Assessing chemical control options and their effects on lesser clover leaf weevil, red clover pollinators and evaluating red clover seeding rates for optimal seed production and N fixation in Saskatchewan, Canada

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Abstract

Red clover (*Trifolium pratense* L.) seed production in Saskatchewan is complicated by a series of agronomic and pest management issues. These include damage from the lesser clover leaf weevil (*Hypera nigrirostris* F.; LCLW), insecticide toxicity to bee community and unidentified optimal red clover seeding rate and biological N fixation. The research presented in this thesis had two objectives. First, to evaluate the effectiveness of the insecticides deltamethrin and cyantraniliprole in controlling LCLW in red clover in both field and laboratory-based evaluations, as well as to assess potential impacts on the bee community. The second objective was to determine the optimal red clover seeding rate for both seed production and N fixation.

Examined under the field conditions, both insecticides were effective at controlling LCLW. However, deltamethrin provided rapid pest suppression in 24 hours after application. Rearing LCLW larvae on treated plants under laboratory conditions showed similar results where both insecticidal materials significantly reduced LCLW numbers in 10-12 days after treatment. The abundances of LCLW were always negatively related to seed yield regardless of treatment and initial weevil pressure. Seed yield was significantly lower in untreated controls whereas no difference was found between deltamethrin and cyantraniliprole application. In contrast, no yield response was found when LCLW abundance was lower than four larvae per 10 shoots. Unexpectedly, the bee community was not affected by either treatment and was not associated with seed yield.

In this study, red clover was able to compensate for the range of seeding rates between 2.5 and 10.5 kg ha^{-1} without seed yield and N fixation losses, whereas only a 4.5 kg ha^{-1} rate had higher biomass production and N fixation value than a 0.5 kg ha^{-1} rate under adequate precipitation conditions. In contrast, no seeding rate effects were found in a site with limited water availability. Regardless of site, biomass was always positively correlated with N fixation; however, no association was found between seed yield and N fixation.

Collectively, this thesis reveals that for single cut red clover seed production in Saskatchewan, a seeding rate of 4.5 kg ha^{-1} is optimal as it generated greater seed yield and N fixation than 0.5 kg ha^{-1}, whereas higher seeding rates had no explicit benefits. It also demonstrated that LCLW management is likely more important to seed production than the risk of harm to bee the community. Finally, while not fully quantified, results in this thesis indicate that drought and water stress can substantially influence red clover seed yield.

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LIST OF ABBREVIATIONS

LCLW	Lesser clover leaf weevil
N	Nitrogen
MoA	Mode of Action
RvR	Ryanodine Receptors
IRAC	Insecticide Resistance Action Committee
IPM	Integrated Pest Management
IPPM	Integrated Pest and Pollinator Management
EIL	Economic Impact Level
DAT	Days After Treatment
UTC	Untreated Control (pure water)
RCBD	Randomized Complete Block Design
ANOVA	Analysis of Variance
df	Degrees of Freedom
PERMANOVA	Multivariate analysis of Variance
FC	Field Capacity
Ndfa%	Percentage of N derived from atmosphere

1. GENERAL INTRODUCTION

1.1 Introduction.

Red clover (*Trifolium pratense* L.) is an important forage seed crop in Canada. In Saskatchewan, seed production of red clover contributes over $4-6 million annually to the provincial economy in grower sales (SFSDC annual report, 2019), in addition to a significant additional value in seed processing and seed export. Following alfalfa (*Medicago sativa*) seed, red clover is the second most important crop to Saskatchewan forage seed growers. Besides seed production, red clover is widely used as a cover crop in cropping systems or as a nitrogen source on organic farm operations (Kayser et al., 2010). It can also be a productive forage crop in areas with high moisture, particularly because it tolerates acidic soil better than alfalfa (Taylor & Quesenberry, 1996). To maximize red clover seed production, it is crucial to evaluate integrated pest control and agronomic practices.

Seeding rates of red clover for seed production in Canada range from 2.2 to 4.5 kg ha^{-1}. The recommended seeding rates specific to eastern Canada are from 5.6 to 10 kg ha^{-1} (Bowly & Upfold, 1985). However, currently there is no formal seeding rate recommended for red clover seed production in Saskatchewan. While seeding rate has been well studied for many legumes, limited data are available to support relationships of seed production, stand density, N fixation and winter hardiness of red clover for seed production.

There are numerous species of insect pests that can threaten Saskatchewan forage crops. These include insects such as thrips (*Haplothrips* spp.), aphids (*Acyrthosiphon* spp.), lygus bugs (*Miridae* spp.), and multiple weevil species (*Curculionidae* spp.). However, in red clover, the lesser clover leaf weevil (*Hypera nigrirostris* F.; LCLW) is the most important (SFSDC, 2016). Uncontrolled infestation of this pest can lead to significant yield losses resulting from sometimes complete defoliation of the clover shoots and reduced or damaged flowers. Combined, these result in greatly reduced seed yield (Sechriest & Treece, 1963). Management of these weevils is achieved almost exclusively through the application of insecticides and currently deltamethrin is the single chemical registered t in Saskatchewan. Recently, a new class of systemic insecticides (anthranilic diamides), employing a novel mode of action have been developed and commercialized. Cyantraniliprole, an insecticide from this class is one such material class that has succesfully been used for controlling pests from the weevil family *Curculionidae* (Caballero et al., 2015). This insecticide may provide an alternative to deltamethrin in managing LCLW in red clover.

Red clover is a cross pollinated crop and is therefore dependent on insect pollination. Multiple bee species were reported to successfully pollinate red clover (Rao & Stephen, 2009). However, deltamethrin was reported to cause bee mortality and severe sublethal effects due to its toxicity and mode of action. Cyantraniliprole, on the other hand, has not been well studied for effects on bee community and LCLW. Neither material has been studied for effects on the bee community in clover.

1.2. Hypotheses and objectives

The first objective of this study was to evaluate the efficacy of the registered accepted (deltamethrin) and alternative (cyantraniliprole) insecticidal materials in controlling LCLW and the effects of these treatments on the bee community. The second objective of this study was to determine an optimal seeding rate of red clover for both seed production and N fixation. I hypothesized that:

1) Cyantraniliprole, an insecticide with a systemic mode of action (MoA), would provide rapid LCLW control, higher seed yield protection and little to no effect on the bee community, whereas the commercially available contact insecticide (deltamethrin) would have an opposite effect.

2) 0.5 and 10.5 kg ha^{-1} seeding rates will have significantly lower seed yield and N fixation than more moderate rates, whereas the optimum seeding rate will have the highest returns for both criteria.

1.3. Thesis organization

This thesis is organized in manuscript format. In Chapter I, I provide a general introduction to the whole work. In chapter II, I provide an overview of the importance and agronomic management of red clover as well as on red clover pests, pollinators and their management. In chapter III, I present a study where I evaluated the effects of insecticides on LCLW and the red clover bee community. In Chapter IV, I present a field study in which I evaluated the seeding rates for optimal red clover seed production, N fixation and overwintering. In chapter V, I discuss major findings from both chapters three and four, concluding the thesis as a whole and indicating future work pathways. The literature cited in this thesis is located in Chapter VI. Supplemental material is presented in Chapter VII.

2. Literature Review

2.1 Importance and management of red clover

2.1.1 Red clover and its significance in Saskatchewan agriculture

Red clover (*Trifolium pratense* L.) is one of 250 species in the genus *Trifolium* and family *Fabaceae* (Taylor & Quesenberry, 1996). Due to characteristics such as improving soil qualities, high vigor and nutritional values, ease of establishment, and rapid growth, red clover has become a crucial forage crop in the temperate climate zone (Smith et al., 1985). The cultivation of red clover originated in southeastern Europe and Asia minor in what is now Turkey. By the late 1580s red clover was successfully introduced into Western European countries, where it was mainly used as a forage to supplement soil N for the rapidly growing practice of agriculture production. Chorley (1981) reported that an increase of production in European agriculture in the 18th century by approximately 175% was mainly due to the increased cultivation of nitrogen-fixing legumes, particularly clover. Kjærgaard (2003) reports that at the same period of time, 30-50% of the Denmark farmland was covered with red clover. However, with the increased development and use of industrial synthetic fertilizers, red clover fields decreased from 9.5 million hectares in the 1980s to about 6 million ha in 2000 (Rochon et al., 2004).

Presently, in Saskatchewan, interest in red clover cultivation remains high, but primarily in the context of animal forage and seed production. Use as a forage can be attributed to a few important attributes. First, red clover crude protein and overall feed intake is reported to be similar or higher than alfalfa (Fraser et al., 2004; Halling et al., 2002). Second, red clover contains polyphenol oxidase, an enzyme, may reduce proteolysis both in the silo and in the rumen, further increasing the protein supply to the animal (Jones et al., 1995). Third, red clover also contains high concentrations of isoflavanoid and formononetin, which were linked to an increased live-weight gain of finishing lambs. Steinshamn (2008) reported that ruminant livestock diets containing red clover amplified the content of beneficial fatty acids in milk and increased total production of meat and milk. Moreover, the high content of polyphenol oxidase in red clover slows down the degradation of proteins in silage and as a result, reduces N losses from plant material (Sullivan & Hatfield, 2006). The soluble carbohydrate content of red clover along with its digestibility is lower in comparison with forage grasses (Boller et al., 2010). Thus, red clover is frequently grown in pastures and in mixtures with multiple forage grasses to increase the nutritional value of the pasture. In Canada, inclusion of red clover as a companion crop with timothy-grass (*Phleum pratense*) resulted in lower timothy yields in the establishing year, but greater total yield over years (Lemieux et al., 1987). Red clover for seed production can be established either as a pure stand or with an annual cereal companion crop, such as oat (*Avena sativa* L.), wheat (*Triticum aestivum* L.) or barley (*Hordeum vulgare* L.), where red clover seeds are typically harvested on the second year (Taylor & Quesenberry, 1996).

In Canada two types of red clover are grown: early flowering (double-cut) and late flowering (single-cut). The latter is also known as mammoth type (Boelt et al., 2015). In the single cut cultivars, seed is mainly produced only in the second and subsequent years. Single-cut cultivars are winter-hardy in contrast to double-cut and are usually grown in the parkland area of the prairies and northern regions of British Columbia (Fairey, 1985). Following alfalfa, red clover is the most valuable forage seed crop to Saskatchewan forage seed producers. In 2017-2018 red clover seed production contributed over $4 million to the provincial economy (SFSDC annual report, 2019). Canada has traditionally been the top exporter of red clover seeds to the United States and Europe where it is mainly used as a top dressing for winter wheat for plow down purposes (Red clover seed production in Alberta, 2017; ITC, I, 2020; Fig 2.1).

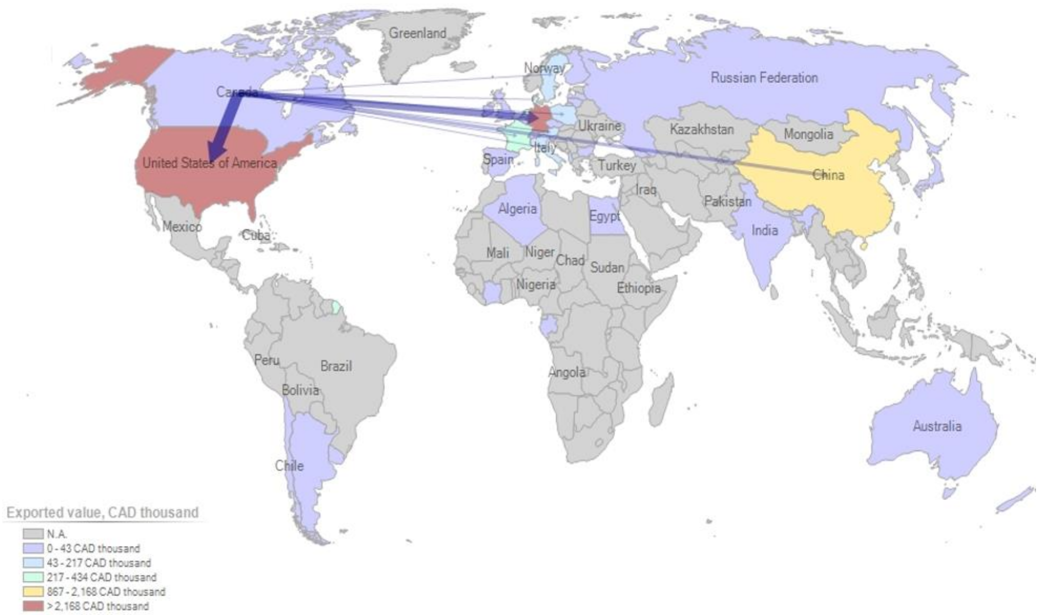

Figure 2. 1. List of importing markets for clovers seed (for sowing) exported by Canada in 2018. Adapted from www.trademap.org.

As an industrial crop, red clover is known for its quick establishment, tolerance to shade, low pH, low soil fertility and poorly drained soil compared to other forage crops (Petkovic et al., 2017; Undersander et al., 2000). However, red clover is considered less drought tolerant than alfalfa based on leaf- to-root weight ratios, and shoot growth rate at low water levels.

2.1.2 Seeding rate, seed yield and seed yield component

On a global scale, red clover seed is mainly produced in the United States (Oregon), Canada, and in most European countries (Kjærgaard, 2003). Seed yield as well as seeding rates of this crop may vary greatly due to climatic conditions and agronomic management practices. Multiple studies have examined the optimal range of red clover seeding rates for red clover cultivated either in grass mixtures or in pure stands for forage production. Schnyder et al. (2010) determined that the optimal seeding rate for red clover grown in grass mixtures in Belgium lies in a range of 12-15 kg ha^{-1}. In Ireland, a seeding rate of 12 kg ha^{-1}of red clover with 2 or 4 kg ha^{-1}of timothy resulted in the most satisfactory stand and forage biomass (Frame et al., 1985). In Canada, growing red clover in a pure stand under a two-cut system at a seeding rate of 10 kg ha^{-1} guaranteed high herbage yields regardless of May or June seeding time (Coulman & Kielly, 1988). Singer and

Meek (2012) indicated a strong relationship between forage biomass and plant number, where 150 plants m^{-2} rate was a critical threshold for optimizing the yield when red clover is interseeded in winter cereals.

Seeding rates for red clover seed production in Canada, particularly in Saskatchewan are less studied and vary significantly among provinces. In Eastern Canada, as little as 3 kg $ha^{-1}$of red clover seed can produce high seed yields; however, it is recommended to sow 8-10 kg $ha^{-1}$to ensure a good stand (Bowly & Upfold, 1985). A seeding rate of 2.2 kg ha^{-1} is recommended in Alberta. If poor seeding practices are followed, though, higher seeding rates (4.4 – 6.7 kg ha^{-1}) are recommended for improved establishment (Red clover seed production in Alberta, 2017). Pankiw et al. (1977) reported that only very low rates less than 0.5 kg ha^{-1} and rates above 8 kg ha^{-1} resulted in significantly lower seed yield. In the same study red clover was able to compensate for row spacings between 15 and 60 cm and for the range of seeding rates between 1 and 5 kg ha^{-1}. However, there is limited data available on seeding rates and seed production in Saskatchewan.

Seed yield of red clover can vary greatly ranging from 200 to 800 kg ha^{-1} (Petkovic et al., 2017). Red clover seed yield consists of multiple components that are categorized as primary components: the number of florets per flower head, the number of flower heads per unit area (often recorded as number of flower heads or shoots), the number of seed florets, and seed weight. In addition, there are secondary or composed components, such as seed yield per inflorescence and the number of seeds per inflorescence (Amdahl et al., 2017). It is common to evaluate seed yield components, especially in breeding contexts, on single plants since they are more practical and cheaper to manage in contrast to a dense clover canopy. Nevertheless, forage legumes may vary in their response to competition in a dense plant canopy compared to single plants (Fairey & Hampton, 1997). For instance, in alfalfa a poor correlation between seed yield and seed yield per plant was found in a dense canopy (Annicchiarico, 2006). In a recent study, Vleugels et al. (2016) reported that while seed weight per plant, thousand seed weight and number of unripe flower heads per plant are explanatory traits for seed yield, the number of ripe flower heads seems to be the most relevant factor to determine potential seed yield in the majority of red clover cultivars.

2.1.3 Biological nitrogen Fixation and N cycling

Soil nitrogen (N) is a major factor that limits plant growth and production in most agricultural ecosystems throughout the world (Bloom, 1997). Even substantial increase in the use of synthetic nitrogen fertilizers in modern intensive agriculture, the amount of N removed from the soil in the forms of crop harvest, runoff, erosion, leaching and denitrification has been calculated to greatly surpass the N inputs in Canadian prairies (Doyle et al., 1992). However, due to rising concerns about environmental degradation, increases in the prices of N fertilizers, and loss of natural resources, there is increasing interest in determining the potential for biological N fixation and verifying its contribution to the N fertility of soils in agricultural systems (Adjesiwor & Islam, 2016; Peoples et al., 1995). Legume crops fix atmospheric N by forming symbioses with a variety of soil bacteria known as *Rhizobium* species, which together with nitrogenase enzymes, convert N in the air into ammonia (NH3) in root nodules, whereas the nodules are formed as a result of the *Rhizobium* infection in the plant roots (Zahran, 1999). In this way, legumes are able to meet almost all of the N requirements for growth and reproduction as well as transfer fixed N to subsequent crops through plant residuals, and consequently reduce N depletion from the soil N pool (Carlsson & Huss-Danell, 2003).

Among N-fixing plants, forage legumes have the advantage that a larger proportion of the N accumulated generally is available for return to the soil N pool via leaf fall, grazing animals and root turnover compared with grain legumes where much N is harvested with the seeds (Peoples, Herridge & Ladha, 1995). In addition, Warembourg et al. (1997) indicated that more than a half of symbiotic N that is located in above-ground red clover tissues is constantly recycled from one generation of leaves to the next, and only about 25% of the annual harvest is stored in seeds. Reported quantity of N derived from fixation by legumes shows large variation (Ladha, 1996). For instance, Carlsson and Huss-Danell (2003) reported a range in the amount of 0-373 kg ha^{-1} N derived from fixation by red clover.

The great variation in estimates of N fixation by legumes can be attributed to both internal and external factors, such as crop factors, environmental factors and agronomic practices (Peoples et al., 2012). Red clover varieties significantly differ in the number of nodules, plant biomass, root attributes, shoot and root N concentration (Thilakarathna et al., 2017). Lipsanen and Lindstrom (1985) found that temperature is one of the most important environmental factors influencing Rhizobium - clover symbiosis. Temperatures below or above the optimum can significantly reduce Rhizobium activity and consequently the amount of fixed N. Moreover, environmental factors including: precipitation, soil water and mineral N conditions, other soil nutrient content such as soil P, K and Co, soil pH, residual sulfonylurea herbicides, pests, and soil salinity, can greatly affect N_2 fixation by legumes (Peoples et al., 2012; Høgh-Jensen, 2013; Bakken et al., 2004). Overall, N fixation has a negative correlation with soil N availability and high doses of N fertilizer in particular. Knight et al. (1995) found that applying urea at the rate of 100 kg ha^{-1} completely suppressed N fixation in legumes, suggesting an impact of N source on water use efficiencies. In general, environmental factors impact legume N fixation largely by regulating the soil, chemical, biological and physical conditions that are critical for accumulation of plant biomass, root nodulation and accessibility of plant to soil nutrients and water (Bordeleau & Prévost, 1994). Agronomic management practices can also indirectly affect the N fixation of legumes by regulating growing conditions (Xie, 2017). Multiple studies evaluated the seasonal dynamics, distribution and fate of nitrogen in red clover where strong associations were found between leaves biomass and N fixation (Warembourg et al., 1997). However, there is a lack of studies on relationship among red clover for seed yield, seeding rates, and their N fixation capacity.

2.1.4 Stable isotopes as a technique to identify the nitrogen fixation

There are several methods available to measure N fixation (Bergersen, 1980). These include: increment in N yield and plant growth, nitrogen balance, and acetylene reduction, while other methods use N isotopes, or examine the relative natural abundance (Hardarson & Danso, 1990). Among these methods, ^{15}N isotope methods are typically more costly but are considered more precise in agricultural studies if applied appropriately (Khan et al., 2002). The first use of N isotopes in agricultural experiments was done by Norman and Wekman (1945), who introduced a procedure that enabled researchers to better understand N transformations and its cycling within a plant, including N use efficiency (Myrold & Bottomley, 2008; Post, 2002). This method involves the growth of N-fixing and non-fixing reference plants in soil fertilized with N enriched inorganic or organic fertilizers. Typically, ^{15}N-labeled fertilizer includes inorganic salts such as: NH4Cl, KNO_3, $(NH_4)_2SO_4$, NH_4NO_3 and ^{15}N marked ureaform fertilizer (Chalk, 1985; Brown & Volk, 1966; Witty, 1983). This technique is based on differential dilution in the plant of N-labelled fertilizer by soil and fixed nitrogen (Dittert et al., 1998). Two assumptions that are central to the validity of this method include: the legume crop of interest and the reference crop take up an approximately identical proportion of fertilizer-derived enriched N and soil N during the growing season, and (2) the reference crop does not take up N derived from the atmosphere and released by the legume crop (Hardarson & Danso, 1993). Consequently, growing the appropriate reference crop with a similar lifecycle and N uptake is essential for accurate estimation of legume N fixation. Overall, this powerful technique may be utilized to determine the influence of agricultural practices such as crop rotations, tillage systems, seeding rates and nutrient recycling, where labelled ^{15}N can be traced in both N-fixing and non-fixing plant material.

2.1.5 Effects of drought stress on seed yield and N fixation.

Scant information about water requirements and crop stress response for red clover seed production is available. Overall, red clover is reported to be less drought tolerant than alfalfa and other forage legumes. Kendall et al. (1988) evaluations of drought effects on alfalfa and red clover cultivars' shoot weight, root weights, shoot-root ratios and the number of roots revealed significant differences between species under the same drought conditions, yet cultivars within two species did not differ.

In the study, red clover shoot growth was inhibited more than alfalfa shoot development at low water levels, and the reverse was true at high water levels. The highest red clover seed yields in the Western US, particularly in Oregon were reported to be achieved by regular frequent irrigation that kept plants growing vigorously throughout the vegetative and seed setting periods (Olivia et al., 1994). Proper irrigation management was also reported to increase flower density, floral fertility and as a result seed yield production of white clover (Clifford, 1987), and alfalfa (Cohen et al., 1972). Similarly, growing red clover under drought stress reduced the duration of season-long bud and flower production, stem length, potential seed yield, and floral fertility (Olivia et al., 1994).

As described in previous sections, N fixation by legume species and, consequently, the potential contribution of N fixation to N soil pool is influenced by multiple abiotic and biotic factors. However, in arid and semi-arid ecosystems, soil water availability is generally the primary determinant of plant growth (Loik, 2004), and can negatively affect nodulation and symbiotic N fixation in legumes (Zahran, 1999). From studies of multiple legumes, it has been reported that drought stress has a negative impact on nitrogenase activity in legume nodules (Sprent 1972), presumably due to: (1) reduced activity of the main enzyme involved in sucrose hydrolysis in nodules, which leads to a depletion of substrates for bacteroid respiration (González, 1998), (2) change in carbohydrates supply (reduction of photosynthesis) (Huang et al., 1975) or (3) sensitivity of nodule functioning to dehydration (Davey & Simpson, 1990). As a result of declining nitrogen fixation under drought conditions, a 30-40% depression in N content can be observed in stressed legume leaves and pods (Streeter, 2003).

2.2 Red clover pests, pollinators, and their management

2.2.1 Clover pests

Despite extensive crop protection efforts, primarily in the form of chemical control, proportional crop yield losses to pests have remained constant or even increased in the last century (Oerke & Dehne, 2004). Red clover grown for seed production is vulnerable to numerous insect pests, including generalist feeders such as grasshoppers, crickets, armyworms and other caterpillars, and miscellaneous beetles (Taylor & Quesenberry, 1996). However, their attacks are sporadic, and other plant species are typically more attractive to many of these insects than red clover (Smith et al., 1985). Nevertheless, multiple insect species prefer red clover as a primary or alternative host and can cause significant damage. Worldwide as well as in Saskatchewan, pea aphids (*Acyrthosiphon pisum* Harris) concentrations on clover leaves and stems are reported to cause severe damage by feeding on plant sap, but the aphids may be even more destructive as vectors of viral diseases such as Alfalfa mosaic virus, Bean yellow mosaic virus and Peanut stunt virus (Gorz et al., 1979). The red clover casebearer (*Coleophora deauratella* Leinig and Zeller) was recently identified in Western Canada and reported to substantially reduce seed yield production, especially in the 2nd year stand (Evenden, 2010; Kelsey, 1958). The damage is mostly caused by casebearer larvae that feed on flower's reproductive structures and available nectar and can destroy 2-3 seeds/day as they develop (Chynoweth, 2018; Landry, 1991). In Canada, as in many other countries where clover is widely cultivated, weevils (beetles in the family Curculionidae) present the biggest threat to sustainable clover establishment and seed production. Different weevil species may dominate in a certain region, the most destructive of them are reported to be: clover leaf weevil (*Hypera punctata* F.), clover head weevil (*Hypera myles* F.), clover seed weevil (*Protapion trifolii* L.), clover stem weevil (*Apion virens* Hrbst.), *Apion fulvipes* Geoff., and lesser clover leaf weevil (*Hypera nigrirostris* F.) (Hanssen & Boelt, 2008; Latsinger & Apple, 1984; Taylor & Quesenberry, 1996; Lundin et al., 2016). In Saskatchewan, LCLW remains the major destructive pest in red clover fields.

2.2.2 Lesser clover leaf weevil

The habits of LCLW have caused it to be overlooked by many investigators since the damage might be inconspicuous. Often thought to be of little economic importance (Latsinger & Apple, 1984), LCLW is reported to have caused significant reductions in red clover seed yield production globally (Lundin et al., 2017; Markkula & Tinnila, 1956). LCLW has a worldwide distribution (Hansen & Boelt, 2008). It was reported in the United States and Southern Canada in 1875, but not until 1985 were the first LCLW identified in Saskatchewan (MacNay, 1958; Weiss & Gillott, 1993). Canadian studies of LCLW biology revealed that adults overwinter preliminary in red clover fields beneath plant debris and begin to feed, mate and oviposit in late April to early May (Weiss & Gillott, 1993). The lesser clover leaf weevil is approximately 4 mm long. Newly emerged, adults are usually light brown, but after about a week, they become bright green, whereas overwintered adults tend to be more blue-green or sometimes nearly black. There are four instars of LCLW larvae and all of them have a white to grey colour with a prominent black head capsule (Detwiler, 1923; Weiss & Gillott, 1993).

Both larvae and adults of LCLW feed on red clover, but the major damage is usually caused by larvae. Typically after hatching larvae migrate to the axils between two stipules or to the flower head where they eat both seeds and the green parts of the plant, and in many cases the clover shoot and flowers are completely destroyed (Sechriest & Treece, 1963). Markkula and Tinnila (1956) reported that severe infestation with LCLW could cause complete defoliation of clover plants and greatly reduce seed yield. Similarly, Weiss and Gillott (1993) reported that densities of third and fourth instar LCLW larvae greater than three per five shoots could damage up to 50% of clover buds and flower heads. In addition, LCLW larvae reach its peak when red clover fields are in the late bud to mid-bloom stage.

In order to be a suitable host, a plant species should be accepted as food by both the adults and larvae during their development. Markkula and Tinnila (1956) reported that LCLW could feed or oviposit on multiple true clover species including: red clover (*Trifolium pratense*), white clover (*T. repens*), alsike clover (*T. hybridum*), crimson clover (*T. incarnatum*), Persian clover (*T. resupinatum*), strawberry clover (*T. fragiferum*) in addition to alfalfa (*Medicago sativa* L.). Red clover, however, had the highest acceptability and feeding preference by both adult and larvae stages (Table 2.1) (Sechriest & Treece, 1963).

Clover species with morphological characteristics such as large stipules and flower heads were found to be more acceptable to the LCLW. This preference may be linked to the plentiful food supply and protection provided to the insect within the stipules and big flower heads (Sechriest & Treece, 1963).

Table 2. 1. LCLW tolerance (0 - No feeding, or oviposition; 1 – No feeding, or oviposition; 2 – Moderate feeding, or oviposition; 3 – severe feeding or oviposition). Adapted from Sechriest & Treece, 1963.

Crop	Acceptability to Larva	Acceptability to Adult	Preferences for Feeding	Preferences for Oviposition
Red clover	3.0	2.7	3	3
White clover	1.2	2.7	2	2
Alsike clover	—	—	2	2
Yellow Sweet clover	0.0	1.0	1	1
Crimson clover	1.7	1.3	2	2
Alfalfa	0.2	0.7	1	1
Persian clover	0.7	1.0	1	1
Strawberry clover	2.8	1.0	—	—

2.2.3 Red clover pollination

Red clover is self-sterile and outcrossing pollination for each single flower is required to achieve a successful seed set (Proctor et al., 1996). In red clover, each fruit, or more specifically pod, can produce one seed. Seed set under natural conditions varies from 34 to 65%, which is 37 to 107 seeds per head out of 50-200 flowers (Hegland, 2014; Retallack & Willison, 1990). Bees are recognized as the primary pollinators of red clover, and honey bees (*Apis* spp.) together with leafcutter bees (*Megachile* spp.) are frequently used for pollination services as native bees might be in short supply. Overall, honey bees with a stocking rate 1-2 colonies per hectare and wild bees such as bumble bees (*Bombus* spp.) are considered to be the most important pollinators (Free, 1993), Although, there has been considerable disagreement over the relative value of various bee species to red clover pollination (Bohart, 1957). Honey bees' pollination of red clover was considered ineffective primarily due to their short tongues relative to the long corolla tube of red clover florets (Darwin, 1859; Pammel & King, 1911), yet Inouye (1980) reported compelling evidence that corolla tube length does not repel honey bees from red clover pollination nor does it reduces their effectiveness. However, honey bees are significantly slower at foraging on red clover than long tongued bumblebees, and there are still concerns about red clover pollination by honey bees as their efficacy was reported to depend on the amount of alternative foraging sources in the vicinity (Inouye, 1980; Peterson et., 1960). Hawkins (1969) found that seed yield in red clover was strongly correlated with the number of long-tongued bumblebees such as *Bombus pascuorum* and *B. hortorum*, but no correlation was found with the number of honey bees or short-tongued bumble bees such as *B. terrestris* (Hawkins, 1969; Vleugels et al., 2016). Westgate and Coe (1915) found that the efficiency of one bumble bee could be equated to the productivity of two and a half honey bees. Besides shorter flower handling time, bumble bees are typically better pollinators of red clover due to: (1) activity at temperatures below 14°C, which is the limiting temperature for honey bees activity (Vicens & Bosch, 2000; Sapir, 2017), (2) the ability to forage under harsh weather conditions such as wind and rain (Lundberg, 1980; Tuell & Isaacs, 2010), and (3) foraging for pollen rather than nectar, which results in more pollen transfer to the flower's pistils with each visit (Hagbery & Nieh, 2012).

2.2.4 Lesser clover leaf weevil control

Besides abiotic factors there are multiple causes that can influence LCLW densities in red clover. The cultural practice of burning of red clover fields in spring can destroy overwintering LCLW adults but may also reduce the population of parasites and predators limiting potential biological control of the weevil (Taylor & Quesenberry, 1996). Additionally, the LCLW has several natural enemies and parasites, which were linked to significant pest reduction. The pupae are attacked by *Bracon mellitor* (Hymenoptera: Braconidae), which is an external feeder on the LCLW and may pupate near the feeding site and crawl to different pupation sites, *Bathyplectes* (Hymenoptera: Ichneumonidae) can feed internally on the LCLW larvae and pupae within the cocoon, and *Dibrachoides dynastes* (Hymenoptera: Pteromalidae) also pupates within the cocoon of the host (Sechriest & Treece, 1962; Weiss & Gillott, 1993). However, no biological control is commercially available for LCLW control.

In Saskatchewan, LCLW control is accomplished almost exclusively through the application of insecticides. Currently, and for over a decade, deltamethrin (Decis®, Poleci®) from the pyrethroid family of insecticides (IRAC Group 3A) has been the only material registered for pest control in red clover for seed production (Saskatchewan Ministry of Agriculture, 2019). Intensive use of pyrethroids and the limited availability of compounds from other chemical classes leads to intense selection pressures being imposed on insects, resulting in resistance to pyrethroid insecticides (Coelho, 2009; Nauen, 2005). Multiple studies have already reported pyrethroid resistance in populations and species of weevils worldwide (Ramoutar et al., 2009; Ribeiro et al., 2003). Moreover, the use of pyrethroid insecticides is problematic as they can have fatal effects on bees and other beneficial insects (Gromiszs, 1994).

Even though most research into the impacts of insecticides on bees has focused on honey bees due to their extensive use in commercial pollination globally, and concerns over widespread honey bee loss in the USA and Europe (Baron et al., 2014; Potts, 2010), recent studies have demonstrated sublethal effects of insecticides on native pollinators and bumble bees' health and foraging ability (Gill et al., 2012; Laycock, 2012). Applying pyrethroid insecticides, in particular deltamethrin, at recommended field doses was reported to cause very high bee mortality of up to 100% at 24 hours after treatment (Stanley et al., 2015a) or to cause severe disorientation (Vandame, 1995). Reduced bee numbers, activity or their ability to orient in red clover fields can negatively affect seed yield production since earlier reports indicated absence of seed set due to lack of bees (Braun et al., 1953; Rao & Stephen, 2009).

Currently, 95% of insecticides used consist of active ingredients that interfere with one of five processes: (1) acetylcholine receptor function, (2) γ-aminobutyric acid (GABA) receptor function, (3) mitochondrial respiration, (4) chitin synthesis or (5) sodium channel function (Nauen & Bretschneider, 2002; Narahashi, 2002). Even the most recent broadly commercialized insecticides act upon previously exploited targets, which contributes to pest resistance (Cordova et al., 2006). One of the most used systemic classes of insecticides in Canada (Neonicotinoids, IRAC Group 4) were demonstrated to impair bees' cognitive functions such as learning and memory, to impair foraging ability and to disrupt communication (Stanley et al., 2015b; Tsvetkov et al., 2017). These findings led to the ban of the neonicotinoids imidacloprid, thiamethoxam, and clothianidin in numerous European countries (Jactel et al., 2019). In light of these bans, the need for suitable, well-studied replacements has become imperative.

Recently, insecticides employing a new mode of action, anthranilic diamide ryanodine receptor (RyR) activators, have been developed to control a broad spectrum of pests. These anthranilic diamides, also called “anthranilimides” or “diamides”, were developed by DuPont (Selby et al., 2017). Cyantraniliprole is the second insecticide with xylem-systemic properties in the new diamides class that was identified as an essential alternative to neonicotinoids due to its rapid control over a broad spectrum of pests and new systemic mode of action.

The anthranilic diamides have a novel mode of action, regulating the ubiquitous RyR, which are responsible for calcium release from intracellular stores. Cyantraniliprole selectively activates and keeps the calcium channels open in insect muscles, which causes depletion of the calcium stores impaired feeding regulation, paralysis and, consequently, death (Jeanguenat, 2013; Selby et al., 2017). Anthranilimides are designed to target insects over mammals (Zhang et al., 2015). Cordova (2006) revealed that anthranilic diamides exhibit >500-fold differential selectivity toward insects, over mammalian receptors that implies a low risk of toxicity to humans. Diamides are being widely used on a range of pests in the orders: Coleoptera, Lepidoptera, Diptera, Hemiptera, and Thysanoptera (Selby et al., 2017). In addition, Cyantraniliprole demonstrated effective pepper weevil (*Anthonomus eugenii* Cano) control (Caballero et al., 2015; Qureshi & Kostyk, 2020), and had low acute bee toxicity (Cordova et al., 2006).

Yield gaps (difference between maximum potential yield and obtained yield) may be amplified where pest damage and its control impact pollinators or pollination. Even though multiple research focused on the need to reduce the non-target effects of pesticides on bees, the ways in which pests and bees are managed remain largely uncoordinated. Focusing exclusively on either pest management or promoting integrated pest management (IPM) as a specific response to bees decline can affect sustainable crop production as well as pollinators and their ecosystem service to crops (Pimentel & Peshin, 2014). Therefore, developing complex integrated pest and pollinator management (IPPM) practices is crucial for achieving high yields in cross pollinated crops. Egan et al. (2020) indicated the major IPPM elements can utilize: (1) pest-resistant and pollinator-attractive cultivars, (2) pollinator-friendly cultural, physical, and mechanical control, (3) pollinator-friendly cultural, physical, and mechanical control, (4) bioactive natural products, and (5) conventional pesticides. Besides following IPPM actions, multiple common practices were verified to be critical for reducing pollinators losses and poisoning during insecticides spraying. These practices involve: preventing application of insecticides that are toxic to bees on crops in bloom, applying insecticides when bees are least active, minimizing insecticide drift, use insecticides that are the least hazardous to bees and beneficial insects.

3. Assessing chemical control options and their effects on the lesser clover leaf weevil (*Hypera nigrirostris)* and red clover pollinators in Western Canada.

3.1 Introduction

Clovers (*Trifolium* spp.) are important protein rich forage and green manure crops that provide a significant nitrogen supply and great weed control (den Hollander et al., 2007; Warembourg et al., 1997; Stockdale et al., 2001). Red clover (*Trifolium repens* L.) for seed production is an integral part of the economy of Western Canada, where a high proportion of the seed is exported (ITC, I, 2020; Coulman & Kielly, 1988). Seed yield of red clover may vary greatly and range from 200 to 800 kg ha^{-1}, whereas the potential yield can reach over 1000 kg ha^{-1} (Boller et al., 2010; Vasiljevic et al., 2005). There are multiple factors that might cause such high variability in yield. However, it is well-documented that satisfactory seed yield is highly dependent on sufficient pollination and insect pest control (Braun et al., 1953; Hansen & Boelt, 2008).

Among multiple insect pests, weevils (*Apion*, *Hypera*, or *Sitona* spp.) have been major pests to reduce red clover seed production (Lundin et al., 2012), with the (LCLW), *Hypera nigrirostris* Fabr (Coleoptera: Cucurlionidae) being one of the most destructive insects in Canada. Both larvae and adults of this species reside, feed, and reproduce on red clover shoots, and can cause severe defoliation and substantial reduction in seed yield (Dickason & Every, 1955). Red clover seed yield can be reduced by more than 50% if the larvae count is six larvae in every 10 stems (Gillott & Weiss, 1993). In a one-year study by Everly (1952), each additional adult per 100 stems can decrease the yield by 2.9 kg ha^{-1} mostly by feeding on young leaves in low portion of the plant. LCLW is distributed throughout Europe, the Unites States, northern Africa, and Western Asia Minor. Since 1985, it has been reported in most of Canadian provinces (Sechriest & Treece, 1963). The main host plants for LCLW include true clovers (*Trifolium pratense*, *T. repens, T. incarnatum, T. medium, T. hybridum*), among which red clover was found to be the most preferred species by both adults and larvae (Sechriest & Treece, 1963). Adult LCLW predominantly overwinter in the fields they infest, beneath plant debris, but also at the base of trees and ditches from where they crawl to the closest host crop fields in spring (Gillott & Weiss, 1993; Sechriest & Treece, 1963). In Canada, LCLW has only one generation with spring and early summer oviposition. Eggs are laid in the stipules and stems and are normally found singly or in small clusters of two to four eggs. Larvae are usually restricted in movement among feeding sites and typically crawl from stipules to clover buds and flowers in the second half of July. LCLW larvae mostly damage the plant by feeding on newly emerged clover buds and eating all of the green parts of the plant along the way. As a result of this feeding behaviour, clover shoots are often destroyed and lodged (Markkula & Tinnilä, 1956). The complete development from egg to adult beetle takes on average 4–6 weeks, depending on the temperature (Sechriest & Treece, 1963).

Commercial farms in Canada manage LCLW almost exclusively through the application of pyrethroid insecticides, and for over a decade deltamethrin (Decis®) has been the single material registered for the suppression of the LCLW in Saskatchewan. The intensive field application of pyrethroids, unfortunately, increases the potential for the development of pesticide resistance in insect pests (Rinkevich et al., 2013).

While only deltamethrin is registered for use on red clover in Western Canada, insecticidal products, such as cypermethrin, cyfluthrin, and thiacloprid, are registered for use in LCLW control in red clover for seed production in other countries. Of these, many are quite toxic to bees and belong to the neonicotinoid group of insecticides (IRAC Group 4A) that are currently being banned worldwide due to their toxicity to bees with sublethal and lethal effects (Raine, 2018; Jensen, 2015). In contrast, cyantraniliprole (Exirel®) is the second insecticide with xylem-systemic properties registered in the new anthranilic diamide class of chemistry (IRAC MoA group 28) that was discovered by DuPont[tm] (IRAC, 2014). The anthranilic diamides have a novel mode of action, targeting and activating ryanodine receptors (RvR) to release calcium ion stores from insect muscle cells, resulting in impaired regulation of muscle contraction, paralysis, and consequently, death (Jeanguenat 2013; Selby et al., 2013; Cordova et al., 2006;). In both field and laboratory trials, cyantraniliprole treatment resulted in high insect mortality and suppressed feeding damage from pepper weevils (Caballero et al., 2015; Stansly & Kostyk, 2010). Similarly, a single cyantraniliprole treatment resulted in significantly increased potato yield and decreased percentage of defoliation due to greatly suppressed numbers of colorado potato beetle larvae (Kuhar & Doughty, 2018). It is not known, however, if cyantraniliprole is effective against LCLW in clover or in commercial production. In addition, effects of these insecticide treatments on bees or on red clover seed yield remain largely unstudied.

Red clover is self-incompatible and therefore cross-pollination is a critical factor for seed production. Bees are recognized as an essential source of red clover pollination (Taylor & Quesenberry, 1996). Among bee species known to pollinate red clover, various bumble bee (*Bombus*) species are reported to be the most effective (Inouye, 1980; Darwin, 1859). However, honey bees (*Apis*) and native bees are also capable of providing effective and nearly maximum pollination of red clover (Rao & Stephen, 2009). Previous studies have found that the number of bees, particularly a lack of effective individuals, is a major factor limiting seed yield, while high seed yields are often associated with a higher number of bees per unit area (Braun et al., 1953). Nevertheless, both native and managed bee populations are declining and are being affected in clover fields and worldwide by intensive use of insecticides (Hladik et al., 2016; Rundlöf et al., 2015; Thompson, 2003). For example, field doses of deltamethrin were reported to significantly reduce bee populations from 50% to 100% in 24 hours after treatment application in both laboratory and semi-field condition (Sharma & Abrol, 2005; Stanley et al., 2015a). At the same time, reduced pollinator visitation may occur in clover fields when no insecticide is applied, mostly due to clover flower damage caused by weevils (Lundin et al., 2017). Thus, besides examining efficacy of the pest management treatments, information is needed on whether pollinator abundance in red clover fields is changed under various insecticide treatment conditions.

The aim of this project was to evaluate the effects of insecticide treatments, LCLW and pollinators on red clover seed yield, and to understand their possible interactions. In particular, we examined three aspects: 1) insecticide efficacy in controlling LCLW utilizing in-field and laboratory-based evaluations at multiple time points; 2) effects of insecticide treatments on bee abundance and 3) the impacts of LCLW and bee abundance on red clover seed yield.

3.2 Materials and methods

3.2.1 Study sites

Studies were conducted in 2018 and 2019 in red clover fields using cv. "Altaswede" cultivar in landscapes dominated by agricultural crops. Across the two field seasons, the experiments were performed at a total of seven locations (Table 3.1). Six commercial fields were located near Nipawin in Northern Saskatchewan, and were seeded by growers for seed production. The distance among commercial fields within each year varied from 1 to 115 kilometers. All Northern Saskatchewan commercial sites were selected due to continuous reports of LCLW damage. One red clover field was grown in Clavet (Livestock and Forage Center of Excellence), Saskatchewan using the same cultivar. The layout of the experiment was a Latin Square design with four replications where the plot size was 4 m x 4 m with 0.5 m buffers between plots. Selected plots from a larger on-going experiment were used in Clavet where Randomized Complete Block Design (RCBD) with four replications was applied. To ensure adequate pollination, either honey bee (*Apis mellifera*) colonies or leafcutter bee (family *Megachilidae*) huts were placed by growers at a distance no farther than 500 meters from the experimental plots on all sites except three sites in 2019, where the apiaries were located within 3 km. Both pests and bees may be greatly affected by the landscape composition around a given field (Bianchi et al., 2006; Kennedy et al., 2013). Therefore, we assessed red clover growth stages among all our locations (Table 3.2) and crops grown in neighboring fields within 1.5 km. Land use was dominated by agriculture and the main cultivated field crops were canola, alfalfa, faba beans and wheat.

Table 3. 1. Summary of data collected from each field site in Saskatchewan in 2018 and 2019.

Site Number	Year	Location	Field Status	Data collection
1	2018	Carrot River	Commercial	LCLW field sampling x3, LCLW larvae rearing, Bees x3.
2	2018	Arborfield	Commercial	LCLW field sampling x3, LCLW larvae rearing, Seed yield, Bees x3.
3	2019	Clavet	Research Farm	LCLW field sampling x2, Seed yield, Bees x3.
4	2019	Love	Commercial	LCLW field sampling x3, Seed yield, Bees x3.
5	2019	Snowden-North	Commercial	LCLW field sampling x3, LCLW larvae rearing, Seed yield.
6	2019	Snowden-South	Commercial	LCLW field sampling x3, LCLW larvae rearing, Seed yield, Bees x3.
7	2019	Carrot River	Commercial	LCLW field sampling x3, Seed yield, Bees x3.

3.2.2 Pesticide application

In both field seasons, three different weevil control strategies were applied: (1) untreated control (pure water) (UTC), (2) deltamethrin (Decis®) and (3) cyantraniliprole (Exirel®). Application took place on June 14 in 2018 and on June 27 in 2019. All treatment applications were conducted using a CO2 backpack sprayer equipped with four TeeJet extended range flat spray nozzles (XR11002; TeeJet Technologies, Springfield, IL, USA) at 40 PSI when the wind speed did not exceed 8 km h^{-1}. In both years Decis® was applied using the highest recommended rate in Canada (250 ml ha^{-1} mixed with 150L water ha) that is equivalent to (12.5 g of deltamethrin mixed with 150L water ha^{-1}). There is no recommended rate for LCLW control using Exirel®, so it was applied at a rate estimated as slightly below the highest rate for controlling other species of weevils (Coleoptera, Curculionidea) (1400 ml ha^{-1} mixed with 200L water ha^{-1}) that is equivalent to (140 g of cyantraniliprole mixed with 200L water ha^{-1}). For the untreated control pure water was used at the same rate as Decis®. Additionally, water sensitive paper cards were used at all sites to estimate droplets size and the coverage uniformity.

3.2.3 Weevil sampling

To estimate the efficacy of insecticide treatments on the number of LCLW in red clover, two different methods were used at all plots of all treatments. In the first method 20 clover shoots were haphazardly collected within 36 hours before treatment and 24 hours after treatment for rearing LCLW larvae for the next 10-12 days under laboratory condition. Collected clover shoots were dipped into rooting hormone (Indole-3-butyric acid 0.4%) and placed in two plastic cups (diameter 6.35cm, height 12.06 cm) filled with 50 ml of agar solution and caged in plastic containers (width, 34 cm; height, 11.7 cm) with two small meshed nets on the sides to ensure that no condensation formed. Rearing containers were kept in growth chambers with 20°C and 70% relative humidity for LCLW development. All rearing boxes were opened after 10-12 days to identify and count living LCLW larvae and adults. In the second method, 10 clover shoots were haphazardly collected from all plots and all sites for field counting of live LCLW larvae within 36 hours before insecticidal sprays were applied to plots, 24 hours after treatment application and 12 days after treatment (DAT). However, in the 2018 season, due to unfavorable weather conditions the last LCLW sampling was performed in 18 DAT, but it was counted and analyzed as 12 DAT time.

Table 3. 2. Summary of collected data, dates, locations, and red clover growth stages in both 2018 and 2019 years.

Year	Data collection	Location	Time	Date	Red clover growth stage
2018	LCLW, Bees	Carrot River	Pre-treatment, Post 24h	June 14-16	8% flowering
2018	LCLW, Bees	Carrot River	12 DAT	July 2	25% flowering
2018	LCLW, Bees	Arborfield	Pre-treatment, Post 24h	June 14-16	15% flowering
2018	LCLW, Bees	Arborfield	12 DAT	July 2	50% flowering
2019	LCLW, Bees	Clavet	Pre-treatment, Post 24h	July 28-30	50% flowering
2019	LCLW, Bees	Clavet	12 DAT	August 9	90% flowering
2019	LCLW, Bees	Love	Pre-treatment, Post 24h	June 27	10% flowering
2019	LCLW, Bees	Love	12 DAT	July 8	28% flowering
2019	LCLW	Snowden-North	Pre-treatment, Post 24h	June 27	Pre-flowering
2019	LCLW	Snowden-North	12 DAT	July 8	20% flowering
2019	LCLW, Bees	Snowden-South	Pre-treatment, Post 24h	June 27	Pre-flowering
2019	LCLW, Bees	Snowden-South	12 DAT	July 8	20% flowering
2019	LCLW, Bees	Carrot River	Pre-treatment, Post 24h	June 27	5% flowering
2019	LCLW, Bees	Carrot River	12 DAT	July 8	20% flowering

3.2.4 Bee sampling

To estimate the effect of insecticide treatments on the abundance and diversity of pollinators, the two most commonly used methods for sampling pollinators were used at all plots of all treatments in six sites over two field seasons. Sampling was performed between June 14 and September 23. Sampling was conducted when daytime temperatures were at least 13 °C. Blue

Vane Traps (Springstar Inc., St. Louis, MO, USA) and "bee bowls" (blue, yellow, and white cups) painted with UV fluorescent paints were filled with a combination of water and blue Dawn™ dish detergent to break water surface tension and deployed into the fields at a fixed 1-meter distances between traps and 2 meters from the edge in each plot at the plant canopy level for 24 hours. Pollinators were collected three times during field seasons including prior to insecticide treatment and 24 hours post-treatment. All collected bees were identified to the genus level since only 10% of bees did not belong to *Apis*, *Bombus* or *Lasioglossum* genera. Other non-bee pollinators were not identified.

3.2.5 Seed yield

To assess potential seed yield of red clover under the insecticide treatments all plots from commercial farm fields were sampled in the last week of September in both 2018 and 2019 field seasons, before the usual harvesting date chosen by the farmer. A systematic random sampling technique was applied. Two sub-samples were collected from each plot using 50 cm^2 square shaped frames. All inflorescences were carefully hand-harvested and dried in a hot air seed dryer. All samples were processed through a seed belt thresher followed by three levels of sieves.

3.2.6 Statistical analyses

Data analyses and generation of graphs was performed using R version 3.5.2 (2018) statistical software (RStudio Team, 2018). Treatment effects were declared significant at α=0.05. Data from the laboratory LCLW rearing from 20 clover shoots were analyzed using a linear mixed effects regression model and analysis of variance (ANOVA). The total number of LCLWs counted was considered to be the response and the fixed effects were insecticide treatment (deltamethrin, cyantraniliprole, UTC), time (pre-spaying, 12 days after spraying) and year (2018, 2019). Both replication and site were included in the model as random effects with replication being nested in site. Least squares means were calculated for each insecticide and compared using Tukey's HSD.

Data from the field trials were examined using Kruskal-Wallis tests to examine the effect of sites on the total number of LCLW from 10 clover shoots. All sites were grouped into two categories: high LCLW pressure sites and low LCLW pressure sites (see results). Data from the high LCLW pressure group were analysed similarly to the lab-based LCLW rearing experiment whereas data from the low LCLW pressure group was examined using a generalized linear mixed model performed with the template model builder (Brooks et al., 2017) in order to include the same random factors (replication, site) while also applying a Poisson probability distribution. Differences in seed yield were assessed using a linear mixed effects regression model and ANOVA for both groups, with the fixed effect of treatment and the random effect of replication and site. Seed yield from the Love site in the low LCLW group was analyzed separately due to vast differences in yield scale using the same model.

Bee community data was analysed using permutational multivariate analysis of variance with distance matrices (PERMANOVA) performed with the Adonis function in the vegan package of R (Oksanen et al., 2013). The model contained treatment, time, year, site and their interaction as fixed factors. To obtain a representative sample of the bee community and to eliminate certain bee biases towards shape and color, for each plot the bees collected in bee cups were summed with the vane traps to generate a single value which was analyzed as a sum of bees per plot where each plot had one bee bowl of each colour and a vane trap. Collected bees from post spraying and full bloom sampling times were generated into one value for each plot to evaluate Pearson correlation with red clover seed yield.

3.3 Results

3.3.1 Site conditions

The LCLW densities varied significantly among seven fields over two years (Table 3.3). In particular, there was a lack of LCLW pressure throughout the 2019 season in Clavet, so LCLW mortality evaluations and seed yield responses to pest abundance were not performed in post-spraying times. This location, however, was included to assess effects of insecticides on the pollinator community. The remaining sites were grouped into two categories where pest pressure was either below (low LCLW pressure sites) or above six LCLW larvae per ten clover shoots (high LCLW pressure sites), based on reports claiming this threshold as dangerous for red clover seed yield in Canada (Gillott & Weiss, 1993).

Table 3. 3. Summary of statistical results for initial LCLW larvae number among seven sites and seed yield grouped by LCLW pressure. LCLW larvae and seed yield numbers presented by means and standard deviations. Means in the yield (kg ha-1) row with different letters present a significant difference at 0.05 level. Means in the LCLW larvae per 10 shoots column with different letters present a significant difference at 0.05 level. NA denotes no results due to very high variance and herbicide overspray.

Group	Site	Year	LCLW larvae per 10 clover shoots	Yield (kg ha^{-1})		
				Treatment		
			Initial count	UTC	Deltamethrin	Cyantraniliprole
High LCLW pressure	Carrot River	2018	6.5±1 *ab*	NA	NA	NA
	Snowden South	2019	10.25±0.9 *ab*	99.3±32.6 *a*	234.7±43.5 *b*	202.1±60.1 *b*
	Snowden North	2019	11.75±2.8 *a*			
Low LCLW pressure	Arborfield	2018	4.5±0.5 *bc*	499±102.4 *a*	583.3±24.4 *a*	587.1±89.5 *a*
	Carrot River	2019	3.25±0.2 *c*			
	Love	2019	2.75±0.9 *c*	135.6±44.2 *a*	126.1±19.9 *a*	145.3±28.7 *a*
	Clavet	2019	0±0 *d*	116.7±15.8 *a*	118.7±46.9 *a*	89.6±58.4 *a*

Note: Sites were grouped into two categories where pest pressure was either below (low LCLW pressure sites) or above six LCLW larvae per ten clover shoots (high LCLW pressure sites), based on reports claiming this threshold as dangerous for red clover seed yield in Canada (Gillott & Weiss, 1993).

3.3.2 Assessment of insecticide efficacy

Two methods were employed to evaluate insecticide efficacy. In the first method 20 shoots were collected from each field plot and reared in the laboratory. Overall, 1,079 *H. nigrirostris* individuals were reared and identified from 2,260 red clover shoots over the 2018 and 2019 seasons. The number of LCLW reared from clover shoots did not differ significantly between years, but differed significantly among treatments (Table 3.4). At 10-12 DAT the number of LCLW was greater in the untreated control compared to the deltamethrin and cyantraniliprole treatments, but did not differ statistically between the insecticide treatments (Fig. 3.1). The number of LCLW was significantly affected by time when LCLW larvae were counted (pre-spraying, 10-11 DAT) and there was a significant interaction between treatment and time (Table 3.4). There was no three-way interaction of treatment, time and year; however, interactions between year and treatment along with year and time were significant (Table 3.4).

In the second evaluation method we identified 1,257 LCLW larvae from continuous sampling over two field seasons. The LCLW pressure differed significantly between years only in the high LCLW group while treatment, time and their interaction statistically affected the LCLW abundance in both low and high LCLW pressure groups (Table 3.4). There was no interaction of year and treatment, year and time or year, time and treatment (Table 3.4). In both pressure groups deltamethrin and cyantraniliprole had a significant effect on LCLW field populations at 12 DAT application, whereas only deltamethrin significantly reduced the LCLW population in 24 hours after treatment (Fig. 3.2).

Table 3. 4. Statistical results with P-values, degrees of freedom (df), F-value and Chi square (χ^2) for tests of the effects of treatment (Deltamethrin, Cyantraniliprole, UTC), Time (Pre-spraying, 24 hours and over 12 DAT), year (2018, 2019) and their interaction on LCLW larvae reared in laboratory conditions and continuously sampled fields with low and high weevils pressure. Bold values denote statistical significance at the p < 0.05 level.

	Method								
	Field Sampling						**LCLW lab rearing**		
	High LCLW pressure			**Low LCLW pressure**					
	F-Value	df	P-value	χ^2	df	P-value	F-Value	df	P-value
Treatment	21.73	2	**<0.001**	15.56	2	**<0.001**	7.59	2	**0.001**
Time	45.59	2	**<0.001**	11.85	2	**0.002**	89.96	1	**<0.001**
Year	7.7	1	**0.019**	0.480	1	0.488	0.83	1	0.375
Treatment*Time	6.71	4	**<0.001**	17.69	4	**0.001**	10.9	2	**<0.001**
Treatment*Year	2.19	2	0.117	0.561	2	0.755	6.37	2	**0.002**
Time *Year	1.38	2	0.255	2.791	2	0.247	46.23	1	**<0.001**
Treatment*Time*Year	1.11	4	0.353	3.691	4	0.449	3.04	2	0.053

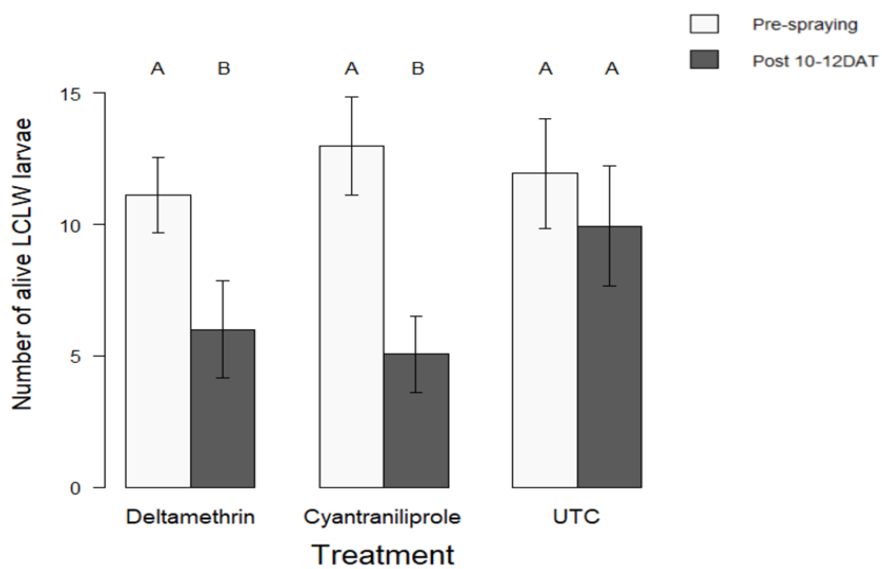

Figure 3. 1. Number of living LCLW reared from red clover shoots over 10-12 days from insecticides and UTC treated 20 clover shoots. Error bars are standard errors of the mean. Treatment conditions denoted with different letters are significantly different at p<0.05 level.

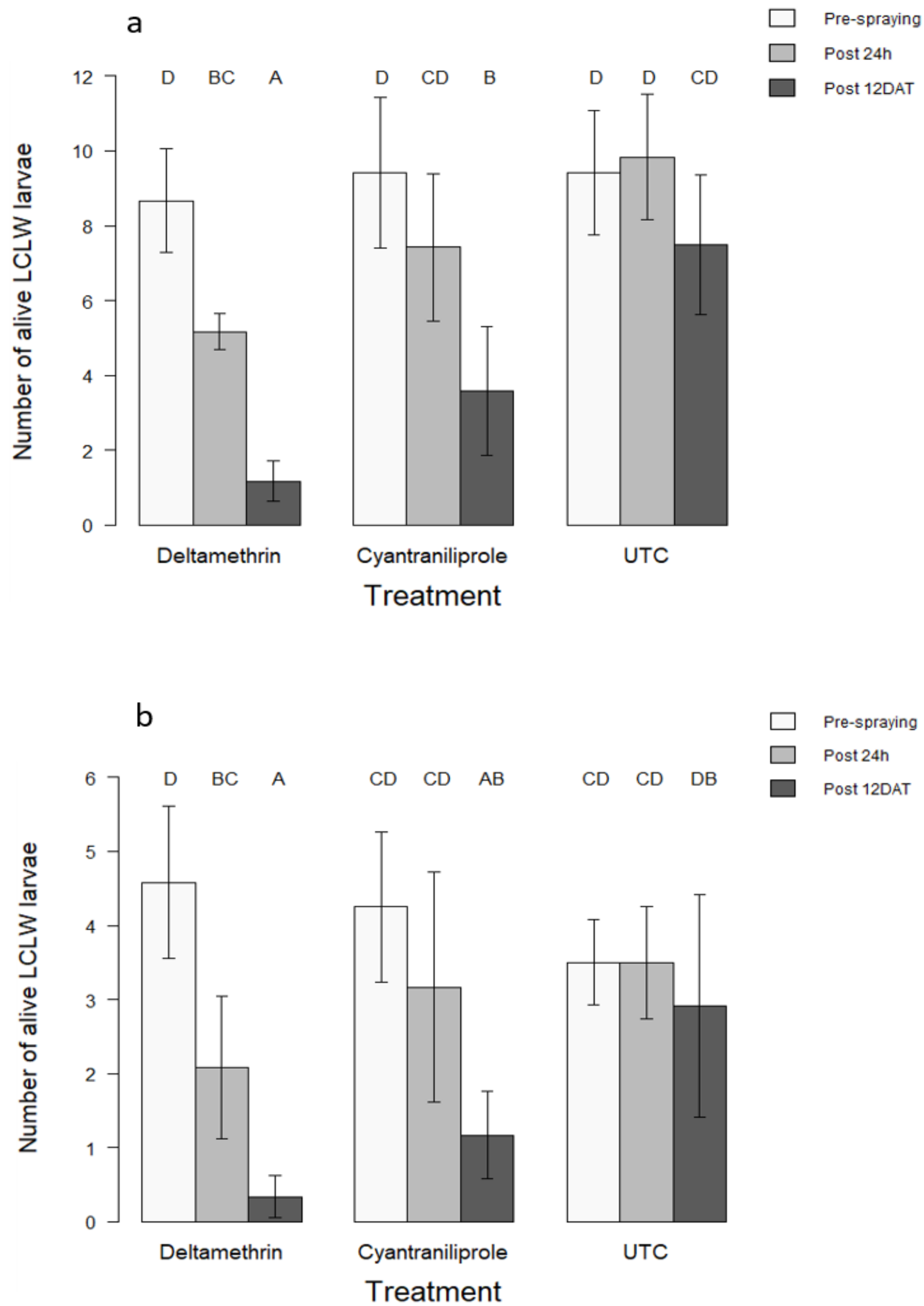

Figure 3. 2. Insecticide treatment effects on LCLW larvae identified from 10 clover shoots before treatment, 24 hours and 12 DAT from groups with (a) high LCLW pressure sites and (b) low LCLW pressure sites. Error bars are standard errors of the mean. Treatments and sampling periods denoted with different letters are significantly ($p<0.05$) different.

3.3.3 Seed yield

The seed yield did not differ statistically among treatments in the low LCLW density sites, but the number of LCLW larvae per plot at 12 DAT was significantly negatively related to seed yield (Fig. 3.3; Fig. 3.4). However, in the group with high LCLW pressure the seed yield was significantly affected by treatments where a similar association between seed yield and number of LCLW in 12DAT was found (Fig. 3.4). The seed yield was statistically higher in both the deltamethrin and cyantraniliprole treatments compared to the untreated control, but did not differ between insecticide treatments (Fig. 3.3; Table 3.3).

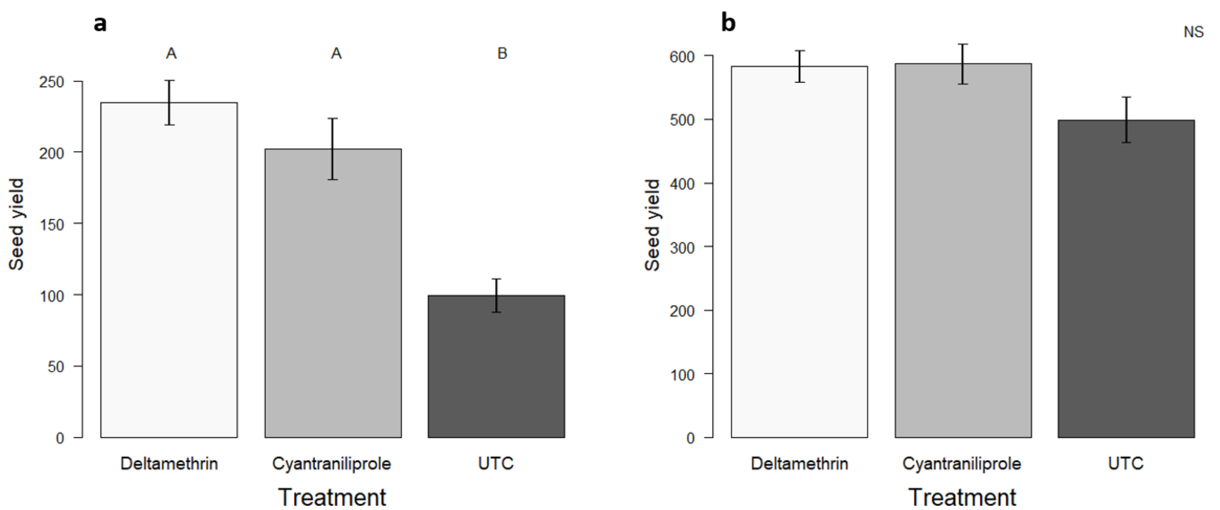

Figure 3. 3. Insecticide treatment effects on seed yield (kg ha^{-1}) from groups with high LCLW pressure (a) and low LCLW pressure sites (b). Error bars indicate standard errors of the mean. Treatments denoted with different letters are significantly ($p<0.05$) different.

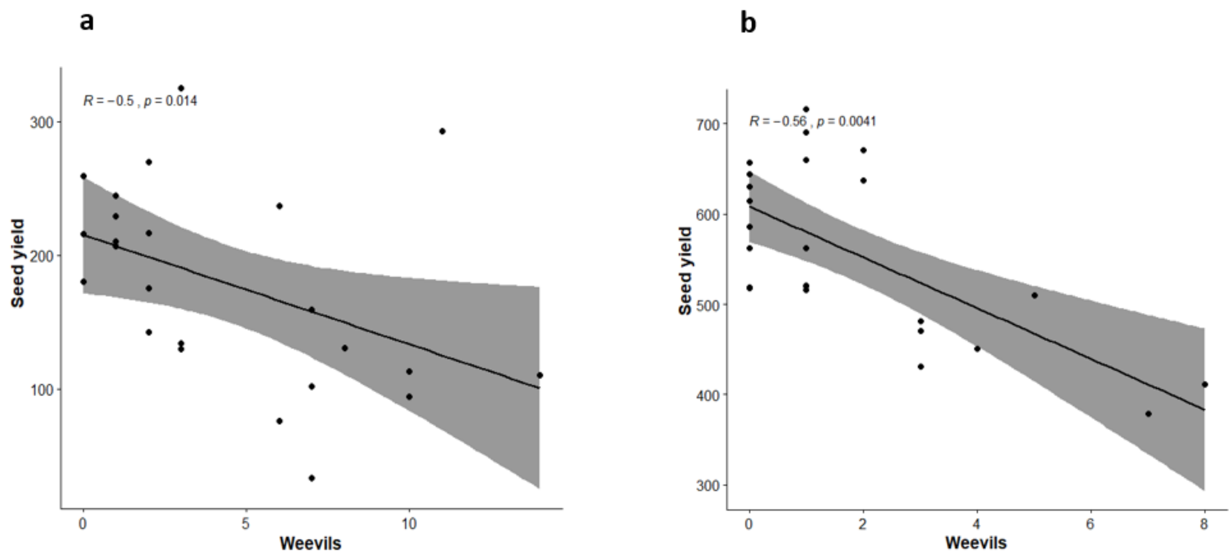

Figure 3. 4. Seed yield (kg ha^{-1}) in relation to LCLW abundance assessed in 12 DAT application from groups with high LCLW pressure (a) and low LCLW pressure sites (b).

3.3.4 Bees

In 2018 and 2019 combined, 1,451 bees were collected in total (Table 3.4). However, the overall bee community differed with respect to year and site (Table 3.5). In 2018, 787 bees were collected from a total of 24 plots. Of these specimens, 25% were honey bees (*Apis)*, 17% were bumble bees (*Bombus)*, 57% were in the genus *Lasioglossum* and less than 1% of the bees collected were other species of solitary bees (Table 3.5). In 2019 a total of 667 bees were collected in a total of 48 plots; 22% of the individuals collected were honey bees, 18% bumble bees, 13% leafcutter bees (*Megachile)*, 17% in the genus *Lasioglossum*, and less than 10% were other solitary bees (Table 3.5). In the current study, bees were classified as belonging to 14 different genera: *Apis*, *Bombus, Lasioglossum, Halictus, Dufourea, Colletes, Anthophora, Megachile, Hylaeus*, *Melissodes*, *Andrena*, *Sphecodes*, *Agapostemon* and *Coelioxys*. The total number of bees did not differ significantly between insecticide treatments from 24h before to 24h following insecticide applications and there was no interaction between treatment and year, treatment and site or treatment and time (Table 3.6). However, there was a significant effect of time (pre-spraying, 24 hours after spraying), year, site and the interaction of time and year along with time and site. There were no three-way interactions (Table 3.6). The total number of bees per transect was not statistically associated with red clover seed yield among four sites, and only in one site the association was significant (Fig. 3.5).

Table 3. 5. Bee community collected during three sampling times in red clover for seed production in 2018 and 2019 among six sites.

Genera	2018			2019		
	Arborfield	Carrot River	Snowden South	Love	Carrot River	Clavet
Apis	147	54	37	49	44	14
Bombus	65	66	27	74	13	33
Lasioglossum	226	222	90	5	92	17
Halictus	1	0	0	0	1	4
Dufourea	1	0	0	1	0	0
Colletes	4	0	0	0	0	0
Anthophora	0	0	5	4	0	0
Megachile	0	0	1	87	2	0
Hylaeus	0	0	2	0	0	0
Melissodes	0	0	0	19	4	10
Andrena	0	0	2	6	4	14
Sphecodes	0	0	0	2	0	0
Agapostemon	1	0	0	0	0	0
Coelioxys	0	0	0	1	0	0
Total	445	342	164	248	160	92

Table 3. 6 ANOVA table for PERMANOVA on total number of bees with respect to treatment (deltamethrin, cyantraniliprole, UTC), time (pre-spraying, 24 hours after spraying) year (2018, 2019) and all interactions. Bold values denote statistical significance at the $p < 0.05$ level.

	Total number of bees			
	df	Mean Squares	F value	P Value
Treatment	2	0.06	0.43	0.942
Time	1	0.56	3.95	**0.004**
Year	1	2.87	20.13	**0.001**
Site	4	3.08	21.6	**0.001**
Treatment*Time	2	0.25	1.76	0.065
Treatment*Year	2	0.07	0.51	0.879
Time*Year	1	0.7	4.95	**0.001**
Treatment*Site	8	0.18	1.3	0.124
Time*Site	4	0.86	6.0	**0.001**
Treatment*Time*Year	2	0.2	1.45	0.179
Treatment*Time*Site	8	0.2	1.43	0.058

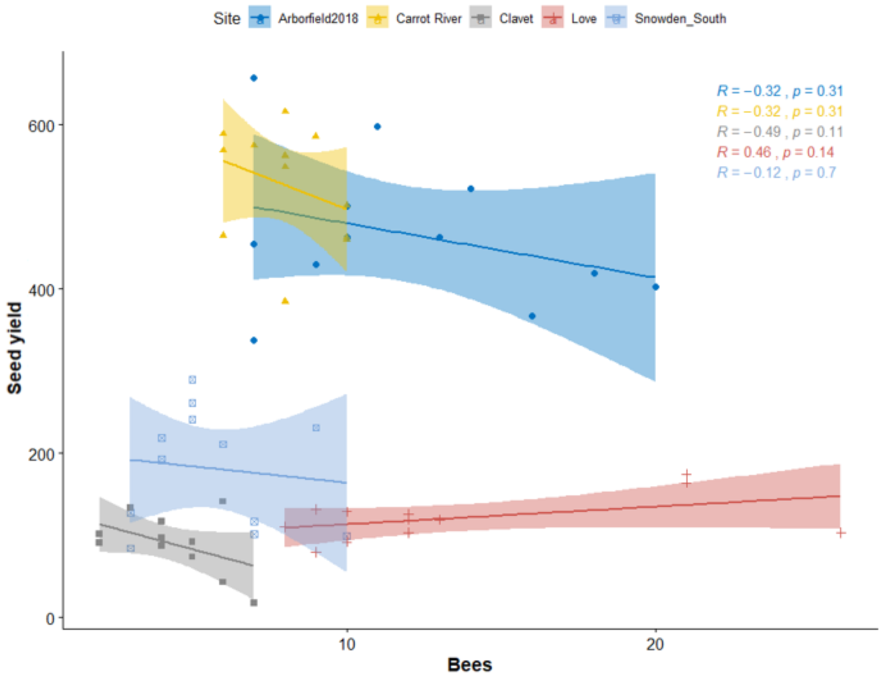

Figure 3. 5. Relationships between the total number of bees per transect from post treatment period (24 hours after spraying and 12DAT) and seed yield (kg ha^{-1}) from four sites.

3.4 Discussion

3.4.1 Insecticide efficacy

LCLW was susceptible to both insecticides in comparison with the untreated control. Despite the significant reduction in LCLW numbers reared from insecticide-treated clover shoots in a controlled environment, the mortality rate reached 50 - 60% at maximum and most LCLW larvae developed into adults. The significant interaction of treatment and time indicates that LCLW numbers are affected over time due to continuous feeding on given treated plants. The meaning of the significant interaction of time and year, as well as treatment and year, is not clear, but it may be explained by potential differences associated with transporting the red clover shoots and LCLW (over 450 km) and consequently, it could affect clover stress response mechanisms differently. These interactions may also reflect some initial unspecified differences in plant condition, such as tissue water content, winter damage and plant nutrient availability in 2018 and 2019 that subsequently might affect LCLW larvae longevity. While the lab rearing trials mostly examined ingestion as a route of exposure, other routes of exposure can be important for complete insecticide efficacy evaluation. For instance, systemic insecticides were reported as more toxic to the eye gnat, *Liohippelates collusor* (Diptera: Chloropidae) when exposed through ingestion versus contact (Jiang & Mulla, 2006). Similarly, cyantraniliprole was over 400× more toxic to corn earworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae) via ingestion compared to contact (Bird, 2016). In field conditions cyantraniliprole can have a significant negative effect on insects' ovulation, oviposition, mating rate and overall lifespan (Kamimuro et al., 2019; Knight, 2010). Thus, it is crucial to investigate pest susceptibility and the efficacy of both systemic and contact insecticide materials under field conditions.

The second insecticide evaluation method where LCLW pressure was evaluated using in-field counts at multiple time points revealed similar results to the lab rearing method. Treatment, time and their interaction had significant effects on LCLW numbers in both high and low LCLW pressure sites. However, at the high LCLW pressure sites year had a significant effect on LCLW numbers. This result may be related to the initially higher variability in LCLW abundance between sites and 2018 and 2019 seasons since in the high LCLW pressure sites larvae number varied on average from six to 12 LCLW per 10 shoots in comparison with three to four LCLW in the low-pressure sites. Despite the finding that mortality rate was significantly higher at 12 DAT versus 24 hours after treatment and pre-spraying conditions in both insecticide treatments and groups, only deltamethrin significantly increased LCLW mortality in 24 hours after treatment. Similarly, Stevenson and Barszcz (1996) reported that deltamethrin can increase carrot weevil, *Listronotus oregonensis* (Leconte) mortality by almost 50 % in 24 hours and an additional 5% in the following 24 hours. Therefore, in field conditions deltamethrin exhibited a high speed of action, enabling fast control of LCLW that can be associated with the substantially higher LD_{50} values when compared to cyantraniliprole. Insecticides with modes of actions that exhibit a high speed of action and extended control are important tools at times when an attack by LCLW is acute, particularly at the point when the larvae migrate from stems, leaves and stipules to the flowers as this causes losses in potential seed yield (Gillott & Weiss, 1993). Even though deltamethrin suppressed LCLW numbers within 24 hours unlike cyantraniliprole, both insecticidal materials effectively lowered the pest densities over longer periods of time when compared to untreated controls.

3.4.2 Seed yield

Similar to white clover, red clover yield components such as number of flowers per area unit, seeds per flower, one thousand seeds weight and the number of florets per flower are highly correlated with the seed yield (Amdahl et al., 2017; Lundin et al., 2017). Thus, in this study experiments we focused on the red clover seed yield rather than on individual yield components. In this research, the effect of pest management treatments on red clover seed yield differed depending on the number of LCLW present in the fields. When LCLWs varied from three to four individuals per 10 shoots (low LCLW pressure group) in the field, no seed yield response was observed in any treatment. Consequently, application of either deltamethrin or cyantraniliprole would not benefit seed yield when the number of LCLW larvae per 10 shoots is below four. In contrast, when the pest pressure was above six LCLWs per 10 shoots, seed yield was significantly higher in both the deltamethrin and cyantraniliprole treatments. This indicates that the potential LCLW economic threshold lies in a range between four to six LCLW larvae per 10 clover shoots. Even though visible stem and leaf damage was noticed on clover plants at densities below four LCLW larvae per 10 shoots, it seems that red clover can compensate for the damaged parts later in the season.

In 2020, in Canada, Decis® application cost about \$27 ha^{-1} whereas Exirel® cost approximately \$185 ha^{-1} ("Farmers Business Network (FBN), 2020) and the price of red clover seed is about \$2.2 per kg ("Peace Region Forage Seed Association", 2021). The seed loss due to LCLW damage can reach up to 45% or 115 kg ha^{-1} if no insecticide is applied. From this, it can be calculated that application of either Deltamethrin or Cyantraniliprole saves 115 kg ha^{-1} of red clover seeds, which is \$255.5 per a ha^{-1} when the LCLW pressure is high. Even though application of Deltamethrin would be a six times cheaper option, including and rotating other alternatives such as Cyantraniliprole need to be considered in order to prevent LCLW tolerance, and protect the seed yield.

Regardless of the treatment effect on yield, a strong negative association was present between LCLW number at 12 DAT and seed yield. This finding is in agreement with previous studies that determined that yield components and seed yield are highly correlated with LCLW presence in red and white clover fields (Hansen & Boelt, 2008; Lundin et al., 2017). In order to protect white clover seed yield in Denmark (Hansen & Boelt, 2008) recommended examining the number of LCLW adults in early spring to predict the potential number of larvae later in the season.

In their model of economic damage under average seed yield expectation (500 kg ha^{-1}), it was reported that 0.7 LCLW larvae per one meter will cross the economical damage threshold. This model, however, did not agree with the findings in our study since red clover seed yield was not statistically different even when the LCLW density varied from three to four individuals per 10 shoots. This may be due to differences in clover's growth type and possibly different stress response mechanisms since white clover grows relatively short and spreads using stolons, whereas red clover grows mostly tall and upright. It is possible that red clover compensates for LCLW damage more efficiently than white clover does. Although, it is also likely that LCLW oviposition, feeding and behaviour varies between white and red clover and therefore damage may also vary (Sechriest & Treece, 1963).

3.4.3 Bees

We evaluated the effects of three different treatments on the bees present in multiple commercial red clover fields along with the potential association of bees and seed yield. Unexpectedly, the total number of bees and bee community was not affected by either insecticide treatment and was statistically the same as the untreated control plots. Results from this study are in contrast to previous findings where densities of bees were significantly affected by several field insecticide applications (Lundin et al., 2017). The absence of a significant result in this study may be due to several reasons. Treatment application was mostly done right before or shortly after sunset, which is supposed to reduce bees' exposure to the chemicals. Secondly, most of the fields were sprayed in the pre-flowering and early flowering vegetative stage, and this would limit direct contact to the insecticides. Furthermore, both the systemic and contact insecticide's residues were likely too low by the flowering stage and were not absorbed by bees through the pollen or nectar in an amount that would result in mortality. Besides, the presence of insecticide residues in green parts of a plant does not necessarily imply the presence of residues in either pollen or nectar, which might affect bees (Alix & Vergnet, 2007). An alternative but not mutually exclusive explanation is that the scale of treatment plots was relatively small and not large enough to measure the effects of treatment on the bee community as bees from the non-exposed external environment could access the plots within the 24 hour span from application and sampling. The small plot size might be one explanation for the weak association between the total number of bees and red clover seed yield in one of the five sites. Under field conditions, larger plot sizes could potentially reveal a stronger effect. In addition, we sampled bees from the field traps rather than counting them directly on the flowers and it is unclear if direct counts would make a significant difference in our study.

Thomas (1996) and Holm (2004) report that adequate clover pollination occurs when the weather is sunny, the temperature is >20°C and the wind velocity is below 6.5 m/s for up to five successive days after the flowers reach full bloom vegetative stage. These conditions were met in both the 2018 and 2019 seasons. Consequently, lack of (or excessive) pollination in these study sites cannot justify the significant differences in seed yield. Additionally, the total number of bees in 24 hours and 12 DAT applications did not correlate with seed yield at any site or in any year.

Overall, we found variation in bee numbers between two years and among six sites. This variation was most likely the result of differences in latitude and landscape since the same sites were not sampled in 2018 and 2019. Interestingly, bee abundance differed before and after fields were sprayed. The specific meaning of this is unclear since the difference between sampling time was only 24 hours and this effect was found for all sites. Two potential scenarios seem possible to rationalize this phenomenon. Bees could have biphasic dose-response whereby exposure to low doses of insecticides stimulated and attracted them to the post-treated plants (Cutler & Rix, 2015). Second, even minor variation in air humidity, light intensity and short shower at one site could either synergistically or individually affect bees' activity within 24 hours of sampling times (Abou-Shaara et al., 2012; Peat & Goulson, 2005). Lack of a three-way interaction between treatment, time and year or treatment, time and site indicates that absence of treatment effect was consistent among locations in both years.

Despite reported decreases in pollinator stocks of managed bees for crop pollination (Aizen et al., 2009; Garibaldi et al., 2011), almost all growers in 2018, 2019 trials introduced either honey bees or leafcutter bees near the red clover fields. In addition, bumblebees and solitary bees (i.e *Lasioglossum* spp.) had either similar or substantially bigger community size than honeybees. Therefore, in total native bees were almost 3 times more common than managed honey bees in red clover for seed production. This finding suggests that red clover fields might have enough native bees to provide adequate pollination. Besides, the efficiency of bee pollinators can vary between bee genera and species. Inouye (1980) together with Taylor and Quesenberry (1996) reported that bumblebees are more effective at clover pollination than honey bees partly due to a longer proboscis and quicker flower handling that consequently affects the number of flowers visited per unit of time.

3.5 Conclusion

Taken together, our results show that both insecticide treatments in this study are effective for LCLW control in red clover seed fields, though deltamethrin appeared to act more quickly than cyantraniliprole. Overall, there was no observed difference in pollinator abundance; however, insecticides significantly affected LCLW mortality and consequently red clover seed yield. This finding would suggest that the LCLW effect yield, but there was not a yield effect from a lack of pollinators. This argument can be reinforced by the significant negative association of LCLW density and seed yield, with a quite high yield at pest pressure close to zero. Thus, our results indicate that lack of sufficient LCLW control can be one of the key factors limiting seed yield in Saskatchewan and overall in red clover for seed production, whereas pollen limitation or any other factors seem rare. Although this deserves a follow up study with direct observation on pollinators in larger scale plots. The potential LCLW threshold was found to be in a range of four to six larvae per 10 stems. Future research should investigate LCLW economic thresholds for red clover seed production in North America and examine potential models to predict LCLW abundance by early spring sampling, as well as examine alternative control methods in order to maintain productive yield production and minimize the environmental impacts. Additionally, quantifying flower visitation and duration of bee visits under different insecticide treatments on larger plots would benefit understanding of changes in bee communities.

4. Effect of seeding rates on nitrogen fixation and seed production of red clover

4.1 Introduction

Successful stand establishment is essential for high seed and biomass production of red clover. Taylor (1985) reported that optimum seeding rate for clover seed production differs widely due to the great variability observed in different growing regions. In Canada, current recommended seeding rates for red clover range from 2.5 to 10 kg ha^{-1}, depending on geographical location and associated climate conditions, as well as agronomical practices (Taylor, 1985). In addition, limited information exists about the response of red clover density to soil water availability. Furthermore, the effect of seeding rates on seed yield, and plant biomass is well studied in other legumes, including perennial crops. For example, in birdsfoot trefoil, a change in seeding rate did not affect seed yield production when the crop was sown between 1.1 and 8.8 kg ha^{-1} (Pankiw et al., 2010). Bolger and Meyer (1983) reported that there were no differences in alfalfa yields from stands from 9 kg ha^{-1} up to 22.4 kg ha^{-1}. Hall et al. (2004) found that in seeding rates above 17 kg ha^{-1}, high plant densities experienced significantly higher plant death the first year after planting in comparison to lower densities, making 10 kg ha^{-1} to 17 kg ha^{-1} the optimal seeding rate for plant longevity. There is limited data available, however, to support relationships between stand density, biomass and seed production in red clover.

In comparison with alfalfa (*Medicago sativa* L.), another crucial forage legume species, red clover is described as a rapid spring establisher and known for its superior performance on acid and wet soils. However, red clover is characterized as a short lived perennial due to its poor yield performance and stand mortality over time (Bosworth & Stringer, 1990). The major causes of red clover mortality in stands older than two years include: pest infestation, clover rot susceptibility and winterkill (Taylor & Quesenberry, 1996). In particular, Coulman and Kielly (1988) indicated that in Canada, red clover population densities can be substantially reduced due to winterkill. LCLW and root rot pathogens are one of the major limiting factors that can weaken the plants, increasing their susceptibility to winterkill (Gillott & Weiss, 1993; Fergus & Hollowell, 1960). However, the main components that affect red clover plants directly over winter time are: frost damage, ice cover and low temperatures (Hakala & Jauhiainen, 2007). Plants that have depleted their carbohydrates and were stressed through the winter become especially vulnerable to pathogens (Ylimäki, 1967). Additionally, the Altaswede variety used in this study was reported to have a poor winter hardiness index in comparison to multiple varieties grown locally in Finland (Ravantti, 1980). It is not known, however, how varying plant densities in red clover used for seed production affect winter hardiness, seed yield, plant biomass, and N fixation.

Soil nitrogen is the most limiting macronutrient for plant production worldwide. Even in advanced intensive agriculture, where use of synthetic fertilizers increases substantially, the nutrient deficit continues to be unacceptably high for nitrogen (Doyle et al., 1989). Due to concerns related to application of inorganic N fertilizers, including the production of the greenhouse gas nitrous oxide (N_2O), acidification produced by NH3 deposition, water pollution by nitrate (NO_3), and aquatic eutrophication (Bouwman et al., 2013; Conley et al. 2009), there is growing interest in assessing the potential atmospheric N fixation through the symbiotic relationship between rhizobia and legume crops. Among N-fixing plants, perennial legumes including red clover have several advantages over annual legumes due to greater root and vegetative shoot biomass, multiple succession of leaf generations and higher N concentration in shoots and leaves (Ploschuk et al., 2005; Schipanski & Drinkwater, 2012; Warembourg et al., 1997). Large proportions of the accumulated N in perennial legumes is usually returned to the soil through plant, decomposition, or animal grazing, whereas in annual crops, much of N is harvested with seeds (Peoples et al., 1995). Studies often evaluate N fixation of red clover in the context of intercropping and cover cropping system (Høgh-Jensen et al., 2004; Huss-Danell et al., 2007), however, little information is available on N fixation of red clover in seed production system.

In the conducted study, the ^{15}N isotope dilution technique was applied because of its ability to distinguish between N derived from soil and fixed atmospheric N. In this method, ^{15}N labelled fertilizer is applied to the N-fixing crop and to a corresponding non N-fixing crop that is selected to serve as a reference crop. The atom% ^{15}N of the N-fixing crop and the reference crop is used to calculate the N fixation of the crop of interest (Chalk, 1985). The reference crop plays a critical role in obtaining accurate estimations of the amount of N_2 that is fixed, as the precision of this technique depends on the similarity and the relative availability of soil N to both N-fixing and non N-fixing crops. This is achieved by selecting a reference crop with a similar life cycle, harvesting time and rooting pattern to the legume crop of interest (Chalk, 1985; Hardarson & Hera 2011).

Specific knowledge of red clover seeding rate responses in different locations within Saskatchewan is necessary to determine whether higher plant densities would be beneficial, especially if abiotic stresses such as cold winters and droughts are present. To the best of our knowledge, the effects of multiple seeding rates on nitrogen fixation of single cut red clover were not compared before, nor where the effects of clover densities on winter hardiness. Thus, the objective of this research was to determine the optimal red clover seeding rate for seed production, N fixation and winter hardiness, as well as to evaluate interactions of these components at two different sites.

4.2 Materials and Methods

4.2.1 Field Sites

This research was conducted in 2018 and 2019 at the Livestock and Forage Center of Excellence (LFCE) near Clavet, Saskatchewan (51°57'20.4"N 106°22'48.8"W) and Agriculture and Agri-Food Canada (AAFC) Research Farm at Melfort, Saskatchewan (52°49'46.6"N 104°35'52.8"W). The soil is classified as a mixture of Black with thick Black chernozemic soil at Melfort and orthic Dark Brown soil at Clavet respectively. The fields were previously used primarily for canola (*Brassica napus* L.) production.

4.2.2 Experimental design

To determine an optimal seeding rate for nitrogen fixation and seed production, six different seeding rates (0.5, 2.5, 4.5, 6.5, 8.5, and 10.5 kg ha^{-1} pure live seed) were planted using a commercial seed drill (plot seeder). Red clover cultivar, Altaswede was used in this study. The experimental design was a RCBD with four replications and a non-nitrogen fixing reference crop, crested wheatgrass (*Agropyron cristatum*; cultivar Kirk), was also included at each plot for determining the N fixation. The plot size was 9 x 2.4 m in Clavet, and 8 x 1.2 m in Melfort with a 30 cm row spacing. All rates and reference strips were seeded using plot drill seeder (Hans Ulrich Hege, Waldenburg, D 7112) on May 29th and June 16th in 2018 respectively. Weeds were controlled by mowing and roguing throughout the field seasons, and no fertilizer was applied during the study. Red clover seeds were inoculated prior to seeding with a commercial inoculant (Nitragin Gold, NexusBioAg) containing *Rhizobium leguminosarum* (*biovar* trifolii) according to the manufacturer's recommended rate (189 g of inoculant per 22.7 kg of seeds). At both sites, N fixation in red clover was quantified using the ^{15}N isotope dilution technique (Hardarson & Danso, 1990). The N uptake and fixation rate was determined only in 2019. Plots at Clavet experienced drought in 2018 and 2019. The total precipitation in Melfort was also below long-term means in 2018 and 2019 (Table 4.1).

Table 4. 1. Annual precipitation (mm) in Clavet and Melfort in 2018, 2019. Long-term average for the period from 2000 to 2019 (Environment Canada).

Site	2018	2019	Long Term Average
Melfort	332	312.4	354.2
Clavet	207.7	266.4	362

4.2.3 Seed Yield and Winterkill

To assess seed yield of different red clover seeding rates, all plots were harvested in 2019. Plots at Clavet were harvested in the end of August by straight combining, whereas harvesting in Melfort took place in the mid of September. All seed samples were air dried at 40 °C for approximately 5 days and then processed through a seed belt thresher followed by three levels of sieves.

To examine winterkill associated with different red clover seeding rates, seedling density was quantified using the modified Line-Intercept Method (Cummings & Smith, 2000). In each plot two one-meter rows were randomly selected for measuring growing seedlings in August 2018. The same rows were evaluated for seedling density in summer 2019. To exclude potential errors in discriminating lethal from non-lethal frost damage all post-winter plant counts were done in early June 2019, when the red clover stand was established. Two separated counts were grouped and analyzed as an individual component per plot. The same data was used to determine plant density and seed yield correlations.

4.2.4 Greenhouse Experiment

Additional experiment was carried out in the agriculture greenhouse at the University of Saskatchewan in Saskatoon, Saskatchewan to evaluate the effects of drought on red clover seed yield production among six seeding rates. Similar to field study, the same red clover variety, seed inoculant and six seeding rates were utilized. This experiment was set up as a RCBD with a total of 3 replications: two treatments, which are 100% field capacity and 50% of field capacity (FC) (moderate drought), and six seeding rates for each water treatment. Sterilite plastic containers, 82.6 x 50.2 x 47.3 cm, were used to hold the soil and grow the red clover. Sunshine LA4 soil mixture, which is a product of Canada, was used during this study to fill the containers with 79 L of this mix. The ingredients included; 60-70% Sphagnum Peat Moss, Horticulture Perlite and Dolomite Limestone. To achieve 100% field water capacity (FC) plastic containers with soil were filled and mixed with water to saturation, in 3 hours the same procedure was repeated. The amount of used water was measured and referred to as 100% FC. Based on these measurements, the amount of water for 50% FC was calculated. A fertilizer, 20-20-20 was added to the watering regimes when the red clover was 30 days old and the containers were watered by hand during the course of the study. To ensure successful red clover pollination two bumble bee colonies (B. *impatiens*; Biobest

Ltd) were introduced to red clover cages, which covered all clover containers. Red clover seed was manually harvested in eight months after seeding, which occurred when the total amount of accumulated degree days over 15°C in the greenhouse reached a similar number of days to produce red clove seeds under field conditions.

4.2.5 Nitrogen fixation

When approximately 90% of red clover plants reached height of 10 cm in spring (10 May 2019), 1 × 1 m subplot were randomly selected in the central four rows for N isotope application in each of the clover and reference crop plots. A 10 atom% excess ^{15}N-($^{15}NH_4$) ($^{15}NO_3$) fertilizer was uniformly applied to the subplots at 5.7 kg N ha^{-1} in a liquid form. Prior to application, the N isotope fertilizer was fully dissolved in 9.4 L of distilled water as described by Knight (2012). A plastic 1 × 1m frame was located at soil level to contain the ^{15}N solution within the subplot area. Following the fertilizer application, an additional 4 L of water was applied over the treated area to rinse off the residual fertilizer contained on the frame and leaves into the soil. In fall 2019, whole plants of red clover and crested wheatgrass including seeds, stems, grains, straw and leaves were harvested from two central rows of the subplot on August 20th in Clavet and September 15th in Melfort to determine the dry yield biomass, biomass and N fixation. Crop samples were oven dried (at 65°C) in the laboratory immediately after harvest and grounded using a Wiley mill (Thomas Scientific, Swedesboro, NJ) with a 2mm screen, coffee grinder (Hamilton Beach), and subsequently re-grounded to very fine powder using a roller ball mill at 25 Hz for 2 minutes (Mixer Mill MM 200). The final ground samples were then weighed (2.5+/- 0.5 mg) using a micro-balance (Sartorius Microbalance, CPA2P, Bradford, MA, USA) and encapsulated using 8 × 5 mm tin capsules into an approximately spherical shape with air pressed out of the encapsulated sample. The encapsulated red clover and crested wheat grass samples were then analyzed for atom %^{15}N using a Costech Elemental Combustion System coupled to a Delta V Advantage Mass spectrometer (Isomass Scientific Inc. Calgary, AB). Percentage of N derived from atmosphere (%Ndfa) was calculated according to Hardarson and Danso (1990) as:

$$\%Ndfa = \left[1 - \left(\frac{atom\,\%15N\ excessN{-}fixing\ crop}{atom\,\%15N\ excessnon{-}fixing\ crop}\right) \times\right] 100\% \quad (1)$$

Where atom%^{15}Nexcess = ^{15}N atom% - 0.3663% (2)

Where, atom %^{15}N excess - is the ^{15}N atom% in the sample and 0.36637 – is the natural content of atmospheric ^{15}N in both fixing and non-fixing crops.

Amount of N fixed was calculated according to Hardarson and Danso (1990) by:

$$N\textit{fixed} = \frac{\%Ndfa \times totalNfixing}{100} \quad (3)$$

Total amount of N in legume was calculated as equation (4):

$$\text{Total N} = \%\text{N} \times \text{DM yield} \quad (4)$$

Where, total N - the amount of N (kg ha^{-1}) in the legume, %N – the percentage of N in the legume.

4.2.6 Data analysis

Data analysis and plotting of the graphs were performed using R version 3.5.2 (2018) statistical software (RStudio Team, 2018). Treatment effects were declared significant at $\alpha<0.05$. Seed yield and nitrogen fixation data were analyzed only from 2019, due to lack of yield and yield components in the seeding year of single cut red clover. Kruskal-Wallis test was conducted to examine the effect of sites on seed yield, N fixation and biomass. Data of seed yield was transformed using log transformation prior ANOVA analyses, and data present in the thesis were back – transformed. Mixed models were used for all regression analyses, including seeding rate as the fixed factor and block as a random effect. For plant mortality analysis, seeding rate, plant seasonal counts (winterkill) and site were included as fixed effects and block as a random effect. For greenhouse data analysis, seeding rate and treatment (100% and 50% FC) were included as fixed effects, whereas replication was included as a random effect. Data distribution normality was tested using Shapiro-Wilk's test and the homogeneity of variance was tested using the Levene's Test. Differences between means were compared using the least square means (Tukey's test) procedure. Correlations presented were done by applying Pearson correlations.

4.3 Results

4.3.1 Seed yield

Overall, the two study sites (Melfort and Clavet) were different for seed yield (χ^2=34.5, df=1, $p<0.0001$) and total biomass (χ^2=34.51, df=1, $p<0.0001$). Compared to Clavet, red clover produced 88% higher seed yield and 80% higher forage biomass at Melfort. At the Melfort site, seed yield differed significantly among seeding rates (F=6.19, df=5, p<0.001), where 0.5 kg ha^{-1} rate produced the lowest yield (Table 4.2. Fig. 4.1). Seed yield was always the highest for seeding rate from 2.5 to 10.5 kg ha^{-1}, ranging from 643 to 835 kg ha^{-1}. Seed yield was similar among the various seeding rates at the Clavet site (F=0.42, df=5, p=0.82) averaging at 56 kg ha^{-1} (Table 4.2; Fig. 4.1). There was a significant positive association between seed yield and aboveground forage biomass in both Clavet (R=0.56, p=0.005; Fig. 4.2) and Melfort (R=0.58, p=0.003; Fig. 4.2). Despite the significant correlation, only the 4.5 kg ha^{-1} seeding rate had significantly higher biomass weight than 0.5 kg ha^{-1} rate in Melfort conditions.

In the greenhouse experiment, red clover see yield was significantly affected by seeding rates (F=5.21, df=5, p=0.003) and water treatment (F=27.69, df=1, p=0.013); however, no interaction between treatment and seeding rate was observed (F=1.59, df=5, p=0.2). Drought treatment caused 60% seed yield reduction, whereas only 0.5, 2.5 and 4.5 kg ha^{-1} differed from 10.5 kg ha^{-1} rate.

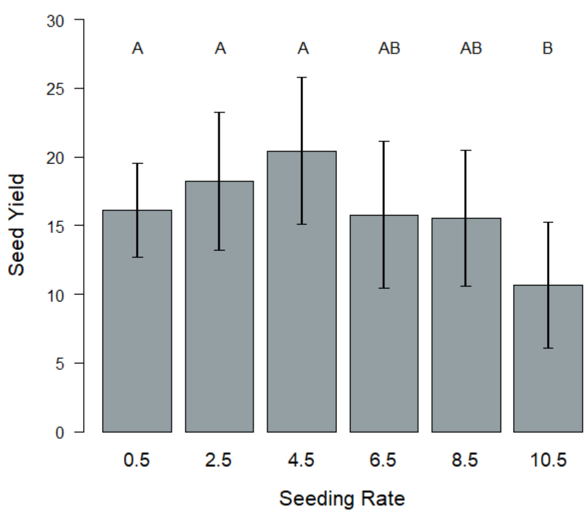

Figure 4. 1. Distribution of red clover seed yield (grams per container) among six seeding rates grown in greenhouse condition. Error bars are standard errors of the mean. Treatments denoted with different letters are significantly p<0.05 different.

Table 4. 2. Summary of statistical results from Analysis of Variance for red clover seed yield, forage biomass and nitrogen fixation in Clavet and Melfort, SK. Seed yield, nitrogen fixation and seedling numbers presented by means and standard deviations. Means in the same column with different letters present a significant difference at 0.05 level according to Tukey's test.

Seeding rate ($kg\ ha^{-1}$)	Clavet				Melfort			
	Seed yield ($kg\ ha^{-1}$)	N fixation ($kg\ ha^{-1}$)	Ndfa%	Biomass ($kg\ ha^{-1}$)	Seed yield ($kg\ ha^{-1}$)	N fixation ($kg\ ha^{-1}$)	Ndfa%	Biomass ($kg\ ha^{-1}$)
0.5	55.5±30.8 *a*	1.2±11 *a*	0.01±6.3 a	1100±105 *a*	427.9±42.1 *a*	271.1±61.5 *a*	71.1±5.9 a	5450±611 *a*
2.5	88.1±16.9 *a*	18.6±51.4 *a*	5.4±25 a	1470±544 *a*	718.9±69.3 *b*	382±125.7 *ab*	78±14.5 a	8170±1633 *ab*
4.5	89.8±46.6 *a*	43±10.3 *a*	23.9±11 ab	1735±485 *a*	877.9±209.7 *b*	427.4±46.7 *b*	87.5±21 a	7880±1033 *b*
6.5	92.8±44.9 *a*	43.9±7.9 *a*	27.3±5 ab	1365±261 *a*	711.5±150.9 *b*	369±57.8 *ab*	74.9±6.2 a	7020±1078 *ab*
8.5	94.4±28.5 *a*	53.3±25 *a*	35±16.25 b	1310±189 *a*	759.3±178 *b*	390.3±97.9 *ab*	74.6±3.4 a	7690±2413 *ab*
10.5	99.7±68.9 *a*	42.1±18.4 a	25.5±10 ab	1387±398 *a*	923.4±112 *b*	325.3±39.5 *ab*	74.1±4 a	6810±1080 *ab*

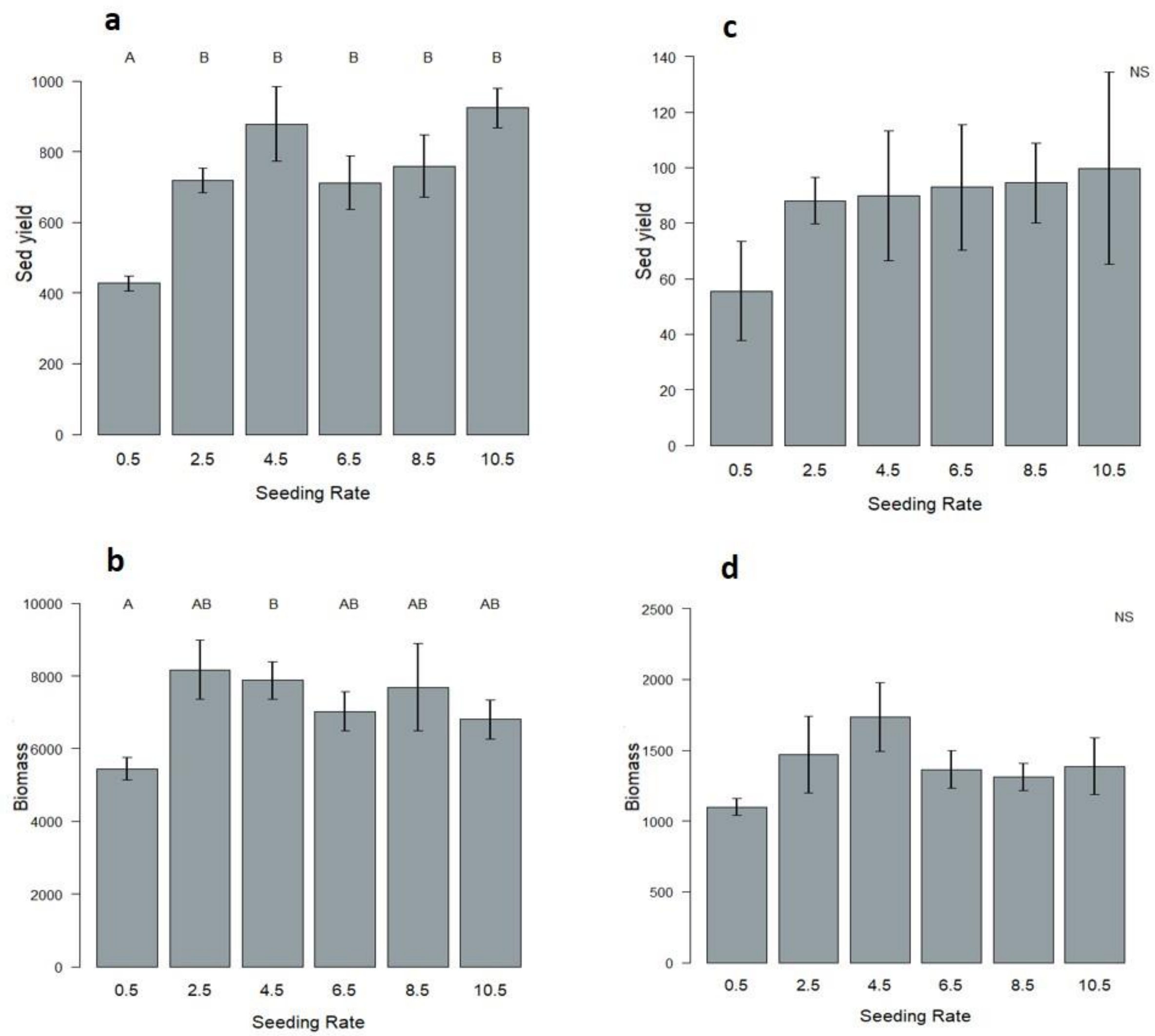

Figure 4. 2. Distribution of red clover seed yield and forage biomass among six seeding rates at Melfort (a, b) and Calvert site (c, d). The standard errors of the means are presented by vertical bars. NS indicates not significant difference at P ≤ 0.05. Treatments denoted with different letters are significantly p<0.05 different.

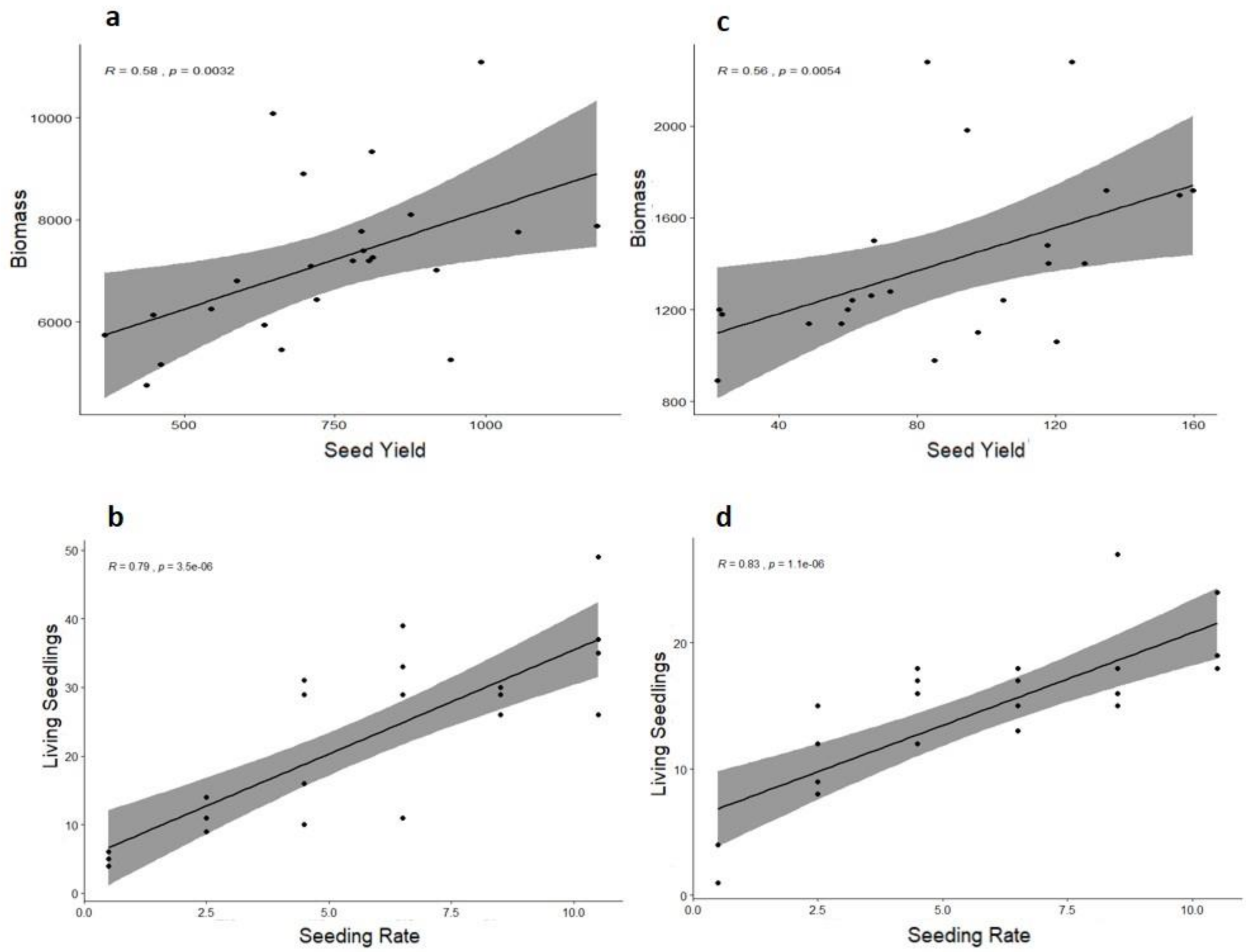

Figure 4. 3. Correlation between biomass (kg ha^{-1}) with seed yield (kg ha^{-1}), and living seedlings (plants per m^2) with seeding rate (kg ha^{-1}) for both Melfort (a, b) and Clavet (c, d).

4.3.2 Plant mortality rate

Averaged across all seeding rates, the number of living seedlings two months after planting varied between the two study sites (Table 4.3). In each site, the plant densities generally remained ranked according to the seeding rate and seeding rate had a significant effect on plant density (F=48, df=5, p<0.0001; Fig. 4.2). However, the interaction of seeding rate and site had no effect on seedling density (F=1.8, df=5, p=0.11). The total number of red clover seedlings in fall 2018 and spring 2019 (winterkill) did not affect clover seedling density at either site (F=0.2, df=1, p=0.62), and there was no interaction between seeding rate and winterkill on the number of living seedlings (F=0.36, df=5, p=0.85).

Table 4. 3. Significance of P values from analysis of covariance of sites, winter-kill, seeding rate, and their interaction. Bold values denote statistical significance at the $p < 0.05$ level.

Source of variation	Plant Density (plants m^{-2})			
	Degrees of freedom	Mean squares	F-Value	P-Value
Site	1	14.41	37.48	**<0.001**
Seeding rate	5	18.67	48.58	**<0.001**
Winter-kill	1	0.05	0.2	0.62
Winter-kill × seeding rate	5	0.14	0.36	0.85
Winter-kill × site	1	0.08	0.22	0.64
Seeding rate × site	5	0.71	1.86	0.11
Winter-kill × seeding rate x site	5	0.06	0.16	0.97

4.3.3 Nitrogen fixation

Nitrogen fixation was significantly different (χ^2=34.5, df=1, p<0.0001) between the two sites, averaging 360 kg ha^{-1} in Melfort and 33 kg ha^{-1} in Clavet, respectively (Table 4.2). Additionally, N fixation in Clavet varied widely, from 1.2 to 53.3 kg ha^{-1}, particularly high variation was observed in 2.5 kg ha^{-1} rate. Neither low or high seeding rates statistically affected the amount of N fixed (F=2.2, df=5, p=0.1). However, Ndfa% varied significantly among seeding rates in Clavet (F=3.6, df=5, p=0.02; Table 4.2), particularly 8.5 kg ha^{-1} had higher Ndfa% than 0.5 and 2.5 kg ha^{-1} rates. In contrast, seeding rates significantly affected N fixation in Melfort (F=3., df=5, p=0.04). Similarly to biomass yield, clover plants seeded at 4.5 kg ha^{-1} rate fixed more N than 0.5 kg ha^{-1}, however there were no statistical differences in N fixed among any rates above 2.5 kg ha^{-1} in Melfort (Table 4.4; Fig. 4.3). Among six seeding rates Ndfa% did not vary significantly (F=3., df=5, p > 0.9; Table 4.2) in Melfort and ranged from 71% to 87%. Despite the low red clover N fixation and biomass in Clavet, there was a significant correlation between the total N fixation amount and red clover biomass (Fig. 4.4). The same relationship was found in Melfort (Fig. 4.4). In contrast, there was no association between seed yield and N fixation at either site (Fig. 4.5).

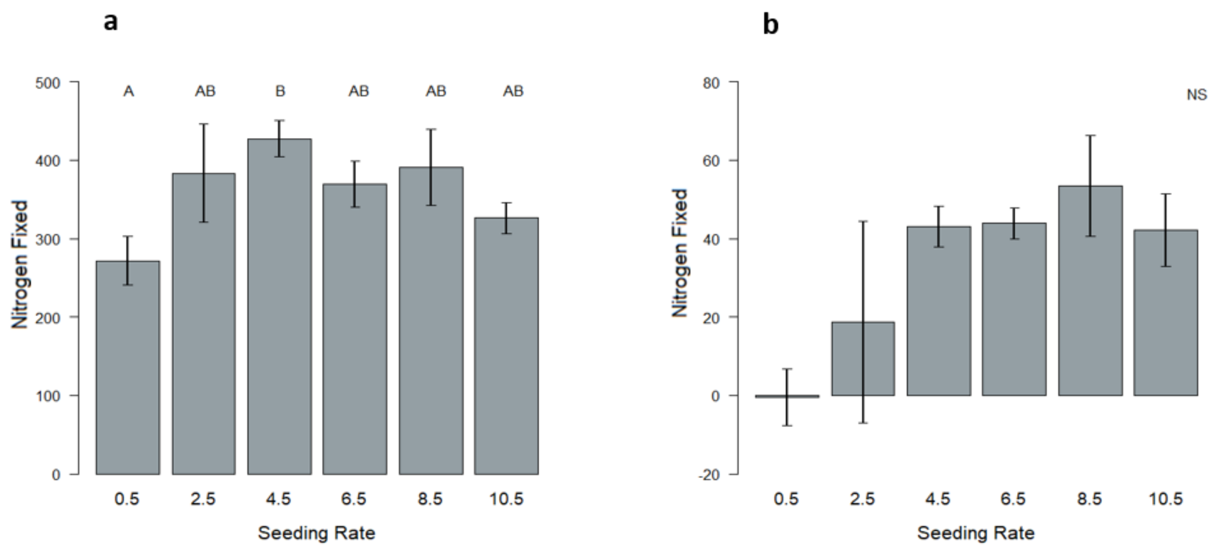

Figure 4. 4. Distribution of total nitrogen fixed (kg ha^{-1}) in six different seeding rates (kg ha^{-1}) in Melfort (a) and Clavet (b), SK Canada. Error bars are standard errors of the mean. Treatments denoted with different letters are significantly p<0.05 different.

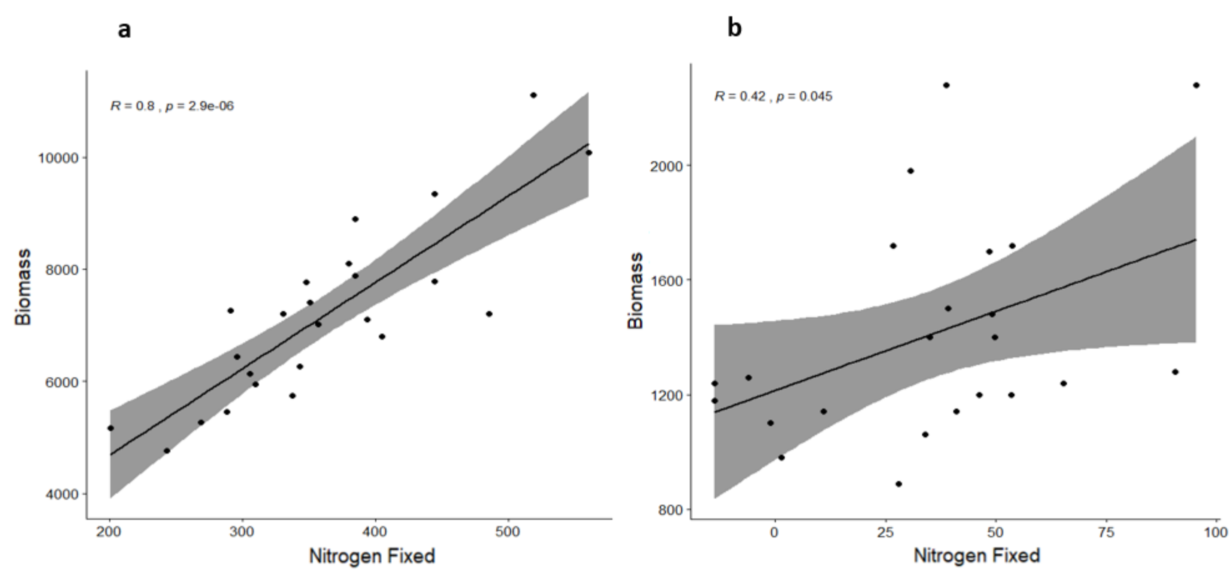

Figure 4. 5. Relationship between total nitrogen fixed (kg ha^{-1}) and forage biomass (kg ha^{-1}) in Melfort (a) and Clavet (b), SK Canada.

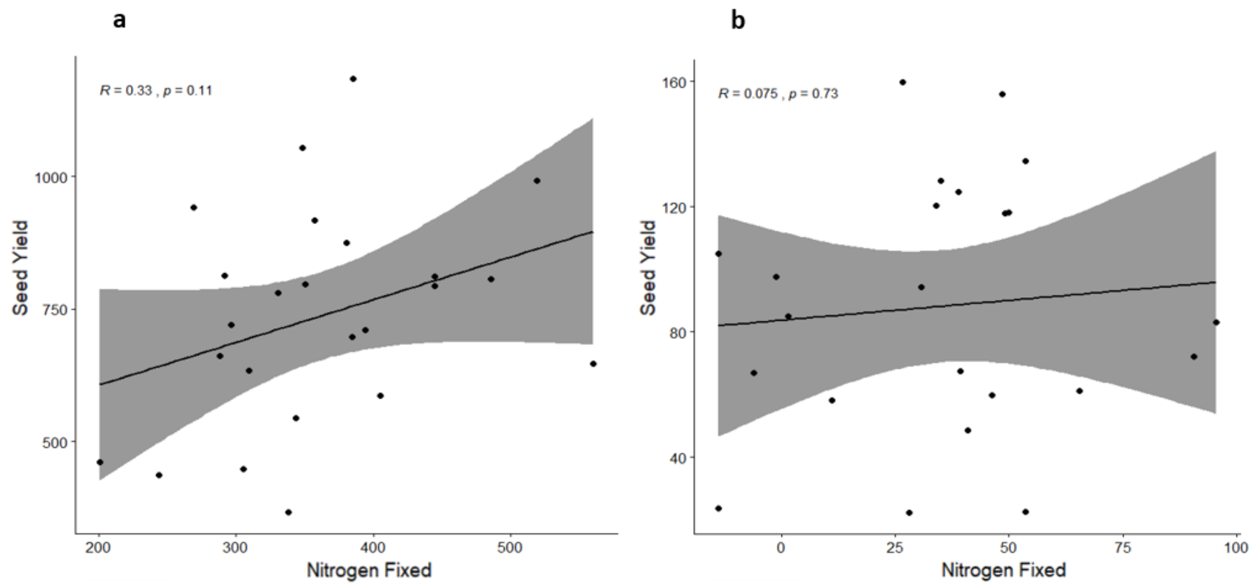

Figure 4. 6. Correlation between seed yield (kg ha^{-1}) and nitrogen fixation (kg ha^{-1}) at Melfort (a) and Clavet (b) sites.

4.4 Discussion

4.4.1 Seed yield and forage biomass.

Seed yield and forage biomass of red clover were greater in Melfort than at the Clavet site. At the Melfort site, red clover seeding rate had an effect on biomass and seed yield, but no seeding rate effect was found in Clavet. This is likely due to differences in soil characteristics and precipitation levels between the two sites. Not only did the Clavet site have less precipitation than Melfort, but it also had 42.7% and 26.5% less annual precipitation in both the 2018 and 2019 years respectively in comparison with the 15 years average. Peterson and Hall (1992) reported that drought stress can significantly reduce red clover herbage yield, which was observed at the Clavet site. Additionally, black soil with higher water holding capacity, such as that at the Melfort site, seemed to be a key factor associated with the 31% increased germination rate, however, we did not aim to conduct an experiment on effects of soil types.

Due to dry growth conditions at Clavet and sandy, well-drained soils, neither seed nor biomass yield was affected by the lowest seeding rate, which suggests that water stress may affect clover water utilization in higher and lower densities differently. Even though seeding rates were positively correlated with the number of seedlings per unit area, lower rates having less plants per area unit could accumulate similar seed and biomass yields compared with higher rates, which resulted in absence of significant differences among the seeding rates. There is limited information available for red clover water management and its effects on yield components. However, it has been demonstrated that besides reduced stem length, number of florets per flower and duration of season-long bud, drought negatively affects the most important red clover yield components, such as floral fertility and flower number per unit area (Oliva et al., 1994).

The presumed impact of the greater precipitation received at the Melfort site in combination with black soil better water holding capacity was probably responsible for the higher yields and overall increased germination rate observed at that site. Even though all seeding rates at the Melfort site accumulated higher yield than the 0.5 kg ha^{-1} rate, 4.5 and 10.5 kg ha^{-1}rates differed the most, resulting in a 51 and 54% yield increase, respectively. This indicates that there is no explicit reason to increase seeding rate above 4.5 kg ha^{-1}. This finding is in agreement with previous studies that determine that optimal clover seeding rate is in a range from 1 to 5 kg ha^{-1} with row spacings between 15 and 60 cm (Pankiw et al., 1977; Taylor & Quesenberry, 1996). In eastern U.S and eastern Europe, a higher range of seeding rates (8 to 15 kg ha^{-1}) with similar thousand kernel weight was claimed to be more productive (Barać et al., 2011; Rincker & Rampton, 1985). Conducted study reveals that overall, under sufficient precipitation, red clover is able to compensate for the differences in seeding rates in a range between 2.5 and 10.5 kg ha^{-1} without seed and biomass yield losses. Despite the differences in yields between sites, there was a significant positive relationship between biomass and seed yield. Similar results were demonstrated in earlier studies for multi-cut red clover, including when clover was under stressed conditions (Oliva et al., 1994; Steiner & Alderman, 2003).

Even though a limited number of replications was done in the greenhouse experiment, revealed findings confirmed that limited water availability can substantially reduce clover seed production regardless of seeding rate. However, it is unknown whether it was triggered by reduced floral fertility, number of florets per inflorescence or water use efficiency. In contrast to seed yield results from field experiment, seeding rate of 10.5 kg ha^{-1} in greenhouse accumulated significantly lower seed yield than 0.5, 2.5 and 4.5 kg ha^{-1} rates. These results could be because in the growing containers red clover deep tap root system at higher rate was more limited per unit of area than under field conditions. Increasing the density to a 10.5 kg ha^{-1} rate under greenhouse conditions would cause greater competition for the limited resources, resulting in a reduction in plant flower production and seed yield. It is important to point out that mechanisms behind drought stress and water use efficiency in red clover for seed production are largely unstudied and both field scale and lab studies related to this issue would be critical for persistent seed production.

4.4.2 Plant mortality rates

Initially in the sowing year, there was a higher seedling density at the Melfort site, but plant number increased proportionally according to the seeding rate at both sites. In certain rows, more plants were counted in the second year than in the seeding year. Since no flowers were produced in the first year, it is evident that a certain percentage of red clover seeds might stay dormant. Rampton and Ching (1966) reported that red clover has high seed viability in comparison with other grasses and legumes, but large numbers of red clover seeds can lay dormant in the soil. Winterkill had no effect on red clover density at any seeding rate at either site. Throughout the winters of 2018 and 2019, there was a consistent cover of snow at both sites. Additionally, a single cut clover in conducted experiments was not cut either in the first or second year and was grown exclusively for seed production, which possibly contributed to a high total nonstructural carbohydrate reserve in the root system. Research in Sweden investigated the effects of several fall cutting dates on overwintering clover and reported that mid-September cutting resulted in poorest overwintering, presumably due to limited regrowth period and reduced total number of total nonstructural carbohydrates in the root system (Taylor & Quesenberry, 1996). Similarly, Anderson (1986) reported that cuts at flowering limit storage of red clover root reserves and decrease both persistence and winter hardiness. Thus, leaving red clover for seed production uncut in the seeding year, when very little flowers are formed, presumably contributes to the plant hardiness despite differences in plant density.

4.4.3 Nitrogen Fixation

Multiple research studies have looked at red clover N distribution within the plant, its fate, seasonal dynamics and the total N derived from the atmosphere (Fernandez & Warembourg, 1987; Warembourg et al., 1997), this study concentrated exclusively on the benefits of optimal seeding rates for nitrogen fixation.

The total amount of N fixed differed greatly between two sites, where almost 90% more N fixed was observed at Melfort compared to Clavet. A significant positive correlation was found between plant biomass and N fixation irrespective of site. This result is in agreement with related research where a large N fixation variation was associated with a geographical site along with significant association between aboveground plant weight and N fixed kg ha^{-1} in different environments (Carlsson, 2003). In general, seeding rate had a similar effect on N fixation and aboveground biomass. No significant differences in N fixation among the seeding rates was found at the Clavet site. However, clover plants seeded at the rate of 4.5 kg ha^{-1} fixed more N than 0.5 kg ha^{-1} rate at the Melfort site. It was found that Ndfa% did not differ statistically among seeding rates in Melfort, which indicates that differences in forage biomass and potentially root biomass weight were the major contributors to the significant differences in nitrogen fixation kg ha^{-1}. In addition, Warembourg et al. (1997) reported a similar percentage of Ndfa%, which ranged from 60 to 90%. Besides low precipitation and biomass during two field seasons, high variability in Ndfa% and N fixation among three seeding rates could also contribute to the absence of seeding rate effect on N fixation in Clavet site. Goergen et al. (2009) and Streeter (2003) reported that soil water availability can drastically affect the symbiotic N fixation activity, number of nodules, as well as the total N content in legume plant tissues. The results from the present investigation did not indicate that N fixation was associated with seed yield, however, N fixation strongly depended on plant biomass. Warembourg et al. (1997) reported that 60 to 90% of N derived from the atmosphere was recovered in the aerial parts of clover and more than 50% of this was constantly recycled from one generation of leaves to the next, and only 25% of the annual harvest was stored in seeds. Red clover has an interesting feature as succession of leaf generations, which may have environmental consequences (Taylor & Quesenberry, 1996). Thus, regardless of seeding rate in drought environments and in a range of 2.5 to 10.5 kg ha^{-1} rates in adequate conditions, red clover can significantly contribute to the N input to the soil by constant leaf succession and remaining plant residues when a single cut red clover is grown for seed production.

4.5 Conclusion

Taken together, provided results indicate that red clover seed yield and N fixation can vary greatly depending on a seeding rate under adequate precipitation conditions, wheras no effect of seeding rate was found under drought conditions. This study revealed that N fixation is highly correlated with the red clover biomass regardless of climate conditions, but is not associated with red clover seed yield. When comparing all six seeding rates, only the 4.5 kg ha^{-1} rate resulted in increased biomass and N fixation, while rates from 2.5 to 10.5 did not differ statistically under adequate precipitation. Moreover, no winter hardiness advantage was found in either clover density or site over this two-year study. Hence, seeding red clover for seed production at a rate of 4.5 kg ha^{-1} would be the most beneficial for both N fixation and seed production. Future research should investigate winter hardiness in plant densities over 3 or more years as well as to investigate red clover water-stress response and its components.

5. General discussion and conclusions

The issues of insect management and insecticide toxicity to pollinators combined with unidentified optimal seeding rates for red clover seed production and N fixation, particularly under drought all pose complications for the red clover seed production industry in Saskatchewan. Therefore, it is crucial to evaluate the effects of insecticides on both lesser clover leaf weevil and red clover pollinators under field conditions as well as to identify an optimal seeding rate for sustainable red clover seed production in Saskatchewan. Previous research has demonstrated that pollinator abundance strongly correlates with red clover seed yield (Braun et al., 1953), whereas limited pest management options can contribute to insecticide resistance in pests (Rinkevich et al., 2013; Schoonhoven et al., 2005). Taylor and Quesenberry (1996) stated that seeding rates of red clover for seed production, as well as red clover performance, can vary significantly depending on climate conditions and agronomical practices. Although it has been postulated that beyond cultivation practices, IPM is a crucial component to sustainable red clover seed yield production (Lundin et al., 2017). Nevertheless, the effects of LCLW pest management practices and their impacts on bees and clover seed production are largely unknown and there is a similar deficiency in agronomic information with respect to the effects of red clover seeding rates on N fixation and seed production.

In Canada, red clover for seed production is included in grain-forage rotations mostly due to its better ability to grow on acid or wet soils in comparison with other forage legumes (Bosworth & Stringer, 1990), high economic value and ability to fix atmospheric nitrogen (Boelt, 2015). In the Canadian prairies, red clover for seed production is cultivated using a recommended seeding rate of 2.2 kg ha^{-1} which is based on research conducted in 1976 in Beaverlodge (Alberta). Pest management in the prairies primarily relies on a single chemical compound (deltamethrin) that has been registered and used for over a decade. In the present study, main focus was on assessing the efficacy of currently available and alternative insecticides and their impacts on the bee community and potential seed yield (Chapter 3), as well as determining an optimal red clover seeding rate for both seed production and N fixation (Chapter 4). Both chapters provide a significant contribution to the management of red clover for seed production.

To evaluate the effects of LCLW management on bees, seed yield and LCLW populations, we compared insecticide treatments in multiple commercial fields across Saskatchewan employing several methods. The initial hypothesis was that cyantraniliprole an insecticide with a systemic mode of action (MoA) would provide rapid LCLW control, higher seed yield protection and little to no effect on the bee community, while the commercially available contact insecticide (deltamethrin) would have an opposite effect. However, my research findings reveal more nuanced effects taking place and overall do not support this hypothesis (Chapter 3). Both insecticides were effective at suppressing LCLW numbers 12 DAT; however, only the contact insecticide (deltamethrin) had rapid control after treatment application. Even though LCLW larvae are usually hidden under clover stipules, results from this study suggest that larvae have direct contact with either the residual insecticide on plant material or spraying droplets of the contact insecticide. In this study, rearing LCLW larvae on treated plants in lab conditions also revealed that feeding exclusively on either systemic or contact insecticide treated plants causes identical LCLW mortality rates in 10-12 DAT. However, only in sites with high LCLW pressure was seed yield significantly reduced in untreated control plots, and no significant yield differences were observed between the two insecticides treatments.

In the entomological study (Chapter 3), no substantial impact of the insecticide applications community on the bee were detected. There are many possible reasons as to why no insecticide effects were found. These factors include the relatively small size of experimental plots, late evening-night spraying when bees are not active and possibly low doses of insecticides. However, the failure to find an effect in this study should not be interpreted to mean that either insecticide is not harmful to pollinators. Multiple studies have concluded that exposure to insecticides might have severe sublethal effects such as reduced reproductive success, immune system and increased queenlessness in social bee colonies (Sandrock et al., 2014; Tsvetkov et al., 2017), but evaluation of these factors under field condition was not performed in this work. In contrast, Lundin et al. (2017) indicated that bee numbers could be higher in conventionally managed red clover fields in comparison with organic fields, mainly due to bees' tendency to be attracted to fields with higher flower density, which was achieved by conventional pest management approaches. In this research, a single application of either insecticide did not influence the abundance or composition of pollinators during the flowering period. Therefore, seed yield was not affected by a lack of pollination service. This information is important because it points towards the importance of LCLW management in maintaining and optimizing seed yield. Further, it illustrates the importance of alternative compounds for LCLW control and avoiding the development of resistance in LCLW.

It also provides evidence that seed yield shortages are likely not triggered by limited pollination but more often by insect pressure. Future research should focus on identifying the economic threshold for LCLW, and the pollinator EIL (economic impact level) along with identifying the effectiveness of various bee species in red clover pollination since over 60% of the bees in this study were native to Saskatchewan and non-managed.

Based on the field research at the Clavet and Melfort sites (Chapter 4), an optimal seeding rate for both red clover seed production and N fixation was determined. Even though the seeding rate of 2.5 kg ha^{-1} could yield higher seed yield than the lowest rate (0.5 kg ha^{-1}) and similar yields to all rates above that, the 4.5 kg ha^{-1} rate seemed to be optimal due to higher N fixation than the 0.5 kg ha^{-1} rate in Melfort. The absence of seeding rate effects on N fixation in Clavet might be associated with the high variability in the three seeding rates that could be triggered by plant water stress. I observed that overall clover development, N fixation, and seed yield differed significantly between the two locations despite using the same agronomical practices. These differences, though, can be explained by climate differences and substantial variation in precipitation levels. Melfort received substantially more precipitation than Clavet during this course of the study. Limited information on crop water stress response is available for red clover seed production. However, my greenhouse experiment corresponded with the findings of (Oliva et al., 1994), where water stress significantly suppressed red clover development and seed production. Although, notably my study has a limited number of replications. Nevertheless, my data suggests that regardless of precipitation level, most red clover N is accumulated in above ground and underground material rather than in seeds, since no significant association was found between N fixation and seed yield. Similarly, Warembourg et al. (1997) found that more than 50% of fixed N accumulates in red clover leaves. In the agronomic study (Chapter 4), I did not find significant winter kill in any plant density. However, clover persistence typically declines in three years from the seeding year or when high pest and disease pressure is present (Taylor & Quesenberry, 1996), which was not observed in either location. Based on this study, it is safe to recommend 4.5 kg ha^{-1} as an optimal seeding rate that would provide less variation in N fixation and stable seed production regardless of water availability. Future studies should focus on quantifying the effects of drought stress and developing varieties better adapted to drought and drought related environmental conditions while maintaining high seed production and N fixation. Along with this, there is a need for designing specific crop rotations where red clover could be interseeded with grain crops and harvested for seed production in the following years. Such rotations would reduce the application of synthetic N, adverse environmental impacts, soil erosion and nutrient losses while maintaining sustainable crop production (Drinkwater & Snapp, 2005; Schipanski & Drinkwater, 2011; Snapp et al., 2007).

Taken together, the results of studies conducted as part of this thesis indicate that red clover N fixation and seed yield, LCLW numbers, and the red clover pollinator community greatly vary within Saskatchewan. Integrating these findings into agronomic practices will reduce potential red clover seed yield yield losses and increase overall biomass and N fixation. Developing a model that would predict LCLW outbreaks based on the number of adults early in spring would greatly benefit forage seed growers and reduce potential threats to the pollinator community. Additionally, developing a better understanding of cyantraniliprole sublethal effects on bees and beneficial insects would contribute substantially to understanding the results of the insecticide trials. Since these studies employed different sites for different experiments, it was not possible to determine how seeding rate or N are associated with bee communities.

In conclusion, this thesis demonstrates that for red clover seed production in Saskatchewan, a seeding rate of 4.5 kg ha^{-1} is optimal as it provided high yield and N fixation while preventing the use of additional costly seed. It also demonstrated that control of LCLW is likely more important to generating yields than the risk of harm to pollination. Finally, while not fully quantified, data in this thesis indicate that drought and water stress can substantially influence red clover seed yield.

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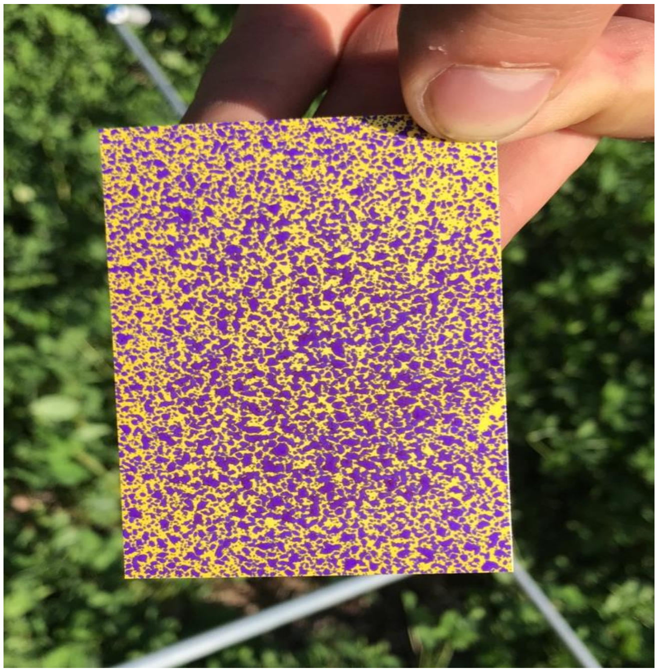
7. Appendices

Appendix A. Size and distribution of insecticides' droplets on the water sensitive paper.



Appendix B. Amount of insecticide droplets below red clover canopy.

Appendix C. Pictures illustrating LCLW rearing in plastic containers out of 20 collected red clover shoots.