individual at a locus

Table 7.2 Simple sequence repeat primers used for alfalfa DNA amplification

Marker name	Linkage group	Primers (5'– 3')		Tm (^{o}C)	Allele size in alfalfa (bp)
FMT13	1	Forward	GATGAGAAAATGAAAAGAAC	50	162-204
		Reverse	CAAAAACTCACTCTAACACAC		
B14B03	2	Forward	GCTTGTTCTTCTTCAAGCTCAC	55	163-215
		Reverse	CTGACTTGTGTTTTATGC		
ATP456	3	Forward	GGGTTTTTGATCCAGATCTT	55	131-173
		Reverse	AAGGTGGTCATACGAGCTCC		
MTIC451	4	Forward	GGACAAAATTGGAAGAAAAA	55	145-181
		Reverse	AATTACGTTTGTTTGGATGC		
MTIC338	5	Forward	TCCCCTTAAGCTTCACTCTTTTC	55	167-194
		Reverse	CATTGGTGGACGAGGTCTCT		
MTIC82	6	Forward	CACTTTCCACACTCAAACCA	50	140-167
		Reverse	GAGAGGATTTCGGTGATGT		
MTIC343	7	Forward	TCCGATCTTGCGTCCTAACT	55	137-167
		Reverse	CCATTGCGGTGGCTACTCT		
MTIC432	8	Forward	TGGAATTTGGGATATAGGAAG	55	175-243
		Reverse	GCCATAAGAACTTCCACTT		

Tm: melting temperature

iii) *G* (Genotypic richness) = the number of four allele genotypes at a locus

iv) H_o (Observed heterozygosity), and

v) H_E (Expected heterozygosity)

I performed Wilcoxon Mann-Whitney test (rank sums) on the genetic diversity indices *A*, *Ai*, *G* and H_E to compare the level of genetic diversity in feral populations and cultivars.

AUTOTET also computed the fixation coefficient (*F*), which is a measure of departure from Hardy-Weinberg expectations. *F* was calculated using the following formula:

$F = 1 - (H_o / H_E)$

In alfalfa, double reduction is low and random chromatid segregation is infrequent (Julier et al. 2003). To account for this, H_o and H_E were calculated based on the assumption of random chromosome segregation for each locus and for each population. I applied Bonferroni's correction (family wise error rate) to test for departure from Hardy-Weinberg expectations for each population for all seven SSR loci studied. Bonferroni's correction was carried out by adjusting the α value for a set of *n* comparisons to α/*n* in order to account for the number of comparisons being performed (Shaffer 1995). An SSR null allele is an allele that fails to amplify to detected levels during PCR reaction (Dakin and Avise 2004) and in my study, the frequency of null alleles was estimated for each SSR locus and for each population as per Brookfield (1996).

$$r = (H_E - H_o) / (1 + H_E)$$

The level of differentiation among the populations was determined based on the genetic differentiation coefficient (F_{ST}), computed using the software SPAGeDi 1.2g (Hardy and Vekemans 2002). F_{ST} represents the amount of genetic variance detected in a population in relation to the total genetic variance found over all the populations compared, and high values of F_{ST} mean a high degree of differentiation among the populations. Cluster analysis was performed by means of UPGMA (unweighted pair-group method using arithmetic average) clustering procedure, using the NTSYS-PC package (Rohlf 2000). Hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992) was carried out using the program 4xNested, which extended AMOVA to autotetraploids

(Jenczewski et al. 1999b). AMOVA was performed after excluding rare alleles in the dataset (alleles with less than five fragments over 690 individuals).

7.3.2 Phenotypic characterization

7.3.2.1 Experimental design and data collection

A field study was established in the summer of 2008 at 'The Point' research facility of the University of Manitoba, Winnipeg, Canada (50°38'N 96°19'W). The site was characterized by a deep clay soil, and a continental climate with warm summers and cold winters. In 2008, the site received a mean monthly precipitation of 16 cm (SD = 1.4 cm), about 11% of which was received in the form of snow fall. The mean monthly soil temperature was 6.7^{o}C (SD = 9.5^{o}C).

The trial included all 23 populations used in the SSR analysis (12 feral populations, 10 alfalfa cultivars and a *M. falcata* germplasm collection) arranged in a randomized complete block design with three replicates. Each population per replication consisted of 20 seedlings space planted with 50 cm separating adjacent plants in all directions.

The seedlings were initially established in small potted trays under room temperature in a greenhouse during the spring of 2008. Once the seedlings were well established, they were cut at about 2cm above the soil surface immediately prior to transplanting to the field.

Phenotypic data were obtained from five (summer/fall 2008 observations) or ten (spring 2009 observations) plants per replication in each population with the exception of winter survival and regrowth ground cover, which were estimated on a per plot basis (i.e. 20 plants). Summer/fall observations were carried out between early August and late September 2008, while the spring observations were conducted in early June 2009. All the data were collected from the same plant except when the plant was dead following the winter. Data on plant height (PTHT), number of shoots (NSHT), days to first flowering (FIFL), days to 50% flowering (50FL), plant dry weight (DRWT) were estimated in summer/fall 2008. In addition, ordinal data on growth habit (1-prostrate to 10-erect)

(GHAB), colonization (1-full to 5-poor) (COLO), flower color (1-purple; 2-variegated purple; 3-variegated yellow; 4-yellow) (FCLR) and pod shape (1-sickle to 5-coiled) (PDSH) were also determined in summer/fall 2008. Data on winter survival (% of plants survived during 2008 winter) (WSUR), rhizome production (% of plants with rhizome/root proliferation) (RHIZ), regrowth height (height of the regrowth following winter) (RGHT), regrowth width (RGWI) and regrowth ground cover (% of the plot area covered during spring regrowth) (GCOV) were recorded in spring 2009. Ground cover was estimated based on digital images of the plots using 'Assess'software (Lamari 2002).

7.3.2.2 Analysis of phenotypic data

The phenotypic variables were analyzed using the Statistical Analysis Software (SAS) version 9.1 (SAS Institute 2003), using a mixed model analysis of variance (ANOVA) (PROC MIXED, Littell et al. 1996). Prior to ANOVA, outliers were removed using the studentized residual values as per Lund's table (Lund 1975). Normality of the residuals was confirmed using the Kolmogorov-Smirnov test. Populations were considered as a fixed effect and replication was considered a random effect. To conform to normality, data on number of shoots plant^{-1} and days to 50% flowering were transformed using square root transformation.

Non-normally distributed data or ranked data should be analyzed using non-parametric tests, which are based on ranks rather than continuous data (Shannon et al. 2009). Kruskal-Wallis test is a non-parametric one-way analysis of variance (Kruskal and Wallis 1952). Therefore, statistical significance of the variables growth habit, colonization, days to 50% flowering, flower color and pod shape were tested using Kruskal-Wallis test (Wilcoxon rank sum) using the NPAR1WAY procedure of SAS. The difference between ferals and cultivated populations was determined using a single degree of freedom contrast.

Phenotypic correlation among the phenotypic variables was performed using the CORR procedure in SAS. Bonferroni's family wise correction was applied to the correlation output, while determining the significance of the effects. I estimated the genetic correlations using the variance-covariance matrices generated by multivariate analysis of

variance (Manova) in PROC GLM of SAS. To derive the important combinations of the set of phenotypic variables, I carried out a principal component analysis (PCA) using the PRINCOMP procedure of SAS. The Euclidean distance matrix of the populations for the phenotypic variables was computed using the DISTANCE procedure of SAS. Because of high levels of variance, the phenotypic data were standardized before performing PCA and the Euclidean distance analysis.

7.4 Results

7.4.1 SSR marker analysis

7.4.1.1 Genetic diversity

The SSR loci used in my study were highly polymorphic. An example of SSR variation detected in my study population is given in fig.7.1. The marker Mtic 432 was excluded from further analysis since it produced a ladder profile, which was hard to score. A total of 119 alleles were detected at seven SSR loci. The number of alleles detected per locus ranged from 10 (for M338) to 22 (for B14B03) with an average of 16.7 alleles per locus. The frequency of the most frequent and infrequent alleles followed a similar pattern across all populations for each of the seven SSR loci. Null alleles were detected at low frequencies (*r*) ranging from -0.046 to 0.189 across all populations for all the SSR loci. Among different loci, *r* was the lowest in FMT13, ranging from -0.03 to 0.038.

High levels of genetic diversity were detected in feral populations. Allelic richness (*A*) calculated for each feral population ranged from 10.43 alleles per locus in SPR3 to 12.0 alleles per locus in HAN 3 and HAN 4 (Table 7.3). Allelic richness within individuals (*Ai*) and genotypic richness (*G*) did not vary substantially among the feral populations (Table 7.3). Assuming chromosome segregation, the mean values of H_E detected for each feral population over seven SSR loci ranged from 0.73 to 0.77, respectively, showing greater levels of within population variability.

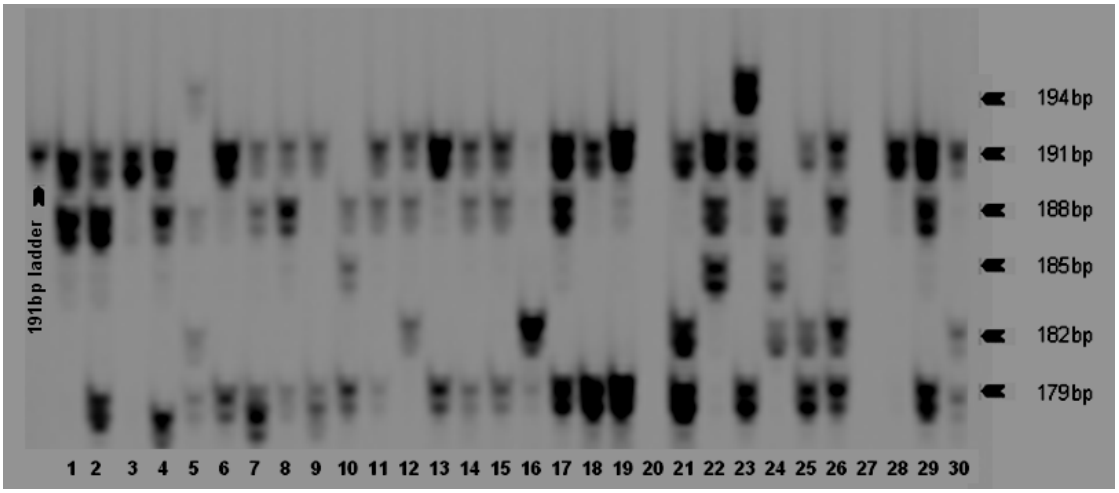

Fig. 7.1 Gel plate showing SSR variation on 30 individual alfalfa plants (locus MTIC338). Black arrows indicate different alleles. The dose of each allele was scored based on its intensity within each lane. For example, the individual in lane 26 had four alleles, each in a single dose; the individual in lane 2 had three alleles, in doses 1/2/1 from the bottom; the individual in lane 13 had two alleles, in doses 1/3 from the bottom, the individual in lane 1 had two alleles, each in double dose; and the individual in lane 28 had one allele in quadruplet dose

Table 7.3 Levels of genetic diversity estimated for feral and cultivated alfalfa populations for all microsatellite loci

Population	*A*	*Ai*	*G*	H_o*	H_E*	*F**	*D*
Ferals							
Springfield1	11.14	2.75	21.71	0.73	0.75	0.020	0.748
Springfield2	11.14	2.78	20.43	0.72	0.74	0.031	0.743
Springfield3	10.43	2.72	22.71	0.71	0.75	0.053	0.749
Springfield4	11.43	2.79	23.71	0.73	0.77	0.053	0.771
Hanover1	11.71	2.73	21.86	0.72	0.75	0.045	0.754
Hanover2	10.71	2.59	20.57	0.69	0.76	0.095	0.762
Hanover3	12.00	2.76	23.29	0.74	0.77	0.045	0.769
Hanover4	12.00	2.78	23.29	0.73	0.77	0.054	0.772
MacDonald1	11.43	2.80	23.29	0.74	0.77	0.047	0.771
MacDonald2	10.71	2.58	22.29	0.67	0.73	0.081	0.726
MacDonald3	11.43	2.67	21.29	0.69	0.73	0.055	0.726
MacDonald4	11.29	2.76	23.71	0.72	0.76	0.054	0.762
All ferals	15.71	2.73	152.71	0.72	0.76	0.063	0.764
Cultivars							
Viking	10.00	2.55	21.57	0.67	0.73	0.083	0.730
Ranger	10.14	2.76	21.14	0.73	0.74	0.011	0.738
Provence	10.43	2.76	21.29	0.73	0.72	-0.022	0.716
Rangelander	11.86	2.83	23.57	0.76	0.78	0.032	0.781
Grimm	10.86	2.68	20.86	0.70	0.73	0.033	0.726
Vernal	11.86	2.63	22.29	0.69	0.77	0.102	0.768
ACLongview	11.29	2.83	22.86	0.75	0.78	0.040	0.780
Beaver	10.86	2.80	22.86	0.75	0.77	0.032	0.770
Algonquin	10.86	2.73	22.00	0.73	0.74	0.021	0.745
Haygrazer	10.43	2.91	21.43	0.78	0.76	-0.023	0.764
All cultivars	15.86	2.75	132.43	0.73	0.76	0.046	0.764
Global	16.71	2.74	222.29	0.72	0.77	0.057	0.765

A - allelic richness (number of alleles detected per locus); *Ai* - allelic richness within individuals (average allele per individual at a locus), *G* - genotypic richness (number of four allele genotypes at a locus); H_o - observed heterozygosity; H_E - expected heterozygosity; *F* - fixation coefficient; *D* - Mean expected gene diversity
*based on the assumption of chromosome segregation

The Wilcoxon Mann-Whitney test did not detect any significant differences for the genetic diversity indices *A*, *Ai*, *G*, H_E between feral and cultivated populations, indicating that the genetic diversity present in feral populations was the same as the diversity observed in cultivars.

The fixation coefficient *F* ranged from 0.020 to 0.095 in ferals and from -0.022 to 0.102 in cultivars. After applying Bonferroni's correction for each SSR loci and for each population, I did not observe great departures from Hardy-Weinberg expectations of random mating equilibrium. One exception to this was the *M. falcata* population which deviated from the equilibrium for all seven SSR loci studied.

7.4.1.2 Genetic differentiation

The values of the genetic differentiation coefficient (F_{ST}) were very low. The calculated F_{ST} over all loci were 0.002 and 0.008 for feral populations and cultivars respectively (Table 7.4). The F_{ST} values computed for each pairwise combination among the 22 populations ranged from 0 to 0.034. The cluster analysis (UPGMA) also showed small differentiation among the populations (Fig. 7.2). Analysis of molecular variance (AMOVA) revealed that 99.8% of the total variation was accounted for by the individuals within feral populations and only 0.2% of the variation was explained by the locations.

7.4.2 Phenotypic characterization

7.4.2.1 Population effects

ANOVA or non-parametric Kruskal-Wallis test revealed highly significant differences among the populations (Table 7.5). In my study, feral populations (as a group) were significantly different from cultivars for all the phenotypic traits except for plant height, pod shape, dry weight and regrowth height. Phenotypic and genetic correlations performed on 14 phenotypic variables measured on individual plants were largely similar

Table 7.4 F_{ST} estimates of genetic differentiation for seven SSR loci and overall loci, evaluated for 12 feral populations and 10 cultivars (excluding *M. falcata*)

Locus	F_{ST}	
	Ferals	Cultivars
MTIC343	-0.0014	0.0043
B14B03	0.0052	0.0063
MTIC82	0.0045	0.0254
ATP456	0.0014	0.0122
MTIC451	0.0008	0.0013
FMT13	-0.0014	0.0027
MTIC338	0.0080	0.0110
Overall loci	0.0022	0.0084

Table 7.5 Estimates of phenotypic variables measured on all the 23 alfalfa populations used in the study

Variable	Over all populations				Ferals	Cultivars	Ferals vs. Cultivars
	Mean (SE)	Chi-square value	F value	*P* value	Mean (SE)	Mean (SE)	(*P* value)
Plant height (cm)	66.1 (1.91)	-	5.92	<0.0001	66.6 (2.43)	65.8 (3.1)	0.6491
Number of shoots[ǂ]	11.1 (0.51)	-	5.09	<0.0001	11.9 (0.68)	10.2 (0.67)	0.0008
Growth habit (1-10)[†]	4.4 (0.32)	55.5	-	0.0001	3.9 (0.28)	4.9 (0.57)	n/a
Colonization (1-5)[†]	3.2 (0.16)	53.3	-	0.0002	2.9 (0.13)	3.4 (0.29)	n/a
Days to first flowering[ǂ]	44.9 (0.76)	-	2.16	0.0149	44.7 (0.81)	46.9 (1.36)	<0.0001
Days to 50% flowering[†]	67.7 (1.07)	41.2	-	0.0078	68.6 (1.42)	66.6 (1.63)	n/a
Flower color (1-4)[†]	1.9 (0.11)	239.9	-	<0.0001	2.0 (0.02)	1.9 (0.23)	n/a
Pod shape (1-5)[†]	4.3 (0.16)	37.3	-	0.0220	4.3 (0.07)	4.2 (0.33)	0.3616
Plant dry weight (g)	83.5 (2.95)	-	4.71	<0.0001	82.6 (4.81)	83.0 (3.49)	0.8908
Winter survival (%)	78.9 (3.85)	-	15.47	<0.0001	86.3 (1.54)	70.6 (7.26)	<0.0001
Rhizome production (%)	57.9 (4.40)	-	24.52	<0.0001	62.7 (2.42)	47.8 (9.15)	<0.0001
Regrowth height (cm)	43.0 (0.74)	-	7.06	<0.0001	43.2 (1.05)	42.3 (1.09)	0.3276
Regrowth width (cm)	66.3 (1.36)	-	4.39	<0.0001	67.1 (1.27)	66.1 (2.48)	0.0484
Regrowth ground cover (%)	85.8 (3.45)	-	21.22	<0.0001	91.6 (1.08)	79.4 (6.73)	<0.0001

[†] Non-parametric analysis using Kruskal-Wallis test

[ǂ] Data square root transformed

Growth habit (1- prostrate; 10 - erect), Colonization (1 - full; 5 - poor), Flower color (1 - purple; 2 - variegated purple; 3 - variegated yellow; 4 - yellow), Pod shape (1 - sickle shape; 5 - coiled)

n/a - estimates were not possible due to non-normal distribution

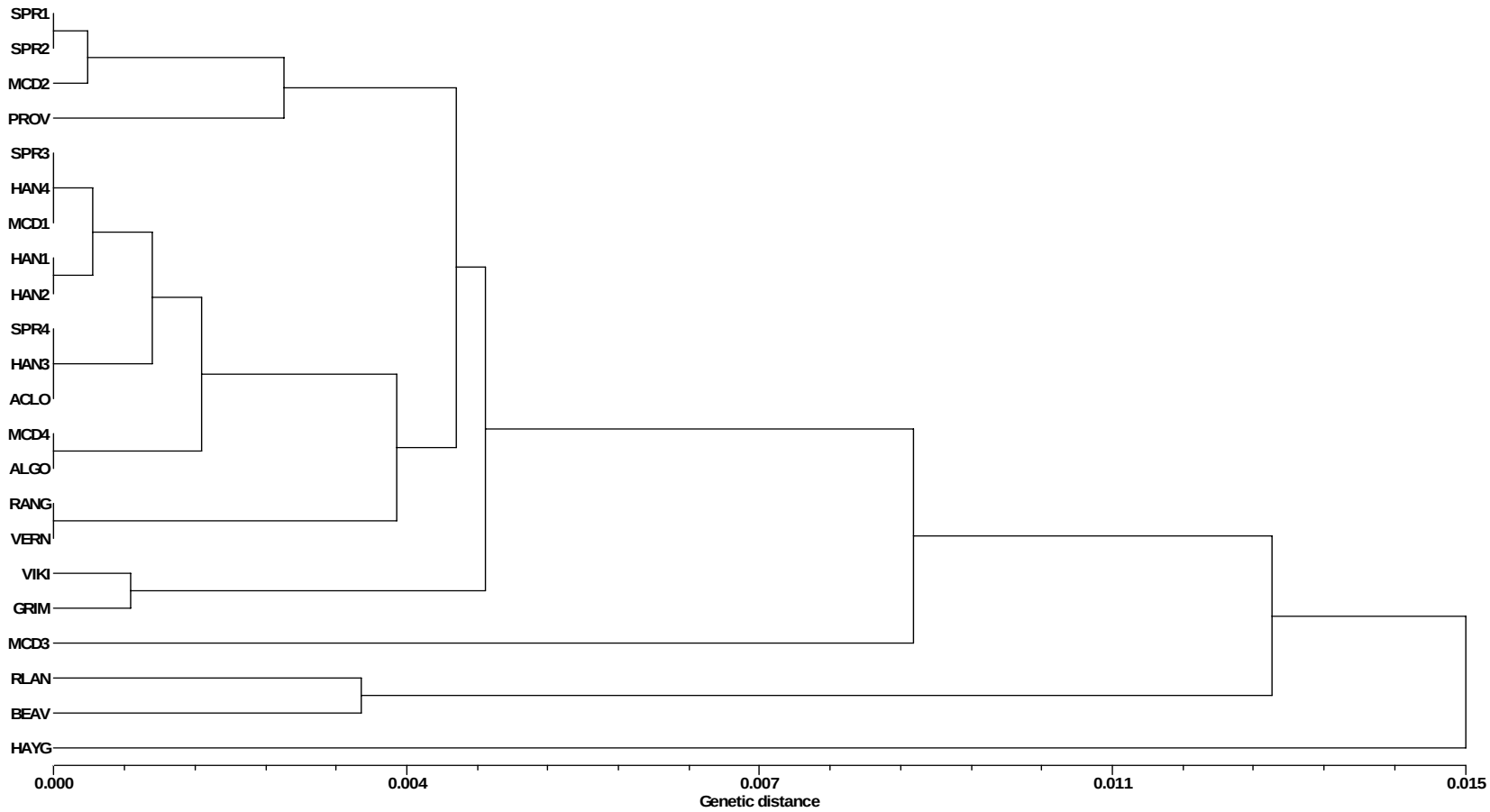

Fig. 7.2 Dendrogram of the genetic distances among different populations, computed based on pairwise F_{ST}

(Table 7.6). In both phenotypic and genetic correlations, the traits such as winter survival, rhizome production, colonization, regrowth ground cover and growth habit were highly associated with each other (Table 7.6).

7.4.2.2 Population differentiation

Phenotypic variables showed substantial differentiation among the populations (Fig. 7.3 a,b). *M. falcata* and Provence were excluded from PCA analysis because the overall variation among the populations was masked by these populations (data not shown). The first three principal component axes explained 65.6% of the total variation (36.4%, 17.9% and 11.3%, respectively).

The phenotypic variables WSUR, RHIZ and GCOV were positively correlated with the principal component1 (PC1) while the variables COLO and GHAB were negatively correlated (Fig. 7.3b). The principal component 2 (PC2) was positively associated with the variables PDSH and 50FL and negatively with RGWI and DRWT.

The fourteen phenotypic variables did not separate feral populations from cultivars in the PCA. Nevertheless, there was a dramatic separation among the populations (Fig. 7.3 a,b). Cultivars were scattered along the first axis but many of the feral populations had greater values on this axis because of high winter survival (Fig. 7.4), rhizome production, colonization and ground cover. Six of the feral populations were closely associated with the three cultivars (RLAND, GRIM and BEAV) that showed greater winter survival in my study (Fig.7.3 a,b). However, the feral populations were scattered along the second axis.

7.4.3 Comparison between SSR markers and phenotypic traits

The relationship between the Euclidean distances measured using phenotypic variables and the distances measured using SSR markers (as per Crochemore et al. 1998) is given in fig. 7.5. No correlation was found. Generally, the values of phenotypic differentiation were greater than genetic differentiation measurement based on neutral SSR markers.

Table 7.6 Phenotypic (lower triangle) and genetic (upper triangle) correlation for 14 phenotypic variables measured in 23 experimental alfalfa populations

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Plant height		0.05	0.24	0.31	-0.38	-0.34	-0.38	0.47	0.69	-0.24	-0.30	0.61	-0.16	-0.17
Number of shoots plant^{-1}	0.04		0.12	0.11	-0.37	0.08	-0.34	0.41	0.04	0.00	-0.01	0.28	-0.42	-0.02
Growth habit	0.23	0.18		0.98	-0.58	-0.54	-0.70	0.64	0.12	-0.79	-0.84	0.59	-0.74	-0.71
Colonization	0.31	0.14	0.95*		-0.65	-0.59	-0.76	0.74	0.17	-0.75	-0.87	0.57	-0.66	-0.64
Days to first flowering	-0.31	-0.23	-0.39*	-0.45*		0.81	0.90	-0.83	-0.42	0.46	0.67	-0.91	0.30	0.30
Days to 50% flowering	-0.26	0.09	-0.36	-0.41*	0.64*		0.63	-0.54	-0.46	0.64	0.72	-0.46	0.26	0.54
Flower color	-0.3	-0.29	-0.63*	-0.66*	0.58*	0.45*		-0.95	-0.27	0.57	0.75	-0.69	0.67	0.44
Pod shape	0.37	0.35	0.54*	0.58*	-0.51*	-0.38	-0.88*		0.29	-0.45	-0.63	0.73	-0.59	-0.31
Dry wt	0.46*	0.01	0.10	0.14	-0.21	-0.37	-0.23	0.25		-0.43	-0.33	0.53	-0.09	-0.34
Winter survival	-0.22	0.07	-0.65*	-0.64*	0.35	0.44*	0.51*	-0.36	-0.32		0.89	-0.39	0.79	0.97
Rhizome production	-0.29	0.04	-0.7*	-0.72*	0.42*	0.44*	0.69*	-0.52*	-0.24	0.84*		-0.51	0.73	0.76
Regrowth height	0.30	0.17	0.30	0.29	-0.25	-0.24	-0.42*	0.41*	0.23	-0.18	-0.32		-0.27	-0.21
Regrowth width	-0.16	-0.22	-0.54*	-0.52*	0.15	0.17	0.52*	-0.4*	-0.12	0.59*	0.54*	-0.07		0.82
Ground cover	-0.13	0.05	-0.61*	-0.54*	0.23	0.35	0.42*	-0.24	-0.25	0.92*	0.72*	-0.09	0.59*	

*The effects are significant at $P \leq 0.005$ (determined using Bonferroni's family wise error correction)

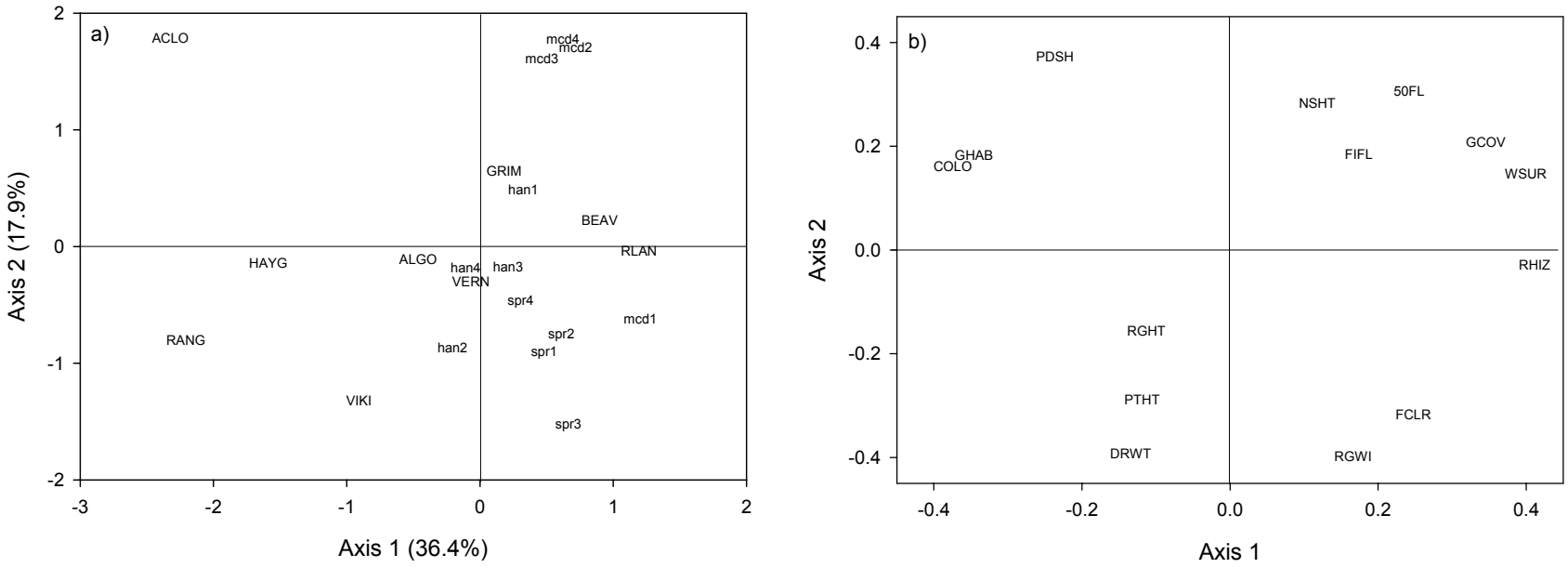

Fig. 7.3 a) PCA ordination of the distribution of 21 alfalfa populations (excluding Provence and *M. falcata*) on the first two axes, and b) the contribution of 14 different phenotypic traits on axis 1 and 2 of PCA. Populations denoted by upper case letters represent cultivars and those with lower case letters represent ferals (The definition of the acronyms can be found in the text).

Fig. 7.4 Differences in the level of winter survivability between feral populations and alfalfa cultivars (Row of plots on the left represent feral populations and the plots on the right represent alfalfa cultivars)

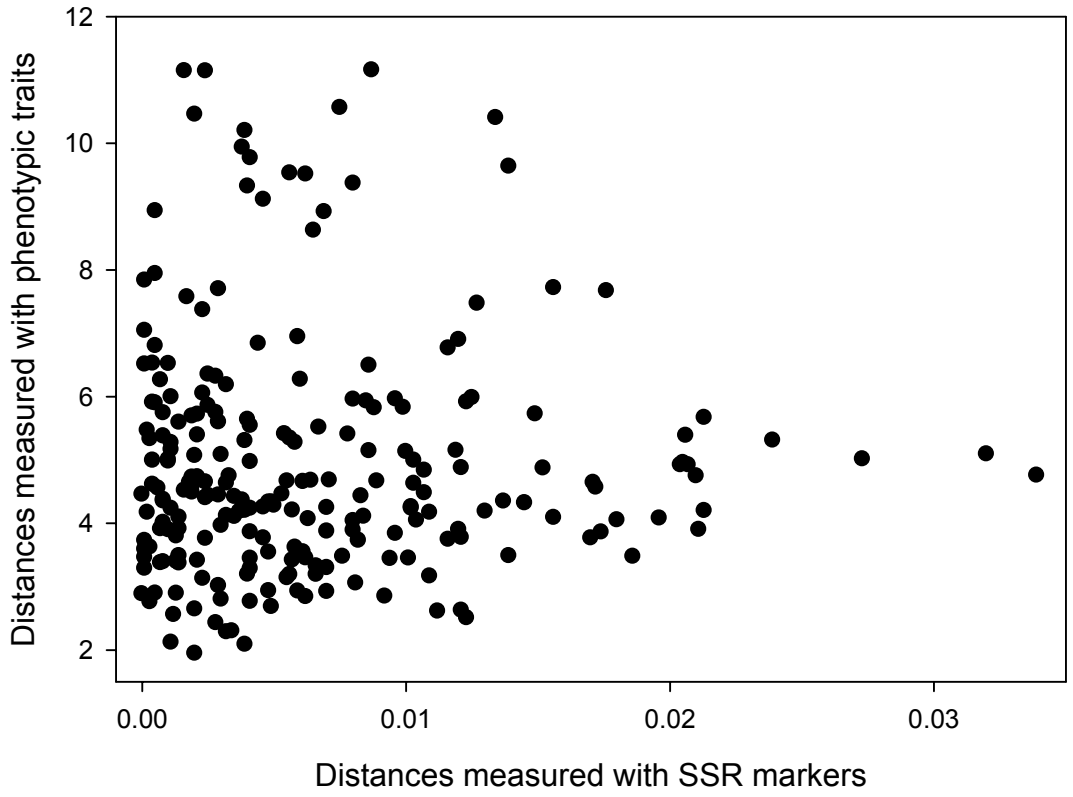

Fig. 7.5 Relationship between the distances (Euclidian distance) measured using seven SSR markers and 14 phenotypic traits for 22 alfalfa populations (excluding *M. falcata*).

In addition, the pattern of differentiation did not coincide for SSR markers and phenotypic traits (Figures 7.2, 7.3).

7.5 Discussion

In this study I present the first investigation of the genetic diversity of roadside feral alfalfa populations. The SSR markers used in this study were highly polymorphic and efficient in revealing the level of genetic diversity present in the populations studied. The genetic diversity detected in feral populations was comparable to that of the agricultural cultivars. High levels of genetic diversity in feral populations indicate that these populations are not experiencing genetic bottlenecks or drift, and may have been large enough to avoid the detrimental effects of genetic drift. In nature, the amount of genetic diversity found in plant populations is often a function of the rate of gene flow (Bruschi et al. 2003). In my study, however, it was hard to confirm the occurrence of gene flow among the populations based on high levels of genetic diversity because my study and several others (e.g. Julier et al. 2000) have demonstrated the existence of substantive genetic diversity within (rather than among) alfalfa populations. As such, gene flow from other populations may not be a requisite for the occurrence of the high levels of genetic diversity I found in feral populations. The preservation of the initial diversity present in the founding individuals may have been sufficient.

In plant populations, genetic diversity facilitates adaptation to a wide range of environmental conditions, while genetic bottlenecks and genetic drift may limit the ability of the populations to evolve and adapt (Jump et al. 2008; Gutierrez-Ozuna et al. 2009). In addition to low gene flow, bottlenecks could also be the result of a small founding population. The high genetic diversity (indicative of limited genetic bottlenecks or genetic drift) I observed in feral populations indicates that alfalfa populations are stable and persistent in roadside habitats. The existence of polyploidy and polysomic inheritance has favorable effects on the maintenance of high genetic diversity and thereby adaptation in alfalfa (Jenczewski et al. 1998; Soltis and Soltis 2000).

I did not find substantial differences between the two groups: feral and cultivated populations, for SSR markers. Further, overall differentiation among different populations was also very low. Low differentiation among alfalfa populations for neutral loci has been reported by other authors (e.g. Flajoulot et al. 2005), and may be due in part to the fact that neutral markers distinguish the populations based on the neutral genetic variation rather than adaptive traits. The majority of alfalfa cultivars that perform well in northern parts of North America were derived from a few accessions and they typically have a narrow genetic history (Manske 2005). This could have contributed to the lack of differentiation among the populations for SSR markers. A lack of distinct separation between ferals and cultivars for neutral loci indicates that feral populations and cultivars share the same genetic history and that the ferals originate from the cultivars. This is further supported by the fact that alfalfa is a crop introduced to North America and it has less than a 150 year history of cultivation in western Canada (Melton et al. 1988). In addition, wild progenitors of alfalfa do not occur in this region of North America. Therefore, farming activities (planting, harvesting and transport) and in many cases intentional roadside planting contributed to the origin of feral alfalfa populations in roadside habitats.

Greater population differences for phenotypic traits, and the lack for neutral SSR loci suggest that there was selection pressure on these populations for adaptive traits, and it is possible to have strong selection forces generate phenotypic differences at some selected loci, without changing the neutral genetic structure (Barton and Hewitt 1985). Similar results were also reported in the wild and cultivated *M. sativa* populations originating from Spain (Jenczewski et al. 1998). In addition, the patterns of differentiation were not similar between SSR and phenotypic markers. This could be attributed to the fact that SSR markers are neutral and forces such as mutation, genetic drift and genetic bottlenecks can only cause differentiation for these markers (Bruschi et al. 2003). Whereas, phenotypic differentiation is based on adaptive traits and the population differences I observed for phenotypic traits could have been the result of environmental selection (Rieseberg et al. 2002). A lack of strong correlation between phenotypic and molecular markers has been reported by other authors (e.g. Pissard et al. 2008).

Some studies have indicated the distinct phenotypic separation of wild or 'Mielga' alfalfa populations from alfalfa cultivars (e.g. Crochemore et al. 1998; Jenczewski et al. 1998; Julier et al. 1995) but similar reports do not exist for feral alfalfa populations. The phenotypic variables I investigated were unable to separate feral populations from cultivars. Nevertheless, there was evidence of selection in the feral populations for traits including high winter survival, high regrowth ground cover, rhizome production, high colonization and prostrate growth habit. My results corroborate Jenczewski et al. (1999a) and Prosperi et al. (2006) who reported the occurrence of natural selection for rhizome production and creeping growth habit in wild alfalfa populations originating from Europe. In my study, regrowth ground cover was highly influenced by the level of winter survival since ground cover was estimated per plot basis during the spring regrowth. In addition, the variables growth habit and colonization were largely inter-related.

Winter survival is an essential trait for alfalfa in western Canada due to the extreme cold winters common in this region. High winter survival may favor the long-term persistence of feral populations in roadside habitats. I noted the regeneration of plants through rhizome/root proliferation even when the crown was dead (personal observation) (Fig. 7.6). Rhizome/root proliferation is a trait associated with drought tolerance and persistence in alfalfa (Heinrichs 1963; Berdahl et al. 1986, 1989; Prosperi et al. 2006). Prostrate growth habit, on the other hand, may aid the competitiveness of feral alfalfa populations over the surrounding vegetation in unmanaged habitats.

High winter survival, rhizome production and prostrate growth are all associated with *M. falcata* (Barnes et al. 1977; Berdahl et al. 1989; Jenczewski et al.1998) and *M. falcata* germplasm has been used to enhance the adaptation and persistence of cultivated alfalfa. Most of the successful cultivars grown in western Canada are hybrids between *M. falcata* and *M. sativa* (known as variegated types) and some cultivars are more persistent over others (Heinrichs 1963; Katepa-Mupondwa et al. 2002). Natural selection in roadside habitats may act in a parallel manner to artificial selection. The level of adaptation and persistence of alfalfa populations may be associated with the degree of genetic material

Fig. 7.6 Regeneration of alfalfa through rhizome/root proliferation

contributed by *M. falcata* (Berdahl et al. 1989). In this regard, natural selection for individuals with high parentage from *M. falcata* may facilitate the persistence of feral alfalfa populations in roadside habitats.

The rate of adaptive trait based change in alfalfa populations may depend on the length of time available for selection pressure to act on the populations (Bousquet et al. 1992; Charlesworth and Wright 2001) and this perhaps explains the differences I observed among the feral populations in terms of the adaptive traits. In addition, gene flow from cultivated populations may slow down the rate of evolution in this regard (Jenczewski et al. 1998). Therefore it is most likely that older feral populations have had better opportunities for evolution than recently established populations. My results are in agreement with Berdahl et al. (1986) who demonstrated high levels of winter survival in alfalfa populations persisting in rangelands for over 50 years. It is also possible that good winter survival results from the establishment of a feral population by seeds from a cultivar, which already has high winter survival. However, strong selection may still be necessary to maintain such morphological integrity because gene flow may influence the divergence of these populations from their original shape (Jenczewski et al. 1998). My results suggest that natural selection may play an important role in shaping roadside alfalfa populations. Furthermore, high levels of genetic diversity may assist the maintenance of evolutionary potential of feral populations as long as the population sizes are sustained (Bruschi et al. 2003; Gutierrez-Ozuna et al. 2009).

Overall, the results of this study show that feral alfalfa populations originate from alfalfa cultivars with high levels of genetic diversity are maintained within individual feral populations. Further, I provided evidence of natural selection for traits that favor persistence in roadside habitats. My results have implications for the confinement of novel traits in alfalfa and establishing co-existence between genetically modified (GM) and non-GM alfalfa fields. Because feral alfalfa populations originate from cultivars, stewardship practices should include the prevention of seed escape during farming activities. Furthermore, intentional planting of alfalfa in roadside environments need to be avoided in areas where GM alfalfa is grown. Given the likelihood of feral alfalfa adaptation to and persistence in roadside habitats, feral alfalfa populations need to be

considered in novel trait confinement protocols because they may act as sources and sinks for novel traits and may aid in trait movement. My findings also have implications for the conservation of plant genetic resources. Feral alfalfa populations may be regarded as a source of genetic material for plant improvement and attempts should be made to identify and conserve these resources.

8.0 General Discussion

I carried out an extensive characterization of feral alfalfa populations occurring in roadside habitats in southern Manitoba, Canada. This is the first study of its kind and my results have broad implications for the confinement of novel traits in alfalfa and establishing co-existence between genetically modified (GM) and non-GM alfalfa.

8.1 Salient findings

The survey revealed the widespread occurrence of feral alfalfa populations in roadside habitats, particularly in alfalfa growing regions. My results suggest that the feral populations I was working with were not genetically distinct from typical commercial alfalfa cultivars and are therefore typical escapes from cultivation. Such escape could happen during farming activities (i.e. planting, harvesting, transport operations, etc.) or through intentional planting in roadsides. Regardless of origin, my results also show that alfalfa can persist well in unmanaged habitats. I found roadside alfalfa seedbank, seedling recruitment and reproductive success of mature plants, indicating that alfalfa is capable of establishing self-perpetuating feral populations in unmanaged natural and semi-natural habitats.

Feral alfalfa populations are subject to different disturbances in roadside habitats. I observed that the reduction in plant density due to disturbances is compensated by an increase in number of shoots and reproductive attributes. It was evident that alfalfa can quickly recover from moderate disturbances in roadside habitats. For example, mowing can reduce/prevent reproduction but in my study populations it did not drive the populations to extinction in the short-term. Herbicide (2,4-D) controlled all the above ground parts but seeds in the seedbank may contribute to new seedling recruitments.

The feral populations I studied were genetically diverse and were indicative of an absence of genetic bottleneck and genetic drift. Further, there was evidence of natural selection for adaptive traits in roadside habitats. In particular, these results indicate selection pressure on the feral populations for traits including winter survivability, rhizome production and prostrate growth habit. Heterogeneous selection for adaptive traits can improve the persistence of alfalfa in roadside habitats.

My gene flow study demonstrated that feral alfalfa populations can act as both sources and sinks for novel traits. The white flower color marker I used for the quantification of outcrossing was sufficient to confirm the occurrence of high levels of gene flow between cultivated and feral populations. High levels of gene flow from hay fields were also detected when flowering was observed in the hay fields as a result of delayed haying.

My study provided compelling evidence that alfalfa is capable of persisting in roadside habitats in the northern regions of the Northern Great Plains of North America, although it is less likely that roadside alfalfa will become invasive. The levels of fecundity, seedbank, seedling establishment and adult survival were sufficient for the long-term persistence of feral populations. In addition, the long life span of alfalfa (>10 years) enables persistence of these populations in roadside habitats.

8.2 Implications for co-existence

The results from this thesis provide evidence that feral alfalfa populations can comprise an important component of gene flow and trait movement in agricultural landscapes. As such, feral alfalfa populations may hinder the successful co-existence of GM and non-GM alfalfa in production systems in agricultural landscapes. Therefore, feral alfalfa populations occurring in roadsides and other unmanaged habitats need to be considered in trait confinement protocols, if there is a need to confine novel traits in alfalfa.

I propose the following stewardship approaches, which consider the management of feral alfalfa populations, for reducing the chances of the adventitious presence (AP) of GM traits in non-GM alfalfa:

8.2.1 Minimizing pollen-mediated gene flow (PMGF)

8.2.1.1 Seed production fields

Because feral alfalfa plants growing in road verges and other unmanaged areas can facilitate GM trait movement, management of these populations is necessary, in particular around alfalfa seed production fields. In Canada, the current isolation distance required for certified alfalfa seed production is 50 meters and for foundation seed it is 200 meters (for fields exceeding 5 acres) or 300 meters (for fields that are 5 acres or less) (CSGA 2003). These isolation distances are designed to achieve variety purity (within limits) but not necessarily genetic purity (or the prevention of GM trait entry). As such, and given the evidence of long distance PMGF in alfalfa (St. Amand et al. 2000; Fitzpatrick et al. 2003), the isolation distances may need to be revisited. However, the appropriate isolation distance may be dictated by the nature of the trait and the threshold of GM-AP allowed in conventional alfalfa.

8.2.1.2 Hay production fields

Hay fields need to be managed properly and cut regularly before flowering. However, bad weather conditions can delay haying operations, resulting in flowering within hay crops and opportunities for PMGF and GM trait escape. This may mean that conventional alfalfa seed producers who have neighbors growing GM alfalfa hay may also need to consider isolation distances in relation to surrounding hay fields. In addition, feral alfalfa plants need to be managed such that seed production is prevented, if haying is delayed and considerable flowering is possible in adjacent GM alfalfa hay fields. However, gene flow from feral populations to hay fields is less of a concern because hay fields are rarely allowed to set seed.

8.2.2 Minimizing seed-mediated gene flow (SMGF)

Seeding and harvesting equipment should be cleaned prior to and after use in any GM alfalfa fields. Alfalfa seed (especially GM alfalfa seed) should be transported in spill

proof containers to avoid seed escape and reduce the establishment of feral GM alfalfa populations in road verges. Because herbivores including deer may likely facilitate alfalfa seed dispersal from feral alfalfa populations (Leach 1956; Kufeld 1973), effective control of feral populations around the GM alfalfa fields can also help prevent herbivore-mediated GM trait escape and reduce the AP of GM traits in the environment.

8.2.3 Sustained stewardship practices

Producers who wish to maintain GM-free crops will need to make conscientious efforts to do so and need to better understand the routes and mechanisms of GM trait movement. Establishing region-wide stewardship practices may be necessary to reduce the potential for gene flow between GM and non-GM alfalfa (Van Acker et al. 2007). Co-operative efforts from GM growers would greatly facilitate the co-existence of GM and non-GM crops, particularly letting neighbours know they are growing GM crops. In addition, all alfalfa growers (both GM and non-GM) should work to identify and control feral alfalfa populations both on their farm sites and along roadsides. Special collaborative programs with municipalities, including weed supervisors would be required in order to facilitate the management of these populations.

8.3 General considerations

My results are based on the study of feral alfalfa populations occurring in southern Manitoba, Canada. In my study, the demography of feral populations was highly variable among populations within this fairly defined and homogenous region. Such variation could be caused by several factors including but not limited to edaphic factors, competition with surrounding vegetation, microclimate and differences in roadside management regimes. In some areas, pest and disease incidence may also affect the growth and reproduction of feral populations in roadside habitats. In addition, it may be vital to recognize that most of the cultivars grown in western Canada are variegated types with considerable parentage from *M. falcata* and as such the dynamics of typical *M. sativa* ssp. *sativa* types in roadside and other unmanaged habitats may be different.

Therefore, it is important to consider the dynamics (and factors affecting the dynamics) of feral populations in specific regions, while taking appropriate management decisions.

Adherence to purpose designed stewardship practices can help minimize the potential of GM trait escape into feral and non-GM alfalfa and increase the chances of successfully achieving co-existence between GM and non-GM alfalfa. However, the stringency to which the co-existence programs need to be designed and implemented depends on the threshold of AP allowed in non-GM crops. A lack of clear threshold for AP of novel traits in conventional products is a cause of concern among technology developers, stakeholders and regulatory authorities and this often results in disputes. Realistic thresholds should be established based on the level of risk posed by the GM trait in question.

Some GM traits may facilitate the persistence of feral GM populations in unmanaged habitats than other traits. Traits favoring adaptiveness such as drought tolerance, salinity tolerance and pest and disease resistance will pose potential risks in this regard and will require strict confinement (Clark 2006), particularly in the regions where feral populations and wild relatives are commonly present. In addition, traits that confer herbicide resistance may be a concern if the herbicide is broad spectrum (e.g. glyphosate) and used to control weeds along roadways, right-a-ways and volunteer alfalfa in subsequent crops. Alfalfa is also being tested as a platform for the production of pharmaceutical enzymes (Bardor et al. 2006; Sparrow et al. 2007). Such second generation GM crops will require stringent regulation and the presence of feral populations will make unconfined release of these types of cultivars challenging.

Furthermore, thresholds should be above zero because zero thresholds may be difficult (or often impossible) to maintain in the context of commercial production, particularly in crops that have high levels of outcrossing and ferality potential (e.g. alfalfa). Therefore, feasible and achievable threshold levels should be established considering the nature of the trait introduced. Landscape level gene flow models will help establish such threshold levels. For alfalfa, it is important to include feral populations in such models and my results will greatly contribute to these models (I expand on the use of these models later in this chapter).

In addition to the need to establish clear threshold levels, clear regulations do not currently exist in either Canada or the US as to who will oversee and enforce co-existence stewardship programs and what will be the penalty for non-compliance or incentive for compliance with such protocols. Developing thresholds and clear co-existence regulations would greatly enhance the widespread acceptance of potentially useful GM traits (see Appendix A for a detailed explanation of this logic). It would be better for international trade if these requirements were addressed under an international framework because the lack of internationally accepted procedures makes it challenging for regulatory authorities and creates uncertainty and risk for farmers, processors and exporters. Appendix B is a detailed analysis of the need for international regulations and co-operative risk assessments, with special reference to GM alfalfa.

8.4 Future Research Needs

In this study I extensively summarized what is currently known about the biology and ecology of alfalfa and its propensity for ferality. I also worked to characterize roadside feral alfalfa populations in a region of western Canada representing the northern region of alfalfa production in North America. And I did all of this work in the context of commenting on trait confinement in commercial alfalfa production and the co-existence of GM and non-GM alfalfa. Although I was successful in revealing several novel aspects of feral alfalfa populations (e.g. its response to mowing, its ability to establish in swards and the nature of its seedbank), we still need more information in order to better manage these populations for confinement of GM traits in the environment. I have identified the following areas for future research:

a) My study provided evidence that alfalfa is capable of establishing self-perpetuating populations in roadside habitats. However, the long-term population dynamics is not clear. The growth, spread and dynamics of feral populations can be predicted better by population dynamic models (Garnier and Lecomte 2006). Matrix population models that incorporate density dependence and environmental stochasticity may be particularly helpful (Garnier et al. 2006). These models will be useful in identifying key life history traits that govern the persistence of the populations and can assist in designing efficient

management programs targeting critical control points in their lifecycle. Such models can be amended to specific regions and can be helpful in developing region specific management recommendations. My results will form a foundation for these models. However, there exist some knowledge gaps in this regard.

i) Soil seedbank: We have little information on the seedbank dynamics of roadside alfalfa populations. More information on seed addition (internal and external) and seedbank persistence (including seed predation) needs to be estimated through more detailed time series experiments.

ii) Seedling recruitment: I studied the seedling recruitment and seedling survival around the established alfalfa plants. Seedling establishment appeared to be affected by auto-allelopathic effects. It will be vital to test seedling recruitment in areas away from established alfalfa plants to know the upper levels of recruitment ability.

iii) Growth and reproduction: Studies on the growth and reproduction of feral alfalfa populations in regions with cultivars, rainfall, pest and disease incidence levels very different from my study region may be helpful for designing location specific management programs.

iv) Seed dispersal: Because the spread of feral populations appears to be influenced by the extent of seed dispersal, studies are required in this respect. There are no data available describing the long tail dispersal of alfalfa.

b) I noticed possible selection in roadside alfalfa with my phenotypic estimation study. More investigations need to be carried out using more phenotypic traits, including seed, root and yield traits. Furthermore, it will be valuable to study the phenotypic traits of feral populations originating from different geographical regions with a range of natural selection pressures including drought, edaphic characteristics and pest and disease incidences. In this way, we may gain a better understanding of the adaptive characteristics and potential persistence of feral populations in a broader range of unmanaged habitats.

c) My study has established the level of gene flow to and from feral populations that are occurring adjacent to the cultivated fields. However, the white flower color marker,

although sufficient for my crude estimation purposes, was not adequate to precisely estimate the gene flow from seed production fields under leaf cutter bee pollination. Precise estimation of gene flow may be necessary for establishing appropriate threshold levels. The use of other markers should be considered in this regard. Further, the level of gene flow from hay fields to feral populations needs to be studied with a range of flowering intensities in the hay fields in order to facilitate relevant management decisions.

To estimate the level of long-distance gene flow mediated by feral populations, large scale gene flow studies are required. This will establish the role of feral alfalfa populations in gene flow at metapopulation level and will also be useful in helping to set practicable threshold levels by providing data for probability modelling.

d) My research provides a conceptual framework for the potential persistence of feral populations in roadside habitats. However, these populations are not GM and the fitness of feral alfalfa populations may be altered by novel traits. Therefore, the likelihood of persistence, spread and invasion of feral alfalfa populations as influenced by the introduced trait required to be investigated before their unconfined release into the environment (Claessen et al. 2005 a,b).

8.5 Concluding remarks

Based on my investigations on roadside alfalfa populations occurring in western Canada, I can confidently say that alfalfa persists in roadside habitats without managed cultivation and can act as a reservoir for novel traits. Therefore, stewardship and co-existence programs need to consider the occurrence of feral populations in novel trait confinement protocols. Strict adherence to stewardship practices can reduce the AP of GM traits and facilitate the co-existence of GM and non-GM alfalfa. The degree to which feral alfalfa populations need to be managed and other stewardship practices should be implemented should depend on the nature of risk posed by the GM trait and the threshold level allowed in non-GM alfalfa. Nevertheless, total confinement of novel traits under practical field conditions is highly unlikely in alfalfa. Therefore, alfalfa is not a suitable crop for traits

that would require strict confinement and in such cases, regulatory approvals should not be granted.

Whether or not GM alfalfa should be approved for unconfined release is a policy decision that needs to be taken considering the level of environmental and human health risks posed by the nature of the trait introduced. If approved based on sound risk assessment and available for cultivation, whether or not GM alfalfa should be grown is a personal choice of the farmers driven by the needs and interests of the farmers concerned. However, regulations and practices should be in place, before unconfined release, to ensure that the interests of both adopting and non-adopting farmers are protected. In particular, more emphasis is vital to protect vulnerable sectors, including organic farming, from the AP of novel traits. Particular consideration to issues related to thresholds, liability and enforcement may greatly enhance the acceptance of GM alfalfa among the stakeholders. The lack of such regulations has caused disputes among the stakeholder groups and resulted in court lawsuits.

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APPENDIX A

The deregulation of Genetically Modified Alfalfa in the United States: Mounting Challenges for Risk Assessors and Policy Makers

A.1 Abstract

Genetically-modified (GM) Glyphosate-resistant (GR) alfalfa (*Medicago sativa* L.) is currently under moratorium in the United States (US). While GR alfalfa can provide operational benefits to farmers, there exist unique challenges with alfalfa in terms of novel trait confinement. Alfalfa is the first perennial, highly outcrossing, insect pollinated GM crop to receive deregulation. It is not clear whether GR alfalfa (and GM crops in general) will gain widespread acceptance among the broad stakeholder groups. Detailed investigation of the US regulatory framework and the perception of stakeholders, and the public suggest that crucial factors were not considered in the deregulation process for GR alfalfa. I suggest that addressing these factors may greatly enhance the broader acceptance and market success of GR alfalfa and GM agriculture in general.

A.2 Introduction

Genetically modified (GM) crops have been legally authorized for commercial production in many countries and commercially grown worldwide for over a decade. The incidences of outcrossing with non-GM crops and adventitious presence (AP) of GM traits in non-GM crops are cause for serious concerns of various commercial stakeholders, public interest groups and the general public (Ellstrand 2001). Our ability to contain novel traits within production fields is far from absolute and there exist numerous examples of novel trait escape and associated unintended effects (APHIS 2009). Strategies for the segregation of GM and non-GM crops can be expensive and often

inefficient (Wilson and Dahl 2006). A notable example is the Starlink® corn case in the US, where GM corn deregulated only for use as animal feed was found throughout the human food supply chain (Bucchini and Goldman 2002). Similarly, experience with herbicide-resistant canola in Canada shows that transgenes will escape cultivated fields and will eventually end up in unintended destinations such as non-GM fields, seedlots, roadsides and waste lands (Friesen et al. 2003; Legere 2004). Likewise, for creeping bentgrass, the escape and establishment of GM traits outside production areas was confirmed during field testing of still regulated products, and this was despite strict confinement efforts (Watrud et al. 2004; Zapiola et al. 2008). Previous evidence suggest that the retraction of transgenes might be impossible once they escape into the environment (reviewed by Marvier and Van Acker 2005). The possibility of transgene escape and presence in unintended destinations raises concerns over the safety of transgenic crop production and has lead to law suits over the deregulation of such products, including the recent lawsuit over the deregulation of GR alfalfa in the US.

The deregulation of GR alfalfa in the US in June 2005 raised concerns among some farmer organizations and subsequently attracted a law suit in February 2006 (see section A.10.1 for some of the criticisms raised by the plaintiffs). In May 2007, a federal district court in the US ordered a permanent moratorium on the sale and cultivation of GR alfalfa in the US, pending the production of a detailed Environmental Impact Statement (EIS) (USDC 2007b). APHIS is currently working on the preparation of an EIS (see section A.10.2 for a synopsis of the regulatory developments regarding GM-GR alfalfa in the US).

US regulators have mounting challenges in regard to this case because several factors are left unaddressed by the current US regulatory framework on GM crops. Broad stakeholder and public opinion suggest that addressing these factors may enhance a broader market and public acceptance.

A.3 Factors not considered by the present regulatory framework in the US

Alfalfa was the first perennial, highly outcrossing, insect-pollinated GM crop in the world to be commercially approved (in the US designed as deregulation). The ecology and

biology of alfalfa greatly favors gene flow (reviewed in Bagavathiannan and Van Acker 2009) (Chapter 3.0) and among the GM crops deregulated to-date it is one of the high risk crops in terms of trait movement (Table A.1). The recent moratorium on GR alfalfa in the US requires that regulators more fully investigate and consider the issues associated with GR alfalfa. In this regard, issues including AP, market risks, stewardship practices, co-existence protocols, enforceability, testing procedures, thresholds, labeling and liability are important to consider. However, the current US regulatory framework does not allow to consider most of these factors in the assessment process, nor is the approach to their consideration standardized.

A.3.1 Adventitious presence

When GM crops are deregulated they are allowed to exist anywhere in the environment and there are no US federal requirements that prohibit the AP of wholly owned deregulated GM products in or on another person's property. In the US, rules requiring the prevention of the GM traits from contaminating neighbor's fields and the environment have never been considered. APHIS has no responsibility to consider the AP of deregulated materials outside of GM crop production fields and that includes AP in conventional and organic farms, even if such AP causes economic harm. Market losses and liabilities associated with the AP of deregulated traits are neither dealt with by APHIS nor by any other US federal agencies (USGAO 2008).

Because economic losses and liabilities can be considered damaging to the human environment (USDC 2007b), broader policies may need to be established regarding AP. In addition, the differentiation of world markets into GM, non-GM and organic sectors demands efficient identity preservation (IP), standard and comprehensive segregation protocols and effective policies on the AP of GM traits, including protection of sectors that may realize economic harm from the AP of GM crops. Achieving the segregation of GM and non-GM crops is a challenge and practical experience suggests that this is generally true in current commodity supply chains even when acceptable AP levels are in the range of 1 to 5%. There is broad uncertainty about the practicality and affordability of managing IP for AP levels significantly below 1% (Van Acker et al. 2007).

Table A.1 Comparison of the ecology and biology of some of the major GM crops approved worldwide

Common name	Scientific name	Life cycle	Type of pollination	Self incompa-tibility	Volunteers	Ferality potential	Other remarks	Gene flow
Alfalfa	*Medicago sativa*	Perennial	Predominantly outcrossing	High	Low – medium	High	Can be weedy/invasive[†]	High
Canola	*Brassica napus*	Annual	Selfing/ outcrossing	Low	High	Medium – high	Seed shattering/ large seed bank	Medium -high
Cotton	*Gossypium hirsutum*	Annual/ Perennial	Predominantly selfing	Negligible	Low	Medium	Can be weedy/invasive[†]	Low - medium
Creeping bentgrass	*Agrostis stolonifera*	Perennial	Predominantly outcrossing	High	N/A	High	Both vegetative propagules/seeds	High
Flax	*Linum usitatissimum*	Annual	Predominantly selfing	Low	Medium	Medium	Hermaphroditic flowers	Low -medium
Lentil	*Lens culinaris*	Annual	Predominantly selfing	Low	Medium	Negligible	Pollination occurs before flower opens	Low
Maize	*Zea mays*	Annual	Selfing/ outcrossing	Low	Low – medium	Low	Staminate and pistillate flowers	Medium
Potato	*Solanum tuberosum*	Annual	Selfing/ outcrossing	Low	Low – medium	Low	True seeds and tubers	Low
Rice	*Oryza sativa*	Annual	Predominantly selfing	Low	Medium	Low – medium	Can be weedy/invasive[†]	Medium
Soybean	*Glycine max*	Annual	Predominantly selfing	Negligible	Low	Negligible	Flowers attract few bees	Low
Sugar beet	*Beta vulgaris*	Biennial	Predominantly outcrossing	High	Medium	High	High levels of self-sterility	High
Sunflower	*Helianthus annuus*	Annual	Predominantly outcrossing	Medium – high	Medium	Low – medium	Recent varieties are self compatible	Low - medium
Wheat	*Triticum aestivum*	Annual	Predominantly selfing	Low	Medium – high	Low-medium	Florets remain open only for short period	Medium

Sources: Agbios (2008); CFIA (2008)

[†]Reference: SWSS (1998)

It is not certain who should cover costs associated with confining deregulated GM traits and to-date the costs have been borne by those who can achieve premiums for ensuring low AP levels. To-date, no GM crops have commanded market premiums which could be used to offset segregation costs. More research is necessary to develop reliable segregation and IP protocols in order to better serve the markets with specific AP requirements.

A.3.2 Market risks

A.3.2.1 Risks to the conventional US alfalfa hay export market

Asynchronous market authorizations of GM crops, food and feed in the various jurisdictions, particularly in the US, Japan and the EU, have long been a threat of market harm and sparked disputes. At the time the deregulation decision was taken on GR alfalfa in the US, market authorizations were not granted by most of the key US hay importing countries, including Japan, which is the destination for about 72% of all US alfalfa hay exports (annual value of about 500 million US$) (APHIS 2005b). Since that time approvals as food/feed have been secured in most of the hay export markets (McCaslin 2008) but the approvals took a long time in some cases (Table A.2).

Some delays may have been due to the fact that many approvals were sought only after deregulation in the US. Furthermore, the approvals varied among countries in terms of the type of usage. Some countries granted approvals for food, feed and environmental release, while others only granted approvals for food and/or feed use.

Even when approvals for GM alfalfa were granted in key hay export markets, market barriers still exist in some importing countries (McCaslin 2008), including for example, extremely low tolerance levels for the presence of GM traits in conventional hay shipments (as low as 0.1%). Importers in Japan have generally expressed preference for non-GM alfalfa due in part to the difficulty in segregating GM and non-GM hay lots and to fickleness of consumers (including Japanese dairies) (NAFA 2008a).

Table A.2 Regulatory status of GR alfalfa in important US hay importing countries

Export destination	Exports (tones)*	% of total export	Status/Year approved			Reference
			Food	Feed	Environment	
Japan	680,769	71.6%	2005	2006	2006	JBCH (2006)
South Korea	128,331	13.5%	2007	2008	Not approved	KBCH (2008)
Taiwan	68,662	7.2%	Approval not required†			-
Canada	39,447	4.2%	2005	2005	2005	CFIA (2005)
UAE	19,864	2.1%	No regulatory process in place†			-
Mexico	8987	1%	2005	2005	Not approved	COFEPRIS (2005)
Hong Kong	1,087	0.1%	Approval not required†			-
China (PRC)	420	0.04%	Approval not required†			-
UK	407	0.04%	No information available			-
Australia/New Zealand	-	-	2006	2006	Not approved	FSANZ (2006)
Philippines	-	-	2006	2006	Not approved	NBCP (2006)

* Export of alfalfa hay, cubes and meal - 2006 statistics (NASS 2008);

† Source: NAFA (2008a)

Some reports indicate that between 10 and 20% of Japanese customers of hay demand GM-free (NAFA 2008a) even though the threshold for AP of approved GM events is 5% in Japan. Exporters fear that shipments could be rejected at a cost to them and these fears can cripple markets.

A.3.2.2 Risks to the conventional US alfalfa seed export market

Most of the approvals secured to-date for GM-GR alfalfa in the US are for alfalfa hay markets, not seed markets. The US alfalfa seed export market is valued at about 43 million US$ annually with about 14 million kg of seeds exported to 63 countries (USDA-FAS 2009). Notable seed importers are Saudi Arabia, Mexico, Argentina, Canada and UAE (USDA-FAS 2009) (Table A.3). Among the seed importing countries, only Canada and Japan had authorized environmental release of GR alfalfa (CFIA 2005), while about 43 countries comprising 81% of the US seed imports have either not yet authorized GR alfalfa for environmental release or not put a regulatory system in place.

US exports of alfalfa seed to the European Union (EU) were valued at 1.8 million US$ in 2005, accounting for about 5% of total US alfalfa seed export revenues (USDA-FAS 2009). Markets in the EU remain sensitive to the AP of GM materials. Because tolerances have not yet been set in the EU for AP in seed, alfalfa seed exports to the EU remain threatened if GM trait is detected at any level (GAIN 2006).

Exporters of conventional alfalfa seed to sensitive markets may be required to declare and/or prove that their seed lots are free from GM material. Previous evidences suggest that total containment of novel traits in commercialized GM crops grown commonly across a broad agricultural region may be impossible (Marvier and Van Acker 2005) and the contamination of conventional seeds in these regions may be inevitable. This triggers concerns of customers in sensitive importing countries, including EU Member States. And accidental AP does in fact happen. For example, the USDA-Foreign Agricultural Service has reported the discovery of an EU-unapproved biotech event in conventional rapeseed sown on about 3500 acres in Germany, even though the seed used to plant this crop tested negative for GM materials (GAIN 2007).

Table A.3 Regulatory status of GR alfalfa in important US seed importing countries*

Import country	Approval for environmental release	Alfalfa seed export value ('000 dollars)			% of total alfalfa seed export value
		Certified	Uncertified	Total	
Saudi Arabia	No	10165	139	10304	23.77
Mexico	No	3173	5249	8422	19.43
Argentina	No	6225	1144	7369	17.00
Canada	Yes	7362	-	7372	16.99
UAE	No†	1937	19	1956	4.51
Italy	No	1170	158	1328	3.06
Peru	No	441	557	998	2.30
Chile	No	426	319	745	1.72
Japan	Yes	236	509	745	1.72
Libya	No	326	400	726	1.68
South Africa	No	363	123	486	1.12
Other countries (35)	No	2192	708	2900	6.70
Total seed export value ('000 dollars)				43,351	

* Alfalfa seeds exported for planting purposes in 2005 (USDA-FAS 2009)

† No regulatory process in place (NAFA 2008a)

Incidents like this point to a need for the development and implementation of special considerations and confinement protocols for producers (and perhaps regions) that wish to meet import requirements in GM sensitive markets (NAFA 2008b), and this may be particularly true for seed producers.

A.3.2.3 Risks to organic markets

Organic crop and livestock production is one of the fastest growing agricultural sectors in the US with about 4.1 million hectares of land dedicated to organic production and about 196,000 organic livestock animals in 2005 (USDA-ERS 2005). In this same year, certified organic alfalfa hay was produced on over 200,000 acres in the US. Organic milk production is also a growing industry in the US with about 87,000 cows currently and a growth rate of 25% per year (USDA-ERS 2005). National organic program (NOP) rules in the US prohibit planting of GM seeds and the presence of GM materials in organic products (Furtan et al. 2007; Demont and Devos 2008; USDA-AMS 2009). Alfalfa is an important feed for organic livestock especially dairy cows. It is also an important legume crop in organic rotations. Threats to the availability of GM-free alfalfa seeds, due to the AP and contamination of conventional seed production fields, may create tremendous challenges and costs for organic producers in the US both in relation to cropping system agronomy and dairy cow nutrition challenges (SOD 2006).

A.4 Stewardship practices, co-existence and enforceability

Various stewardship approaches have been proposed as means of achieving co-existence between GM and non-GM alfalfa (Putnam 2006). Strict adherence to these approaches requires incentives, diligence and enforcement (Van Acker et al. 2007). Stewardship approaches are more practicable if they are based on real management practices and not best management practices. In most cases, total confinement of novel traits and maintaining zero threshold level within production fields is difficult. There are numerous examples of regulated materials escaping even confined field tests conducted under strict protocols and severe vigilance (APHIS 2009). Therefore, co-existence programs aimed at

achieving zero AP levels may likely fail. Success of co-existence programs depends on the establishment of consensual, achievable and enforceable tolerance levels (above zero). In the US there is no governmental oversight of deregulated traits and co-existence is left to the market even when there is known risk of market harm if co-existence fails.

A.5 Testing procedures

Technologies for testing the presence of GM material are neither fully developed nor internationally standardized (Viljoen et al. 2004). Most available testing procedures are not capable of precisely detecting the presence of GM materials, especially at low levels, due to variability of sampling methods used (Woodward 2006). Ongoing issues around testing for the AP of GM traits mean that false negatives and positives remain a problem (Remund et al. 2001). This uncertainty translates into marketplace issues. For example, after the deregulation of GR alfalfa, the US federal seed lab was no longer willing to provide a GM-free declaration for alfalfa seed exporters (NAFA 2008b).

Common AP testing procedures are either protein based (lateral flow test strips [LFTS] and enzyme-linked immunosorbent assay [ELISA]) or DNA based. LFTS are simple to use and inexpensive but can vary in their outcome as they dependent on eyesight and judgment of the analyst. The accuracy of two LFTS developed for detecting the presence of the GM GR trait in alfalfa haystacks, hayfields and seeds was evaluated by researchers in Washington State (Woodward 2006) who concluded that LFTS were not reliable for confirming the presence of the GM GR trait at levels of less than 5%. ELISA tests are more accurate than LFTS but are expensive and require greater levels of skill (Viljoen et al. 2004). DNA based tests are more reliable than protein based tests (Griffiths et al. 2002) but DNA based tests are not available for many GM traits including the GM GR trait in alfalfa.

A.6 Thresholds and labeling

Currently there are no internationally accepted threshold levels or labeling standards for the AP of GM materials authorized for market commercialization, although the expected

threshold for regulated traits is zero (Viljoen et al. 2004). Labeling is not mandatory in most countries. The EU requires a label if approved GM materials are present in non-GM crop, food and feed items above a threshold level of 0.9% (GAIN 2006). This threshold level applies only to food or feed materials and as such there is no threshold level for GM material in seeds leading some to suggest this implies a zero threshold level in seeds (GAIN 2006). If traces of EU approved GM material is found in conventional seed, then it has to be labeled as containing GM material. If AP of unapproved GM material is detected, the crop must be destroyed (GAIN 2006). In Japan and Australia, labeling thresholds for approved GM materials are 5% and 1%, respectively (Viljoen et al. 2004). Conversely, some countries, including China, have not yet established any AP thresholds nor any requirements for feed labeling. In this sort of global context, US alfalfa hay and seed exporters are often concerned that particular shipments could be rejected because of the AP of GM material (NAFA 2008b). What is certain is that exporters bear the market risk.

A.7 Liability

Liability is a key issue in relation to functional field and market co-existence. The liabilities for non-compliance with standards are often not addressed in regulatory frameworks. In the EU, farmers growing GM crops are typically held liable for AP, depending on the co-existence legislation of the individual EU Member State. In the US and Canada, there is no co-existence legislation and no mandatory labeling, and deregulated GM crops are granted unconfined release. These conditions make it difficult for stakeholders affected by the AP of GM material to mount any efforts for recourse if they experience market harm as a result of the AP of GM material. This is not necessarily true for regulated GM material, however, and the Starlink® case in the US did result in payment of damages to a range of affected stakeholders (Marvier and Van Acker 2005). Globally, there are no examples of legislation or regulations which outline the liability for AP. A clear assignment of responsibilities and liability would be required, including a consideration of who is responsible and who pays for expenses related to IP, testing and market loss (Van Acker et al. 2007).

A.8 Implications for other GM crops

The case of GR alfalfa makes visible some more fundamental challenges to the US (and other regulatory frameworks) from GM crops. The lack of a comprehensive regulatory framework creates concerns among farmers, agricultural stakeholders and the public, and it creates uncertainty for exporters to GM sensitive markets. Stakeholders demand a regulatory approach that can deal with the regulation of GM crops in a comprehensive and coordinated fashion. In the US, federal agencies have limited assessment mandates and there is no supreme body that ensures overall assessments are inclusive. The lack of clear policies also hinders the regulatory process. Appropriate polices therefore on AP, market risks, stewardship practices, co-existence protocols, enforceability, testing procedures, thresholds, labeling and liability need to be established and included in the existing regulatory frameworks on GM crops. Genetically modified crops should also be assessed on these factors before regulatory decisions are taken. Governments must consider enacting appropriate laws and establishing or empowering institutions to ensure that these regulations are adequately enforced. Furthermore, co-operative international initiatives are required to establish internationally accepted standards with regard to IP, testing, thresholds and labeling of regulated and deregulated GM materials. Adequate consideration to these regulatory issues may greatly reduce the disputes among the stakeholder groups and favor the widespread acceptance of GM agriculture.

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A.10 Appendix

A.10.1 The criticisms raised by the plaintiffs over the deregulation of GR alfalfa in the US

- Alfalfa is a perennial, highly outcrossing, insect pollinated species and it is more likely that GM traits will escape cultivated alfalfa fields and contaminate nearby alfalfa populations. The strong ferality potential of alfalfa further aggravates this problem.
- The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) violated the National Environmental Policy Act (NEPA) by choosing not to prepare an EIS before the deregulation, despite the fact that the introduction of GM alfalfa might present several challenges for conventional and organic alfalfa hay, seed and honey growers.
- The cumulative effects of the widespread cultivation of GR alfalfa, including potential increases in herbicide usage and related health and environmental risks were largely ignored.
- APHIS failed to consider the increased need to use comparatively harmful herbicides including 2,4-D and Paraquat for stand termination and the control of volunteers and feral alfalfa populations.
- Overall impacts from the introduction of yet another GR crop, including the effect of more GR crops in rotation on the crop and human environment, including the development of more herbicide resistant weeds were not adequately analyzed.
- With regard to animal health, the impact of the mixture of GR alfalfa in rations that may already contain other GM products and its impacts on the intestinal fauna were not sufficiently explored.
- APHIS only evaluates public health and environmental effects, as such there is no consideration of potential economic, socio-economic and other costs associated with the deregulation of GM crops.

- Because three agencies [PHIS, Environmental Protection Agency (EPA) and Food and Drug Administration (FDA)] are involved in deregulation, responsibilities were not clearly proportioned among agencies.
- The1986 EPA guidelines on the reregistration of products containing glyphosate (EPA case no. 0178) identified solono grass, the valley elderberry longhorn beetle and the Houston toad as species under threat in relation to the use of this herbicide. The EPA's 1993 Re-registration Eligibility Decision for glyphosate identified even more species under threat and the list had not been updated since then. In addition, the surfactants used with glyphosate may cause adverse effects such as amphibian mortality. These effects were not considered by EPA.
- The EPA did not adequately consult the US Fish and Wildlife Service with respect to setting tolerances for glyphosate in accordance with Section 7 (a) 2 of the Endangered Species Act.

Sources: CFS (2006, 2007)

A.10.2 Synopsis of the regulatory developments regarding GR alfalfa in the US

- May 2003, APHIS receives petition from Forage Genetics International (FGI) for deregulation of GR alfalfa events J101 and J163 (petition 03-127-01p). Petition was subsequently withdrawn after APHIS' request for additional information.
- April 2004, FGI re-submits petition with additional information requesting the deregulation of GR alfalfa (petition 04-110-01p).
- May 2005, APHIS issues Finding of No Significant Impact (FONSI) report. APHIS decides not to prepare an EIS (APHIS 2005b).
- June 2005, APHIS decides to deregulate GR alfalfa based on FONSI report under 1969 NEPA, as amended (42 USC 4321 *et seq.*; 40 CFR 1500-1508; 7 CFR Part 1b; 7 CFR Part 342) (APHIS 2005a).
- Following deregulation, in September 2005, GR alfalfa was grown on 5,485 farms with over 263,000 acres sown.
- February 2006, lawsuit filed against deregulation decision on GR alfalfa, on the grounds of violation of National Environmental Policy Act (NEPA), the Endangered Species Act (ESA) and the Plant Protection Act (PPA).
- February 2007, Federal District Court judge in San Francisco concludes that APHIS violated NEPA by choosing not to prepare an EIS on GR alfalfa.
- March 2007, court issues preliminary injunction order for the sale and cultivation of GR alfalfa in the US (USDC 2007a) and consequently, GR alfalfa is returned to regulated status (APHIS 2007).
- May 2007, permanent injunction order issued by the court, pending the production of a detailed EIS on GR alfalfa (USDC 2007b).
- July 2007, court issues an amended order detailing the confinement requirements including the storing of GR alfalfa, labeling containers and cleaning equipment associated with GR alfalfa fields that were already established.

- December 2007, APHIS issues a supplementary administrative order that specifies practices that must be implemented by GR alfalfa growers (USDC 2007c).
- January 2008, APHIS publishes a Notice of Intent (NOI) in the Federal Register to prepare an EIS on GR alfalfa.
- September 2008, the ninth circuit court of appeals in San Francisco upholds the injunction.

APHIS is currently working on the preparation of a draft EIS.

APPENDIX B

Transgenes and National Boundaries – The Need for International Regulation

B.1 Abstract

What happens when one nation cultivates a transgenic crop variety but neighboring nations do not? Using alfalfa as a case study, I argue that the potential for international transgene flow is substantial, and therefore, the need for international cooperation in regulatory decisions concerning transgenic crops is imperative. Alfalfa (*Medicago sativa,* L.) is a major forage crop in North America. Recently, genetically modified (GM) alfalfa received a moratorium on further cultivation in the US on the grounds that the approvals were based on inadequate environmental impact assessments. With their deep root system, symbiotic nitrogen fixation, prolific seed production and prolonged dormancy, alfalfa plants are capable of establishing self-perpetuating (feral) populations in unmanaged environments. Given what is known about alfalfa pollen dispersal, such feral populations could facilitate gene flow between GM and non-GM fields. The border between the US and Canada, particularly in farming areas, is very narrow (<10m wide). I surveyed along the US-Canada border and found both alfalfa fields and potentially feral alfalfa plants in the ditches along the border. My survey results provide evidence of the possibility of cross-border transgene flow, suggesting a need for international co-operative risk assessment initiatives between the US and Canada. Such situations could occur for other crops, in other international border regions as well.

B.2 Background

Organisms that are deregulated in one country can be present adventitiously at sites along the border regions of a neighboring country. For example, in the US, genetically-modified (GM) glyphosate-resistant (GR) alfalfa (*Medicago sativa*, L.) has been deregulated and was available for cultivation. But it has not been approved for commercial cultivation in Canada. If GM alfalfa were grown in regions along the US-Canada border, transgene movement could occur from the US into adjacent alfalfa populations in Canada. I argue that there exist possibilities for international gene flow among the alfalfa populations that occur in the border regions between the US and Canada.

Before going into the details of international gene flow in alfalfa, let us detail the current situation of GM alfalfa in the US. Herbicide-resistant alfalfa was deregulated in 2005. It subsequently raised concerns among the conventional and organic growers over the adventitious presence of transgenes and associated market and environmental risks (WORC 2005). On March 12, 2007, Charles Breyer, a federal district judge in the United States District Court for the Northern District of California issued a moratorium on further sales and cultivation of GR alfalfa (Fox 2007). The court stated that the Animal and Plant Health Inspection Service (APHIS) violated the National Environmental Protection Act (NEPA) by choosing not to prepare an Environmental Impact Statement (EIS) before it deregulated GR alfalfa in 2005 (USDC 2007a).

Effective March 12, 2007, USDA returned the GR alfalfa to regulated status in compliance with the court injunction. Further, the defendants' request to reconsider the preliminary injunction was denied by the court on May 3, 2007, and the injunction was made permanent pending the preparation of EIS by APHIS (USDC 2007b). However, alfalfa fields that were planted prior to the injunction in 2007 continue to produce, following the regulations imposed by the court as outlined in the amended order issued on July 23, 2007 (USDC 2007c). On September 2, 2008, the US Ninth Circuit Court ruled against the appeal to lift the injunction on GR alfalfa, and ruled for the injunction to continue in effect.

The ecology and biology of alfalfa is favorable for gene flow. Alfalfa is a perennial, highly outcrossing crop species, which is predominantly pollinated by insects such as honeybees (*Apis mellifera*), leaf cutter bees (*Megachile rotundata*), alkali bees (*Nomia melander*) and bumblebees (*Bombus* spp.) (Rincker et al.1988). Honeybee-mediated long-distance dispersal of pollen from alfalfa seed and hay production fields has been confirmed for distances up to 1000 m (St. Amand et al. 2000). In a similar study, Teuber et al. (2004) found outcrossing levels of 1.5% at 270 m and 0.2% at 1.5 km, and were able to detect outcrossing as far as 4 km, although at a very low level. Under leaf cutter bee pollination, Fitzpatrick et al. (2003) observed outcrossing levels of 1.4% at 152 m, and only 0.28% at 274 m, with no outcrossing at 610 m. In the same study, a single outcrossing event was detected at 804 m, at a very low frequency. These studies demonstrate the long-distance dispersal potential for gene exchange among alfalfa fields. Further, the introduction of pollinator insects for alfalfa seed production in the border regions would increase the opportunity for pollen-mediated gene flow.

Feral alfalfa populations occurring in unmanaged habitats would further enhance the extent of gene flow by acting as bridges for the bees (Putnam 2006). Feral crop species are those from which individuals escape a managed area to survive, reproduce and establish self-perpetuating populations in either natural or semi-natural habitats (Bagavathiannan and Van Acker 2008) (Chapter 2.0). With their deep root system, symbiotic nitrogen fixation, prolific seed production and prolonged seed dormancy, alfalfa plants are capable of establishing self-perpetuating populations in unmanaged environments. Their perenniality, quick regrowth potential, drought- and winter-hardiness likely further contribute to their success in the natural areas and their ability to form effective feral sub-populations within agricultural landscapes (reviewed in Bagavathiannan and Van Acker 2009) (Chapter 3.0). My current research in Western Canada suggests that alfalfa is capable of establishing self-perpetuating feral populations (Bagavathiannan et al. 2009) (Chapter 4.0) and they may act as the sources and sinks for the movement of GM traits from fields of GM alfalfa to fields of non-GM alfalfa.

B.3 Hypothesis and examination

I hypothesize that there exists the possibility for the GR trait to move from GM alfalfa fields in the US to non-GM alfalfa fields in Canada. This movement would most likely happen in situations where alfalfa fields and/or the feral alfalfa populations are located on both sides of the border close enough to allow for effective cross pollination. The width of the US-Canada border area within many of the farmed regions is less than 10 m in most cases (Fig. B.1). Such a distance suggests that international gene flow could occur readily from GM alfalfa in the US to non-GM alfalfa in Canada.

The US and Canada share 2,878 km of land border from the Atlantic to the Pacific Ocean (IBC 2007). The border includes the US states of Maine, New Hampshire, Vermont, New York, Pennsylvania, Ohio, Michigan, Minnesota, North Dakota, Montana, Idaho and Washington. Alfalfa is cultivated in most of these states, and the details on the current existence of GR alfalfa fields in counties adjacent to the Canadian border are presented in Table B.1.

On the Canadian side, alfalfa has been widely cultivated in southern parts of all of the land-border provinces including New Brunswick, Quebec, Ontario, Manitoba, Saskatchewan, Alberta and British Columbia.

I carried out a short survey along the US-Canada border in two rural municipalities (Rhineland and Franklin) in southern Manitoba, Canada to see if there were alfalfa fields and potentially feral alfalfa plants (alfalfa plants outside of cultivated fields) in cross-border locations that might facilitate international gene flow. I drove along border roads (a distance of about 50 km). I found alfalfa fields and potentially feral alfalfa plants in the ditches along the border (Fig. B.2).

In one of the municipalities (Rhineland), within a survey distance of 12 km, I found two alfalfa fields on the Canadian side of the border and two potentially feral alfalfa populations (population sizes of 10 and 15) in the ditch along side the border immediately adjacent to the alfalfa fields. I also found one potentially feral alfalfa population (6 plants) located on the US side only 800m from an alfalfa field. In the other municipality

Table B.1 Occurrence of GR alfalfa fields in counties adjacent to the Canadian border*

State	Border counties with Canada	Counties where GR alfalfa fields occur
Washington	4 (Clallam, Island, San Juan, Whatcom)	2 (Island, Whatcom)
Idaho	1 (Boundary)	No
Montana	11 (Lincoln, Flathead, Glacier, Toole, Liberty, Hill, Blaine, Phillips, Valley, Daniels, Sheridan)	8 (Glacier, Toole, Liberty, Hill, Blaine, Phillips, Valley, Daniels)
North Dakota	8 (Divide, Burke, Renville, Bottineau, Rolette, Towner, Cavalier, Pembina)	5 (Divide, Burke, Bottineau, Cavalier, Pembina)
Minnesota	7 (Kittson, Roseau, Lake of the woods, Koochiching, St. Louis, Lake, Cook)	2 (Kittson, Roseau)
Michigan	2(Chippewa, St. Clair)	1 (St. Clair)
New York	5 (Orleans, Jefferson, St. Lawrence, Franklin, Clinton)	1 (St. Lawrence)
Vermont	4 (Grand Isle, Franklin, Orleans, Essex)	None
New Hampshire	1 (Coos)	No
Maine	1 (Aroostock)	No

Source: (APHIS 2008)

*Details on the exact locations of GR alfalfa fields are not made available to the public by USDA. Therefore the fields may or may not be located closely adjacent to the international border. However, the information provided in the above table is the best available at this time.

Fig. B.1 A photograph of the US-Canada border near Emerson, Manitoba, May, 2007. The ditch along the zero mile road is less than 5 meters wide.

Fig. B.2 Potentially feral alfalfa plants in a ditch along the US-Canada border near Altona, Manitoba.

(Franklin), I found a large uncultivated population outside culticated condition (18 plants) and three smaller such populations (3-5 plants each) in a survey distance of 16 km, but I did not find any nearby cultivated alfalfa fields.

B.4 Discussion

My survey results provide evidence of the possibility of international alfalfa transgene flow from the US to Canada. However if transgenic alfalfa seed or alfalfa plants are found in Canada, the liabilities to such contamination are not clear. Who should bear the responsibility in the event of a transgene contamination? Canadian farmers or the Canadian government? Should US farmers move their fields away from the border? The ability of Canada to limit the risk of cross-border transgene flow via metapopulation dynamics requires knowledge of the location of transgenic crops. Currently, the GPS locations of cultivated GR alfalfa fields in the US are not publicly available in any database. Further, it is not clear whether such information would be revealed to the Canadian farmers through the call centers set up by USDA. The situation in the US has implications for Canada, when there is risk of transgene movement across the border. This perhaps points to a need for international cooperative risk assessment initiatives between the US and Canada, particularly for GM crop species which have a high ferality potential, are outcrossing (and insect pollinated), and are very commonly grown on either side of a shared land border.

B.5 Implications for other nations

I speculate that similar situations of international gene flow might occur for other GM crops, in other border regions as well. In Europe, the commercial approval of GM crops is governed by the European Union (EU) on behalf of its member states, and the issue of international gene flow among the EU member states may be considered insignificant. However, transgene flow across an international border could be a potential concern in other regions of the world, including non-EU member states, Asia, Middle East, South Africa and South as well as North America (Table B.2).

Table B.2 Regions and crops in which international gene flow may be a potential concern*

Country	GM crop (s) / trait(s) approved for environmental release	Crop (s) / trait (s) approved in adjacent countries
Argentina	**Soybean** (glyphosate tolerance); **maize** (glufosinate tolerance, glyphosate tolerance, resistance to European corn borer, resistance to European corn borer + glufosinate tolerance, resistance to lepidopteran pests + glyphosate tolerance, resistance to lepidopteran pests + stacked tolerance to glufosinate and glyphosate); **cotton** (glyphosate tolerance, resistance to lepidopteran pests, resistance to lepidopteran pests + glyphosate tolerance)	**Uruguay-** *soybean* (glyphosate tolerance); *maize* (resistance to European corn borer, resistance to European corn borer + glufosinate tolerance); **Paraguay**- *soybean* (glyphosate tolerance); **Brazil**- *soybean* (glyphosate tolerance); *maize* (glufosinate tolerance, resistance to European corn borer, resistance to European corn borer + glufosinate tolerance); *cotton* (resistance to lepidopteran pests); **Bolivia**- *soybean* (glyphosate tolerance); **Chile**- *soybean* (glyphosate tolerance); *maize* (resistance to European corn borer)
Brazil	**Soybean** (glyphosate tolerance); **maize** (glufosinate tolerance, resistance to European corn borer, resistance to European corn borer + glufosinate tolerance); **cotton** (resistance to lepidopteran pests)	**Uruguay**- *soybean* (glyphosate tolerance); *maize* (all except glufosinate tolerance); **Argentina**- all; **Paraguay**- *soybean* (glyphosate tolerance); **Bolivia**- *soybean* (glyphosate tolerance); **Peru**- none; **Colombia**- *cotton* (resistance to lepidopteran pests); **Venezuela**- none; **Surinam**- none; **French Guiana**- none; **Guyana**- none
Paraguay	**Soybean** (glyphosate tolerance)	**Bolivia**- yes; **Argentina**- yes; **Brazil**- yes
Uruguay	**Soybean** (glyphosate tolerance); **maize** (resistance to European corn borer, resistance to European corn borer + glufosinate tolerance)	**Argentina**- all; **Brazil**- all
Bolivia	**Soybean** (glyphosate tolerance)	**Paraguay -**yes; **Argentina**- yes; **Brazil** – yes; **Peru** – no; **Chile** –yes
Colombia	**Cotton** (glyphosate tolerance, resistance to lepidopteran pests); **carnation** (flower color)	**Ecuador**- none; **Panama**- none; **Peru**- none; **Brazil**- *cotton* (resistance to lepidopteran pests); **Venezuela**- none

Chile	**Soybean** (glyphosate tolerance); **Maize** (resistance to European corn borer); **oilseed rape** (glyphosate tolerance)	**Argentina**- all except oilseed rape; **Bolivia**- *soybean* (glyphosate resistance); **Peru**- none
Honduras	**Maize** (resistance to European corn borer)	**El Salvador**- no; **Nicaragua**- no; **Guatemala**- no
Mexico	**Cotton** (resistance to lepidopteran pests); **soybean** (glyphosate tolerance); **tomato** (delayed ripening)	**USA**- all; **Guatemala**- none; **Belize** – none
USA	**Oilseed rape** Argentine type - *Brassica napus* (glyphosate tolerance, glufosinate tolerance, glufosinate tolerance and fertility restored, oil content); **maize** (resistance to European corn borer, glyphosate tolerance, resistance to European corn borer + glyphosate tolerance, glufosinate tolerance, glufosinate tolerance and fertility restored, resistance to European corn borer + glufosinate tolerance, resistance to corn root worm, resistance to corn root worm + glufosinate tolerance, resistance to corn root worm + glyphosate tolerance, resistance to lepidopteran pests, enhanced lysine level); **cotton** (glufosinate tolerance, glyphosate tolerance, resistance to lepidopteran pests, oxynil tolerance, resistance to lepidopteran pests + oxynil tolerance, sulfonylurea tolerance); **soybean** (glufosinate tolerance, glyphosate tolerance, tolerance to glyphosate + ALS inhibiting herbicides, high oleic acid content); **alfalfa** (glyphosate tolerance)[†]; **flax** (sulfonylurea tolerance); **potato** (resistance to Colorado potato beetle, resistance to Colorado potato beetle + potato virus Y, resistance to Colorado potato beetle + potato leafroll luteovirus); **sugar beet** (glufosinate tolerance, glyphosate tolerance); **papaya** (resistance to papaya ring spot virus); **plum** (resistance to plum pox virus); **tomato** (delayed ripening, resistance to lepidopteran pests); **rice** (Glufosinate resistance); **squash** (resistance to cucumber mosaic virus + watermelon mosaic virus 2 + zucchini yellow mosaic virus, resistance to watermelon mosaic virus 2 + zucchini yellow mosaic virus); **chicory** (glufosinate tolerance and fertility restored); **tobacco** (nicotine reduced)	**Canada** – *oilseed rape* (all); *maize* (all); *soybean* (all except tolerance to glyphosate + ALS inhibiting herbicides); *alfalfa* (glyphosate tolerance) [††]; *potato*(all); *sugar beet* (all); **Mexico**- *cotton* (resistance to lepidopteran pests); *soybean* (glyphosate tolerance); *tomato* (delayed ripening)
Canada[‡]	**Oilseed rape** Argentine type (glyphosate tolerance, glufosinate tolerance, glufosinate tolerance and fertility restored, imidazolinone	**USA**- *oilseed rape* Argentine type (all except imidazolinone tolerance, oxynil tolerance); *oilseed*

	tolerance, oxynil tolerance, oil content); **oilseed rape** Polish type- *B. rapa* (glufosinate tolerance, glyphosate tolerance); **maize** (sethoxydim tolerance, glyphosate tolerance, resistance to European corn borer , resistance to European corn borer + glyphosate tolerance, glufosinate tolerance, imidazolinone tolerance, glufosinate tolerance + fertility restored, resistance to European corn borer + glufosinate tolerance, resistance to corn root worm, resistance to corn root worm + glufosinate tolerance, resistance to corn root worm + glyphosate tolerance, resistance to lepidopteran pests, modified amylase for ethanol production, enhanced lysine level); **alfalfa** (glyphosate tolerance)[††]; **soybean** (glufosinate tolerance, glyphosate tolerance, high oleic acid content); **potato** (resistance to Colorado potato beetle, resistance to Colorado potato beetle + potato virus Y, resistance to Colorado potato beetle + potato leafroll luteovirus); **sugar beet** (glufosinate tolerance, glyphosate tolerance); **flax** (sulfonylurea tolerance); **lentil** (imidazolinone tolerance); **sunflower** (imidazolinone tolerance); **wheat** (imidazolinone tolerance)	*rape* Polish type (none); *maize* (all except sethoxydim tolerance, imidazolinone tolerance, modified amylase for ethanol production); *alfalfa* (glyphosate tolerance) [†]; *soybean* (all); *potato* (all); *sugar beet* (all); *flax* (sulfonylurea tolerance)
Burkina Faso	**Cotton** (resistance to lepidopteran pests)	**Mali**- no; **Niger**- no; **Ivory coast**- no; **Ghana**- no; **Togo**- no; **Benin**- no
South Africa	**Maize** (glyphosate tolerance, resistance to European corn borer, resistance to European corn borer + glufosinate tolerance, resistance to lepidopteran pests + glyphosate resistance); **Soybean** (glyphosate tolerance); **Cotton** (glyphosate tolerance, resistance to lepidopteran pests, resistance to lepidopteran pests + glyphosate tolerance)	**Namibia**- none; **Botswana**-none; **Mozambique**-none; **Zimbabwe**- none; **Swaziland**-none; **Lesotho**-none
Egypt	**Maize** (resistance to European corn borer)	**Sudan**-no; **Libya**-no; **Israel**-no; **Palestine** (Gaza strip)-no
Iran	**Rice** (stem borer resistance)	**Pakistan**- no; **Afghanistan**- no; **Turkmenistan**-no; **Armenia**- no; **Iraq**-no; **Turkey**-no; **Azerbaijan**- no; **Nakhichevanskaya** (autonomous)-no
India	**Cotton** (resistance to lepidopteran pests)	**Pakistan**- no; **China**- yes; **Nepal**- no; **Bhutan**- no; **Bangladesh**- no; **Myanmar**- no

China‡	**Cotton** (resistance to lepidopteran pests); **poplar** (resistance to lepidopteran pests); **papaya** (resistance to ring spot virus); **tomato** (delayed ripening, resistance to cucumber mosaic virus); **sweet pepper** (resistant to cucumber mosaic virus); **petunia** (color altered petunia)	**Mongolia**- none; **Russia**- none; **North Korea**-none; **Vietnam**-none; **Laos**-none; **Myanmar**-none; **India**-*cotton* (resistance to lepidopteran pests); **Bhutan**-none; **Nepal**-none; **Pakistan**-none; **Afghanistan**-none; **Tajikistan**-none; **Kyrgyzstan**-none; **Kazakhstan**-none
European Union‡		
Poland	**Maize** (resistance to European corn borer)	**Ukraine**- no; **Belarus**- no; **Russia**-no
Romania	**Maize** (resistance to European corn borer), **soybean** (glyphosate tolerance)	**Ukraine**- none; **Moldova**- none; **Serbia**- none
Slovakia	**Maize** (resistance to European corn borer)	**Ukraine**- no
Czech Republic	**Maize** (resistance to European corn borer)	Surrounded only by EU states
Spain	**Maize** (resistance to European corn borer)	Surrounded only by EU states
Portugal	**Maize** (resistance to European corn borer)	Surrounded only by a EU state
Germany	**Maize** (resistance to European corn borer)	**Switzerland**-no

Sources: Agbios (2008); GMO-Compass (2008); ISAAA (2008)

*There may be further differences in the event (s) of a particular trait approved among the adjacent countries

‡ Source: (Chen and Qu 2008)

†Deregulated in 2005 but later returned to regulated status after a court moratorium

††Authorized for environmental release but approvals were not yet sanctioned for commercial planting

‡All the GM crops approved for environmental release still have to go through a variety registration process before they can be authorized for commercial planting in Canada

‡Approvals in the European Union is sanctioned by the EU directorate on behalf of its member states. Adventitious presence may be a concern in adjacent non-EU states where the GM crop/trait in question is not authorized.

Thus, it is necessary to consider the implementation of additional regulatory measures for growing GM crops in border regions where international gene flow is a possibility. International co-operation and information sharing among the countries in question could resolve how to deal with this possibility. Furthermore, the possibility of international gene flow should be considered as an essential component in the decision-making processes for the field release of any GM crop.

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