AN EVALUATION OF MANAGEMENT OPTIONS FOR THE CLOVER ROOT CURCULIO (*Sitona hispidulus*), A PEST OF ALFALFA (*Medicago sativa*) IN THE INTERMOUNTAIN WEST

by

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ABSTRACT

An evaluation of management options for the Clover Root Curculio (*Sitona hispidulus*), a pest of Alfalfa (*Medicago sativa*) in the Intermountain West

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Clover root curculio (CRC) larval feeding on alfalfa (*Medicago sativa* L.) roots is associated with secondary plant pathogen infection and can reduce forage quality, yield, and stand life. Given the cryptic nature of the susceptible larval stages, the paucity of contemporary literature, and historical focus on eastern U.S. populations, management options for CRC suppression are limited and new research into management is crucial. Belowground pest management in other systems has occurred through the application of soil-active biological agents. Further, host-plant resistance is integral to pest management in alfalfa; over 100 commercial varieties exist with resistance to pathogens and Hemiptera. In Chapter II, I aimed to collect resident natural enemies of CRC immatures in the Intermountain West. I found that *Beauveria* spp. fungi were prevalent natural enemies of CRC immatures, which can inform future biological control efforts. In Chapter III, I applied commercial biological and chemical insecticides to target larvae at various phenological times. While support for biological insecticides to suppress belowground weevil pests exists, I encountered challenges in testing entomopathogenic nematodes, fungi, and bacteria for CRC larval management and did not observe reductions in immature populations. Additionally, flupyradifurone, a systemic insecticide applied in alfalfa, did not reduce CRC. Evaluating CRC suppression was challenging under field settings, because of their cryptic nature, low population densities at field sites, and potentially aggregated population distributions. Lastly, in Chapter IV, I evaluated commercial root-pathogen resistant alfalfa varieties and CRC-resistant developmental lines for resistance to CRC. I found that the commercial alfalfa varieties with root-pathogen resistance did not confer resistance to CRC. However, one Cornell University developmental line considered resistant to CRC revealed enhanced nodulation and could result in increased root vigor and reduced plant stress, allowing plants to tolerate CRC damage. Further, the soil-less arena for testing CRC larvae was presented as a useful method for quick screening of host-plant cultivars in the laboratory and may be adapted to other belowground pest systems. Overall, this research confirms historic natural enemy data and provides a foundation for development of effective contemporary integrated management of CRC in Intermountain West alfalfa, including soil-active insecticides and host-plant resistance.

(206 Pages)

PUBLIC ABSTRACT

An evaluation of management options for the Clover Root Curculio (*Sitona hispidulus*), a pest of Alfalfa (*Medicago sativa*) in the Intermountain West

Kaitlin A. Rim

The clover root curculio (CRC) is an important forage pest throughout North America. Extensive larval feeding on host roots has been associated with reduced stand establishment, disruption of nutrient and water uptake, increased secondary plant pathogen infection, decreased winter plant survival, and reductions in forage quality and yield. Due to the hidden nature of larvae in the soil, CRC is often overlooked and integrated pest management programs are limited as there is a lack of management options. First, I surveyed northern Utah alfalfa for CRC natural enemies, particularly insect-attacking nematodes and fungi (entomopathogens) that could be used in biological control programs against CRC larvae. I found *Beauveria* spp. fungi most commonly infecting CRC larvae belowground. Next, I used recent information on the CRC life cycle in the Intermountain West to test multiple field applications of soil-active biological insecticides (entomopathogenic nematodes, fungi, and bacteria) and a synthetic systemic insecticide, flupyradifurone. Although these insecticides were compatible with spray equipment and alfalfa production, applications of these products did not reduce CRC larval populations or root damage in my studies. Further, the two application timings tested (before larval peak and during larval peak) did not increase application effectiveness. However, evaluating CRC suppression in the field was challenging because they are hidden in the soil, have patchy distributions, and were observed in low numbers. Lastly, I tested existing root-pathogen resistant alfalfa varieties for potential cross-resistance to CRC, and evaluated alfalfa currently being bred at Cornell University for specific resistance to CRC larvae. Although commercial root-pathogen resistant alfalfa had no effect on CRC, the CRC-resistant alfalfa developed at Cornell University may alleviate the impact of CRC larval damage through increased nodule production. This research provides the groundwork for finding effective and long-term management solutions for CRC in the Intermountain West and will assist in the continued development of insecticide application programs and resistant host plants to ultimately improve alfalfa production.

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Kaitlin A. Rim

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CHAPTER I

BIOLOGY AND MANAGEMENT OF CLOVER ROOT CURCULIO (COLEOPTERA: CURCULIONIDAE)[1]

Abstract

Clover root curculio (*Sitona hispidulus* (F.)) (CRC) has become an economically important pest of alfalfa since the regulatory phase-out of carbofuran, methyl bromide, and other synthetic soil fumigants. Native to temperate Europe, the Middle East, and Asia, CRC was introduced to North America in the early 1900s and is now widespread across the U.S. Adult foliar herbivory is not typically economically important. However, severe root damage by larvae can result in secondary plant pathogen infections, leading to losses in forage quality, yield, and stand life. Monitoring efforts include sweep netting adults and soil sampling around the crown and roots of plants for eggs and larvae. Relationships between CRC densities and root damage as well as economic thresholds have yet to be established. Although several pyrethroids have been registered for use against CRC adults, they are not recommended due to potential non-target effects. Chemical controls have not been registered for use against the cryptic larval stage. This review discusses CRC identification, life history, biology, and ecology. Potential ecological management strategies (e.g., crop rotation, planting dates, resistant host varieties), biological control agents, and insecticide options are also described as key components of an integrated pest management program against CRC in U.S. alfalfa

Key Words: *Sitona hispidulus, Medicago sativa, Trifolium, Sitona* spp.

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Clover root curculio (CRC), *Sitona hispidulus* (Fab.) (Coleoptera: Curculionidae) is one of 11 species of *Sitona* in North America and is an important pest of clovers (*Trifolium* spp.) (Fabales: Fabaceae) and alfalfa (*Medicago sativa* L.) (Fabales: Fabaceae) (Bright 1994). Adults feed on the leaves, causing minimal damage, whereas larval feeding causes severe root damage and affects short- and long-term alfalfa health. In 2017, U.S. alfalfa hay alone generated over US$7 billion on 6.7 million hectares (16.5 million acres; USDA-NASS 2018). However, when alfalfa is grown mixed with other forages, mixed hay production (US$16.1 billion) is about two times greater than alfalfa hay alone and exceeds profits from U.S. wheat production (USDA-NASS 2018). Therefore, reduced alfalfa health due to CRC larval damage can have important economic impacts.

Clover root curculio was first reported in Long Branch, New Jersey in 1875 (Wildermuth 1910) but is native to temperate Europe, Asia, and the Middle East (Hamilton 1889, 1894; Boroumand 1975; Kivan 1995). By the early 1900s, CRC had spread throughout North America and is now found from Alaska to eastern Mexico, though it is less common at these extreme latitudes (Fig. 1-1; Bright 1994, Bright and Bouchard 2008). During early investigations, adult CRC in clover swards were occasionally considered an important pest when populations were high, but larval damage was usually misattributed to other pests or simply overlooked (Wildermuth 1910). The historical shift from forage cropping systems involving short-term clover swards to increasingly larger acreage alfalfa stands, where CRC persist for multiple years, may have increased the severity and range of this pest (Wildermuth 1910). It has also been hypothesized that reduced broad-spectrum insecticide use against eastern alfalfa weevil

(*Hypera postica* (Gyllenhal)) (Coleoptera: Curculionidae)) in the 1970s limited non-target control of CRC, given the subsequent increases in premature alfalfa stand degradation (Gotlieb et al. 1987, Hower et al. 1995) and higher frequencies of alfalfa *Fusarium* (Hypocreales: Nectreaceae) wilt (Leath and Hower 1993). The phase-out of soil-active, broad-spectrum insecticides and synthetic soil fumigants (i.e., carbofuran and methyl bromide) in the early 2000s by the Environmental Protection Agency left growers of a wide variety of crops without chemical control options for soil-dwelling pests like CRC. Thereafter, CRC management shifted toward cultural control practices, but research is still needed to develop an integrated pest management (IPM) approach to mitigate the harmful effects of CRC in alfalfa.

Life Stages and Phenology

Description of Stages

Adult

Adults average 4 mm in length and are between 1 and 2 mm wide (Fig. 1-2A; Wildermuth 1910, Bright 1994). Compared with many North American *Sitona* spp., the eyes of CRC are weakly convex (Bright 1994, Bright and Bouchard 2008). The black cuticle is covered with dark gray, brown, and tan scales making a stripe and checkerboard-like pattern on the elytra (Fig. 1-2A). Long, light brown or white, semi-erect, setae on the elytra are diagnostic of CRC, while most other sitonids lack this feature (Fig. 2B; Bright 1994, Phillips and Barratt 2004, Bright and Bouchard 2008). Adult female CRC are identified by the narrowly rounded apex of the eighth sternite as described by Bright (1994). The alfalfa weevil (Fig. 1-2C) may also be collected along with CRC in alfalfa.

Egg

Eggs are ellipsoid, small (<0.5 mm length), and yellowish-white when laid, maturing to shiny black within a few days if fertilized (Fig. 1-3A; Jackson 1922, 1928a; Bigger 1930). They have a slightly granular, shiny cast stemming from the micro-sculpturing of the chorion (Wildermuth 1910, Marvaldi 1999).

Larva

Larvae are off-white and semi-translucent, legless and grub-like, with a light brown head capsule (Jackson 1920). Development progresses through five instars before pupation (Fig. 1-3B-F; Wildermuth 1910, Leibee et al. 1980a, Tan and Hower 1991). The shape of the head capsule and mandibular anatomy of CRC larvae are used to separate them from larval *Sitona cylindricollis* Fåhraeus (Coleoptera: Curculionidae), but it is unknown whether these characters could be reliably used to differentiate CRC from other *Sitona* spp. found in the United States (Herron 1953, Manglitz et al. 1963).

Pupa

The pupa is cream colored with the head tucked toward the sternum, thus concealed beneath the prothorax in dorsal view (Fig. 1-3G). Gosik and Sprick (2017) further described diagnostic features for CRC pupal identification and Jackson (1920) describes sexing through inspection of abdominal segments. Days before eclosion, the eyes and ends of appendages begin darkening to brown (Jackson 1920, Bigger 1930).

Life History

In North America, CRC is univoltine (Fig. 1-4A; Webster 1915a). Typically, adults emerge during midsummer to feed on foliage, and are most active at the soil surface, possibly due to the cool and humid microclimatic conditions under the plant canopy. Additionally, increased CRC activity has been recorded during cooler night temperatures when summer daytime temperatures are high (Kerr and Stuckey 1956). Thereafter, adults emigrate by crawling to adjoining pastures, field edges, or sheltered wooded-edges (Underhill et al. 1955, Barney et al. 1979, Roberts et al. 1982) to aestivate for 1.5–3 mo. During this time they feed minimally and remain inactive, concealing themselves in crevices in the soil, under field litter, or burrowed in plant crowns (Markkula and Roivainen 1961, Phillips and Ditman 1962, Leibee et al. 1980a). Aestivation for this species may be obligatory as similar behaviors have been reported in Finland where mean temperatures during the warmest months can range from 14 to 20°C (Markkula and Roivainen 1961, Rautapää and Markkula 1966). Adults return to the field early fall, continue feeding, and start laying eggs (Pausch et al. 1980). At this time, post-aestivation adults may disperse to new areas. Fields adjacent to each other may receive founders from crawling populations, whereas adult flight—likely on warm Fall days—may be important for long-distance dispersal to new fields (Prescott and Newton 1963, Leibee et al. 1981, Culik and Weaver 1994).

Adults overwinter within the upper ca. 2.5 cm of soil or on the soil surface under organic matter and debris (Wildermuth 1910, Marshall and Wilbur 1934, Herron 1953, Rautapää and Markkula 1972). Warm temperatures (≥10°C) in early spring stimulate over- wintered adults to feed and oviposit once again (Bigger 1930). Spring flights after overwintering may not occur due to the degeneration of flight muscles over the winter (Jackson 1928a) and CRC dispersal, if any, occurs through crawling.

Although feeding by overwintered adults on fresh leaves in spring is minimally damaging, the root-feeding larvae can cause significant damage (Marshall and Wilbur

1934, Underhill et al. 1955, Wenninger and Shewmaker 2014). First instars feed on nitrogen-fixing root nodules, ingesting the tissues inside and leaving behind the hollowed-out epidermis or consuming it entirely (Bigger 1930, Marshall and Wilbur 1934, Manglitz et al. 1963). As larvae develop, feeding extends to the rest of the root system. Fifth-stage larvae stop feeding and create a pupal cell near the soil surface where they have been reported to remain for anywhere from 8 to 22 d before emerging as new generation adults in the summer (Wildermuth 1910, Bigger 1930, Marshall and Wilbur 1934). Adults feed, mate, and oviposit diurnally throughout their lifespan during times of activity into the following spring (Phillips and Ditman 1962, Rautapää and Markkula 1966, Powell and Campbell 1984).

Phenology Differences

Although typically overwintering as both eggs and adults, discrepancies in CRC phenology throughout the U.S. exist regarding overwintering life stage and duration (Bigger 1930, Phillips and Ditman 1962). In eastern states, both eggs and adults overwinter and adults promptly resume oviposition in the spring (Bigger 1930, Herron 1953, Lau and Filmer 1959, Phillips and Ditman 1962, Kalb et al. 1994). Conversely in the western United States (e.g., Utah), recent research has indicated that CRC overwinter primarily as eggs and few adults survive to oviposit the following spring (Fig. 1-4B; Price 2017). Research is underway to confirm this trend in other western states. In areas of mild winter weather or in the event of a warm winter, adult overwintering may be delayed, and females can oviposit throughout the winter months (Powell and Campbell 1984). Additionally, warm fall weather may initiate egg hatching late in the season and larval overwintering may occur (Leibee et al. 1980a, Quinn and Hower 1985a). While overwintering larvae have been reported in some areas, it is unknown to what extent larvae survive and contribute to spring populations (Folsom 1909, Rautapää and Markkula 1966).

Ecology

Host Plants

Members of the tribe Sitonini are oligophagous on Fabaceae, primarily feeding on Trifolieae (e.g., clovers and alfalfa), Cicereae, Hedysareae, and Galegeae (De Castro et al. 2007). Generally, CRC prefers *Trifolium* spp. over *Medicago* spp. and other legumes, although preference can be variable depending on the host species and plant growth stage (Thompson and Willis 1971, Barratt and Byers 1992). Historically, important clovers in North America and preferred hosts of CRC are red (*T. pretense* L.), white (*T. repens* L.), and alsike clovers (*T. hybridum* L.) (Thompson and Willis 1971, Barratt and Byers 1992). However, since the 1900s, alfalfa's role in U.S. forage production has steadily increased (USDA-NASS 2018), making this host more available to CRC. Clover root curculio has also been reported as a minor pest of soybean particularly when adjacent to infested alfalfa or clover fields (Kogan and Kuhlman 1982). Additional host species of lesser importance include *Lespedeza striata* (Thunb.) (Fabales: Fabaceae) (Phillips and Ditman 1962), black medic (*Medicago lupulina* L.) (Fabales: Fabaceae) (Murray and Clements 1994), and bigflower vetch (*Vicia grandiflora* var. *kitaibeliana* W. Koch) (Fabales: Fabaceae) (Byers and Kendall 1982). Trefoils (*Lotus* spp.) (Fabales: Fabaceae) and crownvetch (*Coronilla varia* L.) (Fabales: Fabaceae) are incidental hosts (Thompson and Willis 1971, Byers and Kendall 1982, Barratt and Byers 1992). While congener *S. cylindricollis* has been reported feeding on sweet clovers (*Melilotus* spp.) (Fabales:

Fabaceae) (Bright and Bouchard 2008), this genus has not been recorded as a major host of CRC. Additionally, economically important pulse crops (e.g., chickpeas, peas, lentils) that are hosts of *Sitona lineatus* L. (Coleoptera: Curculionidae) do not seem to be suitable hosts for CRC (Melamed-Madjar 1966).

Description of Damage

Direct Damage

Adult

Adult feeding damage appears as semicircular notches on leaf edges or as symmetrical or paired holes centered on the midrib when feeding occurs before leaflet expansion (Fig. 1-5A; Folsom 1909, Bigger 1930). A recent study observed greater damage to newer leaves at the top of alfalfa plants which suggests leaf maturity may influence adult feeding behavior (Price 2017). Typically under field conditions, damage from adult feeding is negligible; however, foliar feeding on seedlings can severely reduce stand establishment (i.e., seedling densities; Jewett 1934).

Larva

Larval feeding progresses from nitrogen-fixing root nodules (first and second instars; Fig. 5B) to smaller fibrous and lateral roots (beginning with the second instar), and finally to the main taproot and crown (fourth and fifth instars; Fig. 1-5C; Wildermuth 1910, Bigger 1930, Dickason et al. 1958, Tan and Hower 1991). In most cases, larvae consume entire nodules, sever lateral roots, and girdle taproots (Bigger 1930, Jewett 1934, Marshall and Wilbur 1934). Clover root curculio are especially damaging to seedlings and can sever small young roots, reducing seedling densities by 20–30% in addition to overall stand establishment (Godfrey et al. 1986). Nodule removal by early instars may temporarily interrupt nitrogen fixation, putting plants under nitrogen stress (Quinn and Hall 1992, Murray et al. 2002). Further, due to spring synchrony between early-stage larval activity and peak nodule production, the potential for nodule damage is high (Quinn and Hower 1986a, Pietola and Smucker 1995). Additionally, mid-stage larval feeding (second through fourth instars) on lateral roots interrupts water and nutrient movement and can kill root apices (Jewett 1934, Tan and Hower 1991). Weakening of the root system from lateral root feeding also contributes to winter heaving where plants are upheaved by the combination of frozen water expansion and inadequately anchored roots (Underhill et al. 1955, Holmes and Robertson 1960, Russell et al. 1978). Larger fourth- and fifth-stage larvae feeding on alfalfa taproots can remove 5.7 to 1.9 mg/d, respectively (Dintenfass and Brown 1986). After 2 yr of taproot damage, many plants have greater than 17% surface area damage (Pesho 1975, Quinn and Hower 1986b) which has then been reported to increase to 87% by the third year of damage (Hower et al. 1995). Overall, feeding damage on alfalfa roots can occur as deep as 71 cm in the soil, although feeding is concentrated in the top 25 cm of the root system and crown (Dickason et al. 1968). As alfalfa stands age, the depth of root damage changes marginally, but the severity of the accumulative damage occurring at shallow soil depths increases rapidly (Dickason et al. 1968, Pesho 1975, Cranshaw 1985). Consequently, damage to alfalfa is typically not noticed until the second year (Cranshaw 1985, Godfrey et al. 1987).

Indirect Injury

Physical damage from larval feeding predisposes plants to a suite of diseases like crown rots, root rots, and wilts (Graham and Newton 1959, 1960; Graham et al. 1960;

Newton and Graham 1960; Kilpatrick and Dunn 1961; Leach et al. 1963; Thompson and Willis 1967; Dickason et al. 1968; Hill et al. 1969, 1971). While CRC feeding damage is not necessary for root pathogen infection (Dunn et al. 1964), evidence indicates that mechanical injury creates an infection site. Pathogens like *Fusarium oxysporum* f.sp. *medicaginis* (Weimer) or *Corynebacterium insidiosum* (McCulloch) (Corynebacteriales: Corynebacteriaceae) can colonize the vascular system belowground after CRC damage and cause wilt aboveground (Leath and Hower 1993). When deep CRC feeding lesions occur, inner cortex colonization by *Fusarium oxysporum* Schltdl. or *Fusarium solani* (Mart.) can lead to cortical rots (Kalb et al. 1994). It has also been hypothesized that larvae may vector pathogens, given that numerous pathogenic fungi have been isolated from larval head capsules (Kilpatrick 1961, Leath and Hower 1993).

Clover root curculio larval damage and phytopathogens may interact synergistically to reduce crop yields, plant densities, and stand life (Leach et al. 1963, James et al. 1980, Godfrey and Yeargan 1989). For example, combined CRC and root rot fungal infection in alfalfa reduced second cutting yields by 21%, whereas each pest alone reduced yields by approximately 8% (Godfrey and Yeargan 1987). Additionally, CRC and pathogen synergism may amplify stand decline by reducing the cold hardiness of plants, resulting in increased winterkill (Gotlieb et al. 1987). Lastly, once decay within the crown has begun, secondary invaders (e.g., saprophytes, arthropods, microorganisms) colonize, accelerating the decay process and attracting other pests like the clover root borer (*Hylastinus obscurus* Marsham (Coleoptera: Curculionidae); Leath and Byers 1973, Leath and Hower 1993, Kalb et al. 1994).

Economic Impacts

Yield losses from CRC damage have been reported to range anywhere from 8 to 18% in second-year alfalfa fields (Jewett 1934, Godfrey and Yeargan 1987, Hower et al. 1995). Further, reductions in alfalfa stand densities (crowns/0.25 m2) by 20–30% have been reported to occur within the first 1–2 yr of CRC damage (Dintenfass and Brown 1988b, Godfrey and Yeargan 1989). For example, using density data from Godfrey and Yeargan (1989), yields can be estimated to decrease by 1.3 t/ha in the third year of production. Although first-year yield reductions have been inconsistently associated with CRC, early reductions in stand densities due to CRC damage are persistent through the life of the stand, therefore reducing the long-term economic viability of fields (Dintenfass and Brown 1988b). Godfrey and Yeargan (1989) predicted an 11–15% reduction in stand life attributable to CRC.

Within a season, *Sitona* feeding has been associated with the delayed regrowth after alfalfa harvest, or “green-up” (Goldson et al. 1985, 1987, 1988). This may be a result of reductions in the total nonstructural carbohydrate root reserves important for postharvest regrowth. For instance, 50% reductions in nonstructural carbohydrate reserves were predicted to occur when 2% taproot surface area was damaged by CRC, but increasing to 20% taproot surface area damage could result in complete depletion of these reserves (Dintenfass and Brown 1988b).

Clover root curculio larval damage may also decrease forage competitiveness against weeds due to stand thinning, which can increase weed intrusion (James et al. 1980; Godfrey and Yeargan 1985, 1987; Hower et al. 1995). Weeds do not appear to affect CRC populations, nor does CRC directly affect weed growth (Godfrey and Yeargan 1985, Barney and Pass 1987), but CRC larval feeding can increase the rate of nitrogen transfer from clover to neighboring grasses increasing non-host plant growth (Murray and Hatch 1994).

Currently, monetary losses associated with CRC damage have yet to be determined, but economic impacts of congener *S. discoideus* Gyllenhal (Coleoptera: Curculionidae) have been estimated in New Zealand. A study by Goldson and Muscroft-Taylor (1988) estimated yearly yield increases of 25–1000 kg/ ha and increased profits of NZ$16–300 (~US$10–200) from insecticidal management of *S. discoideus*. Comparing these data to third-year losses (1300 kg/ha) reported in Godfrey and Yeargan (1989), successful CRC larval management could result in similar profit increases. Additionally, the cost of N applications to offset losses due to *S. discoideus* nodule feeding were estimated at approximately NZ$140/ha (~US$100; Willoughby and Eerens 2006). As mentioned previously, nitrogen stress can also occur in CRC-infested fields due to decreased nodulation and nitrogen fixation (Quinn and Hall 1992), and additional N applications may also be necessary. Further, direct costs have been estimated for the alfalfa snout beetle (ASB) (*Otiorhynchus ligustici* (L.)) (Coleoptera: Curculionidae), a flightless weevil with a 2-yr life cycle whose root- feeding larvae can also reduce alfalfa stand establishment and cause significant stand loss (Cornell University Cooperative Extension 2007). Alfalfa snout beetle has been estimated to cause losses of US$336/ha during the first year (stand establishment; Cornell University Cooperative Extension 2007). Thereafter, ASB can reduce profits by approximately US$52/ha and US$366/ha if 50% stand loss occurs during the second year in three-cut, 4-yr rotational systems and four-cut, 3-yr rotational systems, respectively (Cornell University Cooperative Extension 2007). Although ASB has a different life cycle and may be more damaging than CRC, comparisons can be made due to its similar feeding habits. If we adjust the costs reported for ASB to 20% stand reductions after the second year due to CRC based on results in Dintenfass and Brown (1988b) and Godfrey and Yeargan (1989), we tentatively estimate US$20/ha and US$146/ha in three-cut, 4-yr rotational systems and four-cut, 3-yr rotational systems, respectively.

Abiotic Influences

Survival of early-stage CRC larvae is affected by soil texture and moisture levels (Tan and Hower 1991). First-instar movement and survival increases in coarse sand and slightly moist silty clay loam (35% clay) due to larger pore spaces and soil cracking as clay dries but declines with increasing moisture content (Pacchioli and Hower 2004). In fact, excessive soil moisture has been observed to reduce larval populations, while moderately moist (19–27% volumetric water content) conditions significantly increase larval numbers (Godfrey and Yeargan 1987, Pacchioli and Hower 2004). Although the association is weak, first and second instars appear to be more positively correlated with field soil moisture content than larger larvae (Quinn and Hower 1986a, b). Whether soil moisture directly affects larval survival or is mediated through other variables (e.g., changes in entomopathogen communities or nodule accessibility) is not known (Quinn and Hower 1986a).

Monitoring

Due to the cryptic nature of larvae and eggs in soil and adult movement between the soil surface and plant canopy, CRC monitoring is complex and requires multiple approaches throughout the life cycle. Eggs can be monitored by taking soil samples near plant crowns and wet sieving the soil (Fig. 1-6). After washing, remaining particulates are separated from eggs through floatation and filtration using a high-solute salt solution (Aeschlimann 1975, Ng et al. 1977, Quinn and Hower 1985b). This method has high egg recovery rates but is time intensive and requires large numbers of samples for accurate density estimations due to the aggregated distribution of eggs in the field (Quinn and Hower 1986b).

A similar process can be used to locate larvae and pupae from soil core samples (Fig. 1-6), though recovery of first instars can be difficult since they are concealed within root nodules (Lau and Filmer 1959, Leibee et al. 1980b, Quinn and Hower 1986b). Later-stage *Sitona* larvae and pupae can also be collected by placing soil core samples in a Berlese–Tullgren funnel (Aeschlimann 1975). Unfortunately, the time constraints and logistic challenges that arise with these monitoring methods impede their usefulness to growers. Many devices have been utilized in the past to monitor CRC adults including pitfall traps for crawling adults (Pausch et al. 1980, Leibee et al. 1981, Culik and Weaver 1994), sticky traps for flying adults (Prescott and Newton 1963), and emergence traps for newly eclosed adults (Barney et al. 1979, Leibee et al. 1981, Roberts et al. 1982). For research purposes, samples can be taken from the soil surface or foliage with a suction sampling device and can be actively sorted on a heated metal pan to encourage movement or used with a Berlese–Tullgren funnel to passively extract adults (James et al. 1980, Roberts et al. 1982, Goldson and French 1983). Most of these methods require large time commitments or specialized equipment. Sweep net sampling circumvents these shortcomings but is useful only when adults are active in the crop canopy, and less so when adults are on or near the soil surface during summer diapause or after emergence (Thompson and Willis 1967).

Pheromone-baited traps are one future possibility for monitoring CRC. During colonization of spring pulse crops, male *S. lineatus* produce an aggregation pheromone (4-methyl-3,5-heptanedione) that attracts both sexes (Blight and Wadhams 1987, Nielsen and Jensen 1993, Quinn et al. 1999). While sticky or cone traps may be effective during migration (Nielsen and Jensen 1993), recent studies have found pheromone-baited pitfall and pan traps to be superior for *S. lineatus* capture in the summer and fall (St Onge et al. 2018). A homologous aggregation pheromone may exist for CRC and could be used in pest management programs. Although pea leaf weevil pheromone is not widely available commercially, other weevil pheromones, particularly those of *Anthonomus* spp. (Coleoptera: Curculionidae), can be purchased. However, further investigations are needed in order to implement this monitoring technique for CRC.

Management

Cultural Control

Historically, cultural controls thought to manage CRC adults included burning forage stubble in winter, or disking and harrowing after the first harvest (Wildermuth 1910, Webster 1915b). However, overwintering adults could escape burning (Webster 1915b), while disking and harrowing could damage and kill plants (Wildermuth 1910). Regardless, such tactics would be impractical in modern production systems and are not recommended today.

Crop Rotation and Planting Dates

Alfalfa growers should rotate to non-leguminous, non-host crops unsuitable for CRC (e.g., grasses, row crops, sugar beets, potatoes) for at least one season to limit CRC-related damage and temporarily disrupt populations before rotating back to alfalfa or clovers (Baird et al. 1997, Tietz 2012, Townsend 2012, Wenninger and Shewmaker 2014). Consecutive alfalfa rotations are not recommended since larvae can feed on leftover root material in both traditional and no-till operations and survive to damage new plants (Godfrey et al. 1986, Barney and Pass 1987). To prevent fall migration of CRC to newly-planted host crops, new alfalfa fields should not be planted near heavily infested fields if possible (Townsend 2012, Wenninger and Shewmaker 2014). Additionally, current recommendations advocate for spring-sown alfalfa in some areas since well-established plants appear to tolerate more damage from fall-migrating adults (Leibee et al. 1981, Wenninger and Shewmaker 2014). Roots of spring-planted alfalfa also have wider diameters and proportionally wider root layers by their first year of potential larval damage, which may limit deep feeding lesions (Powell and Campbell 1983). Lastly, since CRC root damage is additive, alfalfa stands should be removed at the end of their economic productivity (Dickason et al. 1968, Baird et al. 1997, Seyedbagheri 2012).

Irrigation and Fertilization

In established stands, proper irrigation and fertilization may also limit CRC damage since robust healthy plants may withstand root damage and quickly recover from stress (Wilson and Barber 1954). Low soil moisture levels due to drought or underwatering place additional stress on root-damaged plants with impaired capacity to absorb water (Godfrey and Yeargan 1985). Plants grown at optimum moisture levels are better able to tolerate CRC larval damage; however, over-watered soils can become anaerobic leading to higher incidences of secondary infections (Godfrey and Yeargan 1985, Tietz 2012). Increased nitrogen applications may reduce overall larval establishment by suppressing plant nodulation (Wolfson 1987). However, nitrogen fertilization is generally not recommended for alfalfa and unlikely to be an economically viable long-term solution for CRC management (Wenninger and Shewmaker 2014, McNeill et al. 2016).

Host-Plant Resistance

The use of pest-resistant alfalfa cultivars has become a fundamental component of IPM programs for other pests (e.g., root and stem pathogens, aphids). Research into alfalfa resistance toward CRC has been limited and unsuccessful, likely due in part to the tetraploid genome and polysomic inheritance of alfalfa (Maureira and Osborn 2005). This results in varieties with high genetic variation making it difficult to understand the specific resistance mechanisms in alfalfa (Maureira and Osborn 2005). Currently, there are no modern commercial varieties with resistance to chewing insects like CRC, but research is underway to develop CRC-resistant lines.

Historic screenings for CRC resistance in alfalfa and clovers often failed to identify mechanisms of resistance and have not yielded promising results (Pedersen et al. 1975, Byers and Kendall 1982, Byers et al. 1996). Despite the shortcomings of these studies, we can begin to predict what cultivars, mechanisms, and plant defense compounds may be influential in CRC resistance or tolerance. For instance, increased root cellulosic and hemicellulosic fiber density may be one morphological mechanism to investigate that may increase resistance to larval feeding in *T. repens* (Powell and Campbell 1983). Previous research has documented the toxicity of secondary metabolites such as saponins and tannins to various pests, including Coleoptera in alfalfa (e.g., Bennett 1965, Tava and Odoardi 1996, Nozzolillo et al. 1997). Evaluations by Pedersen et al. (1976) demonstrated that alfalfa plants selected to express higher levels of saponin were positively correlated with CRC resistance. Further research revealed that the potentially resistant Lahontan variety exhibited higher levels of root saponins (Hanson et al. 1963; Pedersen et al. 1975, 1976). Additionally, alfalfa leaves treated with tannic acid resulted in decreased alfalfa weevil activity and feeding as well as increased weevil mortality (Bennett 1965). Initial research for clover resistance to clover root weevil (S. *obsoletus* (=*lepidus*) Gyllenhal) showed reduced adult feeding on varieties high in condensed tannins, but subsequent studies were not able to confirm these results (Fay and Dale 1993, Hardwick 1998, Crush and Ouyang 2007). The paucity of information regarding curculionid larval responses toward saponin-rich and tannin-rich tissues makes research into these metabolites and resistance toward CRC imperative. Plants exist as a part of a diverse community of organisms and can be hosts to many different herbivores both temporally and spatially. As plant responses toward one herbivore could affect a different herbivore feeding on the same plant, plant resistance toward one herbivore could be conferred to other herbivores (Rossi et al. 1998, Wondafrash et al. 2013, Ramirez and Spears 2014). Though current commercial alfalfa lines lack resistance toward chewing insects, resistant lines are currently available to combat nematodes, piercing–sucking insects, and phytopathogens. Research is needed to assess whether available resistant varieties can confer resistance toward CRC.

Natural Enemies and Potential Biological Control

Some natural suppression of CRC may occur through natural enemies (Table 1-1), but not enough for economic control. Enhancing natural enemies through biological control tactics can be a promising alternative when other management options are not available or effective, as is the case with CRC. For example, predaceous beetles have been observed consuming CRC eggs and adults (e.g., Barney et al. 1979, Barney and Armbrust 1980, Quinn and Hower 1987). With few management techniques available, conservation biological control may be integral for CRC management in the future, but more research into the role of these predators in CRC population management is needed. Additionally, many of the classical biological control agents introduced from Europe in the 1900s to manage North American *Sitona* failed to establish (e.g., Loan 1965). However, entomopathogens commonly found infecting all stages of CRC have practical applications in the field and may be more important and efficacious natural enemies of CRC larvae.

Predation

Little is known about generalist predator effects on CRC field populations. Birds and mites have been recorded consuming CRC adults and eggs, respectively (Wildermuth 1910, Lindsey 1939, Aeschlimann 1980), but carabid beetles have been more frequently documented as CRC predators. Among three carabid egg predators collected in Pennsylvania, *Amara aenea* (Coleoptera: Carabidae) DeGeer was recorded consuming seven to eight CRC eggs daily per predator (Quinn and Hower 1987). Likewise, in Illinois, *Cyclotrachelus* (=*Evarthrus*) *sodalis* (LeConte) (Coleoptera: Carabidae), *Pterostichus* (=*Abacidus*) *permundus* (Say) (Coleoptera: Carabidae), *Harpalus pennsylvanicus* DeGeer (Coleoptera: Carabidae) and *Gryllus pennsylvanicus* Burmeister (Orthoptera: Gryllidae) were identified as significant field edge predators for aestivating and migrating CRC adults (Barney et al. 1979, Barney and Armbrust 1980). Further, CRC adult predation cage trials showed *H. pennsylvanicus* and *G. pennsylvanicus* consuming two to three weevils per week, double that of *A. permundus* and *C. sodalis*

(Barney et al. 1979). *Sitona lineatus* predator surveys similarly found high numbers of carabids but interestingly, rove beetles of the genus *Philonthus* (Coleoptera: Staphylinidae) were shown to be efficient egg predators in the lab (60% egg removal in 2 d; Vankosky 2010, Vankosky et al. 2011a). Further investigations into alfalfa predator communities across a broader geographic range as well as laboratory and field studies to clarify the role of predators in CRC population management are needed.

Parasitoids

Three Mediterranean species of egg parasitoids (Hymenoptera: Mymaridae), *Anaphes pratensis* Forster, *Anaphes diana* (Girault) (=*Patasson lameerei* Debaucheis), and *Patasson heterotomus* Mathot, were observed emerging from *Sitona* in the late 1960s and early 1970s (Aeschlimann 1977). The predominant species, *A. diana*, was first introduced to Newark, Delaware from France in 1976 as a biocontrol agent of *Sitona*. By 1984, *A. diana* had also been released in Illinois, Kentucky, and Idaho (Schauff 1984, Dysart 1990) although reported parasitism rates in its native range were low (2–20%; Aeschlimann 1977, 1980, 1986). Ultimately, U.S. introductions of *A. diana* failed for unknown reasons. Since the parasitoid is tolerant of a wide range of temperatures, thermal extremes were not considered to be the main reason for reduced establishment (Bloem and Yeargan 1982, Dysart 1990).

Additionally, three braconid parasitoids, *Pygostolus falcatus* (Nees), *Perilitus rutilus* (Nees), and *Microctonus aethipoides* Loan, and one tachinid fly, *Campogaster exigua* (Meigen) (Diptera: Tachinidae), found parasitizing European populations of *Sitona* spp. and *Hypera* spp. adults, were introduced to North America in the mid-1900s (Munro and Post 1948; Berry and Parker 1950; Loan and Holdaway 1961a,b). Although releases targeted sweet clover weevil, *S. cylindricollis*, these parasitoids were known to also parasitize CRC and other *Sitona* in their native range and under laboratory conditions (Berry and Parker 1950; Loan and Holdaway 1961a,b). Ultimately, these introductions also failed to establish in North American *Sitona* spp. possibly due to poor host synchronization, variation in performance of introduced parasitoid populations, or other unknown factors (Loan and Holdaway 1961a, Loan 1965). Surveys to recover introduced or native parasitoids of adult *Sitona* in Missouri, northern California, and Oregon have also had limited success (Crow et al. 1968, Phillips et al. 2000). Although CRC adults have been parasitized by the native parasitoid *Microctonus sitonae* Mason (Hymenoptera: Braconidae), which regularly infects adult *Sitona scissifrons* Say (Coleoptera: Curculionidae), CRC are not reliable hosts for this parasitoid's development (Loan 1960, 1963). One native tachinid, *Strongygaster* (=*Hyalomyodes*) *triangulifer* Loew (Diptera: Tachinidae), was reported parasitizing CRC but is unlikely to be useful in management due to its broad host range (Loan 1963).

Entomopathogenic Fungi

The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) is known to infect all CRC stages, but the role of this fungus in regulating CRC populations is not well understood (Wildermuth 1910, Kilpatrick 1961, Crow et al. 1968, Aeschlimann 1980). Although, *B. bassiana* infection rates of adults in the laboratory and field can reach high levels (Rockwood 1951, Turner Jr. 1957, Kilpatrick 1961, Crow et al. 1968), others have argued that *B. bassiana* may be acting as a secondary pathogen or saprophyte and is a low-level mortality factor in adult populations (Quinn and Hower 1985c). Other sitonids are similarly affected by *B.*

bassiana (Ellingboe et al. 1957, Aeschlimann 1980, Willoughby et al. 1998). Approximately 30 strains of *B. bassiana* collected from CRC and *S. obsoletus* throughout Europe showed pathogenicity toward *S. obsoletus*, but their virulence varied widely when tested against New Zealand populations of *S. obsoletus* in the laboratory (Willoughby et al. 1998, Goldson et al. 2004, Nelson et al. 2015). *Sitona obsoletus* was additionally infected by a strain of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) (Goldson et al. 2004). Aeschlimann (1980) was able to isolate *M. anisopliae* from larval sitonids, though this occurrence was particularly rare. Nevertheless, *S. obsoletus* larvae were found susceptible not only to *B. bassiana* and *M. anisopliae*, but also to *M. flavoviride* Gams and Rozsypal, *Isaria farinosa* (Holmsk.) Fries (Hypocreales: Cordycipitaceae), and *I. fumosorosea* (Wize) Brown and Smith under laboratory conditions (Poprawski et al. 1985). Whether entomopathogenic fungi could be a useful element in CRC IPM programs has yet to be established.

Entomopathogenic Nematodes

Entomopathogenic nematodes (EPNs) tested against CRC under both laboratory and field settings have shown promising results for CRC management. In the laboratory, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae), *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae), and *S. bibionis* Steiner infect and reproduce in early and late instars, pupae, and even adult CRC. Particularly, late-stage CRC larvae supported higher *S. feltiae* and *H. bacteriophora* infectivity and reproduction (Jaworska and Wiech 1988, Wiech and Jaworska 1990). Additionally, the Oswego strain of *H. bacteriophora* may be especially useful when targeting second to fifth instars and pupae (Loya and Hower 2003). When applied in the field, this strain can reach stable populations quickly, persist for multiple years, and reduce adult emergence and larval root damage (Loya and Hower 2002).

EPNs have been used in the long-term successful control of other belowground weevil pests of alfalfa, where there are also no effective chemical control options. For example, ASB has been successfully managed through the augmentation of locally adapted EPNs in New York (Shields et al. 2009). Native, locally adapted *S. carpocapsae* Weiser, *S. feltiae* and *H. bacteriophora* were successfully isolated from ASB-damaged alfalfa field soil in northern New York, and these species have since been distributed to growers and applied to fields as an effective ASB management strategy (Neumann and Shields 2004, Cornell University Cooperative Extension 2007, Shields et al. 2009). The success of the ASB biological control program has led other researchers to investigate the potential use of EPNs for CRC suppression.

Entomopathogenic bacteria

The most widely used microbial agent that has gained attention in IPM literature is *Bacillus thuringiensis* Berliner (*Bt*) (Bacillales: Bacillaceae), a bacterium that ultimately leads to insect death. *Bacillus thuringiensis* is easily produced in vitro under laboratory settings, can be formulated into various products (e.g., suspensions, powders, granules, etc.), and its genome can be incorporated into plants making it effective for commercial production and large-scale usage (Lacey et al. 2015). One study by Bezdicek et al. (1994) found significant reductions in CRC feeding damage on nodules that had rhizobium enhanced with *B. thuringiensis* spp. *tenebrionis* genes when compared with an unaltered rhizobium. Additionally, different *Bt* subspecies can offer greater control to certain insect families over others. *Bacillus thuringiensis* spp. *galleriae* is a subspecies that attacks beetles and may be effective against CRC given its efficacy against field populations of alfalfa weevil and rice water weevil (Lissorhoptrus oryzophilus Kushel) (Coleoptera: Curculionidae) when applied as a spray suspension or granule, respectively (Asano et al. 2003, Aghaee and Godfrey 2015, Shrestha et al. 2018). Research is currently being conducted on its efficacy toward CRC in the field (K. R., unpublished data).

Chemical Control

Chemical control of CRC is difficult due to year-round population presence, dispersal of adults in and out of fields, and cryptic habits exhibited by all life stages. Historically, CRC management relied on heavy applications of chlorinated hydrocarbons with long-lasting residual activity (e.g., DDT, cyclodienes, and hexachlorocyclohexanes), as well as carbamates and organophosphates. Efficacy against CRC was variable, but even when these chemical controls successfully reduced larval populations and root damage, seed or forage yields and stand longevity were often not improved (Phillips and Ditman 1962, Dunn et al. 1964, Dickason et al. 1968, Neal and Ratcliffe 1975, James et al. 1980, Dintenfass and Brown 1988a). Particularly, applications of chlorinated hydrocarbons yielded inconsistent results in their efficacy against CRC depending on application timing, CRC life stages present, and field age (Underhill et al. 1955, Kerr and Stuckey 1956, Hansen and Dorsey 1957, Turner Jr. 1957, Dickason et al. 1958, Leach et al. 1961, Forsythe and Gyrisco 1962, Dunn et al. 1964, Waters 1964). For example, cyclodienes were reported to provide effective control for anywhere from 1 to 3 yr, and multiple applications were often required for effective long-term control in forage and seed production (Turner Jr. 1957, Dickason et al. 1958, Leach et al. 1961).

Carbamates with systemic activity, such as carbofuran, produced inconsistent results for similar reasons, but were best used against pre-ovipositional adults. Late summer (late August to early September) applications of carbamates coupled with spring diazinon (soil-active organophosphate) treatments were observed to reduce larval populations and root lesions the following year (Godfrey and Yeargan 1987). Targeting pre-ovipositional adults in this manner may have decreased fall oviposition, and thus affected egg and larval densities the following spring. However, accurate timing of fall applications is imperative. For instance, fall (late September) foliar applications of carbamate and organophosphates significantly reduced adult *Sitona* populations in alfalfa but were ineffective in controlling immatures (Barratt 1985). Here, adult reductions did not affect larval populations, as treatments may have occurred after oviposition as evidenced by pre-application egg densities of about 10,000 eggs/m2 with approximately 30% eggs hatched (Barratt 1985).

Since the revocation of carbofuran tolerances in 2009 (EPA 2009), current control options have been limited to shorter residual foliar insecticides targeting the adults. Several pyrethroids are registered for use against CRC adults (Reitz 2018); however, prophylactic control of adults may be ineffective in suppressing subsequent larval numbers and is not currently recommended (Wenninger and Shewmaker 2014). Such extensive, field-wide treatments in spring to control adult CRC could likely have unintended ecological consequences such as reducing important biocontrol agents of alfalfa weevil and aphids. Therefore, research is needed on alternative chemistries as well as adjustments to application patterns and timing that could reduce non-target effects of insecticides. One possible approach would be to band spray field edges when adults are reentering fields after aestivation (Pausch et al. 1980). As for larval management with insecticides, there are currently no soil-active synthetic insecticides registered for management, but research is underway to investigate modern chemistries and appropriate application timings for efficacy against CRC immatures in alfalfa (K. R. and R. F. L., unpublished data).

Insecticidal seed treatments (e.g., thiamethoxam, furathiocarb, benfuracarb, imidacloprid) may be a promising alternative for initial protection against CRC, though research into alfalfa seed treatments against *Sitona* spp. is limited. One study by Rotrekl and Cejtchaml (2008) found insecticidal seed treatments to significantly increase the number of undamaged and minimally damaged (10–25% leaf area loss) alfalfa foliage in both greenhouse and field trials. Additionally, yields (green weight) for insecticide-treated alfalfa seed were typically higher than the untreated control (Rotrekl and Cejtchaml 2008). However, this study failed to assess belowground damage, but studies in field pea (*Pisum sativum* L.) (Fabales: Fabaceae) have investigated these effects. For instance, plants grown from insecticide-treated pea seeds displayed increased nodulation but did not decrease nodule damage or *S. lineatus* larval numbers, and was inconsistently associated with yield increases (Seidenglanz et al. 2010, Vankosky et al. 2011b). Further, thiamethoxam seed treatments only provided protection for the first 1–1.5 mo after pea planting (Vankosky et al. 2011b). More research is needed to determine the effects of seed treatments on CRC during alfalfa stand establishment

Concluding Remarks

Since the cancelation of registrations for soil-active insecticides in early 2000s, CRC has become an increasingly important economic pest in U.S. alfalfa forage production. Although adult feeding damage can occasionally be devastating to seedlings, cryptic larval damage to roots and increased incidences of secondary pathogen infections are most threatening to alfalfa stands. The lack of economic thresholds and effective management tactics for larval CRC makes research into alternative management techniques essential. Because the majority of literature on CRC was published in the mid- to late-1900s, it may be beneficial to update and replicate historic CRC studies with modern techniques. Future research should also focus on developing economic thresholds and predictive degree-day models, investigating diverse types of management techniques like biological control agents and resistant host plants, and increasing the understanding of how chemical control of adults impacts egg laying and future larval damage.

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References Cited

Aeschlimann, J. P. 1975. A method for the extraction of *Sitona* [Col.: Curculionidae] eggs from soil and occurrence of a mymarid [Hym.: Chalicicoicea] in the Mediterranean region. Entomophaga. 20: 403–408.

Aeschlimann, J. P. 1977. Notes on *Patasson lameerei* [Hym.: Mymaridae], an egg parasitoid of *Sitona* spp. [Col.: Curculionidae] in the Mediterranean region. Entomophaga. 22: 111–114.

Aeschlimann, J. P. 1980. The *Sitona* [Col.: Curculionidae] species occurring on *Medicago* and their natural enemies in the Mediterranean region. Entomophaga. 25: 139–153.

Aeschlimann, J. P. 1986. Distribution and effectiveness of *Anaphes diana* [= *Patasson lameerei*] [Hym.: Mymaridae], a parasitoid of *Sitona* spp. eggs [Col.: Curculionidae] in the Mediterranean region. Entomophaga. 31: 163–172.

Aeschlimann, J. P. 1990. The distribution and importance of *Microsoma exigua* Mg. (Dipt., Tachinidae), a parasitoid of adult *Sitona* spp. (Col., Curculionidae) in the Mediterranean region. J. Appl. Entomol. 109: 151–155.

Aghaee, M. A., and L. D. Godfrey. 2015. The efficacy of *Bacillus thuringiensis* spp. *galleriae* against rice water weevil (Coleoptera: Curculionidae) for integrated pest management in California Rice. J. Econ. Entomol. 108: 45–52.

Asano, S. I., C. Yamashita, T. Iizuka, K. Takeuchi, S. Yamanaka, D. Cerf, and T. Yamamoto. 2003. A strain of *Bacillus thuringiensis* subsp. *galleriae* containing a novel *cry8* gene highly toxic to *Anomala cuprea* (Coleoptera: Scarabaeidae). Biol. Control 28: 191–196.

Baird, C. R., R. L. Stoltz, and M. M. Seyedbagheri. 1997. Clover root curculio, vol. 1. University of Idaho Parma Research and Extension Center, Marsina, Idaho, pp. 1–3.

Barney, R. J., and E. J. Armbrust. 1980. Field predation of alfalfa weevil and clover root curculio adults. J. Econ. Entomol. 73: 599–601.

Barney, R. J., and B. C. Pass. 1987. Influence of no-tillage planting on foliage-inhabiting arthropods of alfalfa in Kentucky. J. Econ. Entomol. 80: 1288–1290.

Barney, R. J., S. J. Roberts, R. D. Pausch, and E. J. Armbrust. 1979. Insect predators of the alfalfa weevil and clover root curculio (Coleoptera: Curculionidae) during fall field reentry. The Great Lakes Entomologist. 12: 153.

Barratt, B. P. 1985. An attempt to control *Sitona discoideus* larvae with systemic insecticides, pp. 35–37. *In* Proceedings, Proceedings of the 38th New Zealand Weed and Pest Control Conference, 14–16 August 1984, New Zealand Plant Protection Society, Rotorua, NZ.

Barratt, B. I. P., and R. A. Byers. 1992. Legume seedling feeding preferences of adult *Sitona hispidulus* (F.) (Coleoptera : Curculionidae). Environ. Entomol. 21: 103–106.

Bennett, S. E. 1965. Tannic acid as a repellent and toxicant to alfalfa weevil larvae. J. Econ. Entomol. 58: 372–373.

Berry, P. A., and H. L. Parker. 1950. Notes on parasites of *Sitona* in Europe with especial reference to *Campogaster exigua* (Meig.), pp. 251–258. *In* Proceedings, Proceedings of the Entomological Society of Washington, October, Washington, DC.

Bezdicek, D. F., M. A. Quinn, L. Forse, D. Heron, and M. L. Kahn. 1994. Insecticidal activity and competitiveness of *Rhizobium* spp containing the *Bacillus thuringiensis* subsp. *tenebrionis* δ-endotoxin gene (cryIII) in legume nodules. Soil Biol. Biochem. 26: 1637–1646.

Bigger, J. H. 1930. Notes on the life history of the clover root curculio, *Sitona hispidula* Fab., In Central Illinois. J. Econ. Entomol. 23: 334–342.

Blight, M. M., and L. J. Wadhams. 1987. Male-produced aggregation pheromone in pea and bean weevil, *Sitona lineatus* (L.). J. Chem. Ecol. 13: 733–739.

Bloem, K. A., and K. V. Yeargan. 1982. Effects of extreme temperatures on *Patasson lameerei* survival and oviposition. J. Kansas Entomol. Soc. 55: 37–45.

Boroumand, H. 1975. Key to the groups and species of the genus *Sitona* (Col. - Curculionidae) in Iran. J. Entomol. Soc. Iran. 2: 101–110.

Bright, D. E. 1994. Revision of the genus *Sitona* (Coleoptera : Curculionidae) of North America. Ann. Entomol. Soc. Am. 87: 277–306.

Bright, D. E., and P. Bouchard. 2008. Coleoptera, Curculionidae, Entiminae. NRC Research Press, Ottawa, Canada.

Byers, R. A., and W. A. Kendall. 1982. Effects of plant genotypes and root nodulation on growth and survival of *Sitona* spp. larvae. Environ. Entomol. 11: 440–443.

Byers, R. A., W. A. Kendall, R. N. Peaden, and D. W. Viands. 1996. Field and laboratory selection of *Medicago* plant introductions for resistance to the clover root curculio (Coleoptera: Curculionidae). J. Econ. Entomol. 89: 1033–1039.

Cornell University Cooperative Extension. 2007. Management of Alfalfa Snout Beetle. Cornell University Cooperative Extension, Ithaca, NY.

Cranshaw, W. S. 1985. Clover root curculio injury and abundance in Minnesota alfalfa of different stand age. The Great Lakes Entomol. 18: 93–95.

Crow, W. R., B. Puttler, and D. M. Daugherty. 1968. *Beauvaria bassiana* infecting adult clover root curculios in Missouri. J. Econ. Entomol. 61: 576–577.

Crush, J., L. Ouyang, P. J. Gerard, and S. Rasmussen. 2007. Effect of clover root weevil larvae on four annual forage legumes, pp. 213–217. *In* Proceedings, Proceedings of the New Zealand Grassland Association, Wairakei, NZ.

Culik, M. P., and J. E. Weaver. 1994. Seasonal crawling activity of adult root-feeding insect pests (Coleoptera: Curculionidae; Scolytidae) of red clover. Environ. Entomol. 23: 68–75.

De Castro, A. J. V., M. Á. Alonso-Zarazaga, and R. Outerelo. 2007. Systematics of Sitonini (Coleoptera: Curculionidae: Entiminae), with a hypothesis on the evolution of feeding habits. Syst. Entomol. 32: 312–331.

Dickason, E. A., C. M. Leach, and A. E. Gross. 1958. Control of the clover root curculio. J. Econ. Entomol. 51: 554–555.

Dickason, E. A., C. M. Leach, and A. E. Gross. 1968. Clover root curculio injury and vascular decay of alfalfa roots. J. Econ. Entomol. 61: 1163–1168.

Dintenfass, L. P., and G. C. Brown. 1986. Feeding rate of larval clover root curculio, **Sitona hispidulus** (Coleoptera: Curculionidae), on alfalfa taproots. J. Econ. Entomol. 79: 506–510.

Dintenfass, L. P., and G. C. Brown. 1988a. Influence of larval clover root curculio (Coleoptera : Curculionidae) injury on carbohydrate root reserves and yield of alfalfa. J. Econ. Entomol. 81: 1803–1809.

Dintenfass, L. P., and G. C. Brown. 1988b. Quantifying effects of clover root curculio (Coleoptera: Curculionidae) larval feeding on biomass and root reserves of alfalfa. J. Econ. Entomol. 81: 641–648.

Dunn, G. M., R. A. Kilpatrick, and H. S. Chow. 1964. Effects of dieldrin and methyl bromide on *Sitona* larvae and root rot of white clover. Crop Sci. 4: 276–279.

Dysart, R. J. 1990. The introduction and recovery in the United States of *Anaphes diana* [HYM.: Mymaridae], an egg parasite of *Sitona* weevils [Col.: Curculionidae]. Entomophaga. 35: 307–313.

Dysart, R. J., and R. G. Bingham. 1976. An introduced egg parasite of *Sitona* spp., p. 29. *In* Proceedings, Proceedings of the 18th Forage Insect Research Conference, Ithaca, NY.

Ellingboe, A. H., M. F. Kernkamp, and B. A. Haws. 1957. Sweetclover weevil parasitized by *Beauvaria bassiana* (Bals.) Vuill. in Minnesota. J. Econ. Entomol. 50: 175–177.

(EPA) Environmental Protection Agency. 2009. Carbofuran cancellation process. (https://archive.epa.gov/pesticides/reregistration/web/html/ carbofuran_noic.html). Last accessed 7 March 2018.

Fay, M. F., and P. J. Dale. 1993. Condensed tannins in *Trifolium* species and their significance for taxonomy and plant breeding. Genet. Resour. Crop Evol. 40: 7–13.

Folsom, J. W. 1909. The insect pests of clover and alfalfa. University of Illinois Agricultural Experiment Station, Urbana, IL, Bulletin 1.

Forsythe, H. Y., and G. G. Gyrisco. 1962. Evaluating the control of the cover root curculio larva on alfalfa. J. Econ. Entomol. 55: 906–908.

Godfrey, L. D., and K. V. Yeargan. 1985. Influence of soil moisture and weed density on clover root curculio, *Sitona hispidulus*, larval stress to alfalfa. J. Agric. Entomol. 2: 370–377.

Godfrey, L. D., and K. V. Yeargan. 1987. Effects and interactions of early season pests on alfalfa yield in Kentucky. J. Econ. Entomol. 80: 248–256.

Godfrey, L. D., and K. V. Yeargan. 1989. Effects of clover root curculio, alfalfa weevil (Coleoptera : Curculionidae), and soil-borne fungi on alfalfa stand density and longevity in Kentucky. J. Econ. Entomol. 82: 1749–1756.

Godfrey, L. D., D. E. Legg, and K. V. Yeargan. 1986. Effects of soil-borne organisms on spring alfalfa establishment in an alfalfa rotation system. J. Econ. Entomol. 79: 1055–1063.

Godfrey, L. D., K. V. Yeargan, and R. B. Muntifering. 1987. Digestibility, protein content, and nutrient yields of alfalfa stressed by selected early season insect pests and diseases. J. Econ. Entomol. 80: 257–262.

Goldson, S. L., and R. A. French. 1983. Age-related susceptibility of lucerne to *Sitona* weevil, *Sitona discoideus* Gyllenhal (Coleoptera: Curculionidae), larvae and the association patterns of adult infestation. N. Z. J. Agric. Res. 26: 251–255.

Goldson, S. L., and K. E. Muscroft-Taylor. 1988. Inter-seasonal variation in *Sitona discoideus* Gyllenhal (Coleoptera: Curculionidae) larval damage to lucerne in Canterbury and the economics of insecticidal control. N. Z. J. Agric. Res. 31: 339–346.

Goldson, S. L., C. B. Dyson, J. R. Proffitt, E. R. Frampton, and J. A. Logan. 1985. The effect of *Sitona discoideus* Gyllenhal (Coleoptera: Curculionidae) on lucerne yields in New Zealand. Bull. Entomol. Res. 75: 429–442.

Goldson, S. L., G. W. Bourdot, and J. R. Proffitt. 1987. A study of the effects of *Sitona discoideus* (Coleoptera: Curculionidae) larval feeding on the growth and development of lucerne (Medicago sativa). J. Appl. Ecol. 24: 153–161.

Goldson, S. L., P. D. Jamieson, and G. W. Bourdôt. 1988. The response of field-grown lucerne to a manipulated range of insect-induced nitrogen stresses. Ann. Appl. Biol. 113: 189–196.

Goldson, S. L., M. R. McNeill, P. J. Gerard, J. R. Proffitt, C. B. Phillips, R. P. Cane, and P. J. Murray. 2004. British-based search for natural enemies of the clover root weevil, *Sitona lepidus* in Europe. N.Z. J. Zool. 31: 233–240.

Gosik, R., and P. Sprick. 2017. Description of the larvae and pupae of the eight species of the tribe Sitonin Gistel, 1848 (Coleoptera, Curculionidae) from Central Europe. Zootaxa. 4324: 401–435.

Gotlieb, A., N. Pellett, and B. Parker. 1987. *Sitona*/*Fusarium*/cold hardiness interaction in alfalfa. Phytopathology 77: 1615.

Graham, J. H., and R. C. Newton. 1959. Relationship between root feeding insects and incidence of crown and root rot in red clover. Plant Dis. Rep. 43: 1114–1116

Graham, J. H., and R. C. Newton. 1960. Relationship between injury by the clover root curculio and incidence of *Fusarium* root rot in Ladino white clover. Plant Dis. Rep. 44: 534–535.

Graham, J. H., C. L. Rhykerd, and R. C. Newton. 1960. Internal breakdown in crown of red clover. Plant Dis. Rep. 44: 59–61.

Hamilton, J. 1889. Catalogue of the Coleoptera common to North America, northern Asia and Europe, with the distribution and bibliography. Trans. Am. Entomol. Soc., Proceedings of the Entomological Section of the Academy of Natural Sciences. 16: 88–162.

Hamilton, J. 1894. Catalogue of the Coleoptera common to North America, northern Asia and Europe, with distribution and bibliography. Trans. Am. Entomol. Soc. 21: 345–416.

Hansen, H. L., and C. K. Dorsey. 1957. Effects of granular dieldrin and heptachlor on adult weevil populations in red clover. J. Econ. Entomol. 50: 224.

Hanson, C. H., G. O. Kohler, J. W. Dudley, E. L. Sorensen, G. R. Van Atta, K. W. Taylor, M. W. Pedersen, H. L. Carnahan, C. P. Wilsie, W. R. Kehr, C. C. Lowe, E. H. Standford, and J. A. Yungen. 1963. Saponin content of alfalfa as related to location, cutting, variety, and other variables. USDA- ARS 34-44, Washington, DC.

Hardwick, S. 1998. Laboratory investigations into feeding preferences of adult *Sitona lepidus* Gyllenhal, pp. 5–8. *In* Proceedings, Proceedings of the 51st New Zealand Plant Protection Society Conference, 11–13 August 1998, Hamilton, New Zealand.

Herron, J. C. 1953. Biology of the sweet clover weevil and notes on the biology of the clover root curculio. Ohio J. Sci. 53: 105–112.

Hill, R. R., R. C. Newton, K. E. Zeiders, and J. H. Elgin. 1969. Relationships of the clover root curculio, *Fusarium* wilt, and bacterial wilt in alfalfa. Crop Sci. 9: 327–329.

Hill, R. R., J. J. Murray, and K. E. Zeiders. 1971. Relationships between clover root curculio injury and severity of bacterial wilt in alfalfa. Crop Sci. 2: 306–307.

Holmes, R. M., and G. W. Robertson. 1960. Soil heaving in alfalfa plots in relation to soil and air temperature. Can. J. Soil Sci. 40: 212–218.

Hower, A. A., M. A. Quinn, S. D. Alexander, and K. T. Leath. 1995. Productivity and persistence of alfalfa in response to clover root curculio (Coleoptera: Curculionidae) injury in Pennsylvania. J. Econ. Entomol. 88: 1433–1440.

Jackson, D. J. 1920. Binomics of weevils of the genus *Sitones* injurious to leguminous crops in Britain: Part I. Ann. Appl. Biol. 7: 269–298.

Jackson, D. J. 1922. Binomics of weevils of the genus *Sitona* injurious to leguminous crops in Britain. Ann. Appl. Biol. 9: 93–115.

Jackson, D. J. 1928a. XXVII. - The inheritance of long and short wings in the weevil, *Sitona hispidula*, with a discussion of wing reduction among beetles. Trans. Roy. Soc. Edinburgh. 55: 665–735.

Jackson, D. J. 1928b. The biology of *Dinocampus* (*Perilitus*) *rutilus* Nees, a Braconid parasite of *Sitona lineata* L. - Part I. Proc. Zool. Soc. Lond. 98: 600–611.

James, J. R., L. T. Lucas, D. S. Chamblee, and W. V. Campbell. 1980. Influence of fungicide and insecticide applications on persistence of ladino clover. Agon. J. 72: 781–784.

Jaworska, M., and K. Wiech. 1988. Susceptibility of the clover root weevil, *Sitona hispidulus* F. (Coleoptera, Curculionidae) to *Steinernema feltiae*, *Steinernema bibionis*, and *Heterorhabditis bacteriophora*. J. Appl. Entomol. 106: 372–376.

Jewett, H. H. 1934. The clover root curculio. Kentucky Agric. Exp. Stat. Circ. 42: 13–23.

Kalb, D. W., G. C. Bergstrom, and E. J. Shields. 1994. Prevalence, severity, and association of fungal crown and root rots with injury by the clover root curculio in New York alfalfa. Plant Dis. 78: 491–495.

Kerr, T. W., and I. H. Stuckey. 1956. Insects attacking red clover in Rhode Island and their control. J. Econ. Entomol. 49: 371–375.

Kilpatrick, R. A. 1961. Fungi associated with larvae of *Sitona* spp. Phytopathology 51: 640–641.

Kilpatrick, R. A., and G. M. Dunn. 1961. Fungi and insects associated with deterioration of white clover taproots. Crop Sci. 1: 147–149.

Kivan, M. 1995. Preliminary investigations on *Sitona* Gm. (Coleoptera, Curculionidae) species occurred forage legumes, their host plants and distributions in Tekirdağ province. Türkiye Entomoloji Dergisi. 19: 299–304.

Kogan, M., and D. E. Kuhlman. 1982. Soybean insects: identification and management in Illinois. University of Illinois at Urbana-Champaign. Agricultural Experiment Station. Bulletin 7. Illinois Agricultural Experiment Station, Urbana, IL.

Lacey, L. A., D. Grzywacz, D. I. Shapiro-Ilan, R. Frutos, M. Brownbridge, and M. S. Goettel. 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132: 1–41.

Lau, N. E., and R. S. Filmer. 1959. Injury of clover root curculios to red clover in New Jersey. J. Econ. Entomol. 52: 1155–1156.

Leach, C. M., E. A. Dickason, and A. E. Gross. 1961. Effects of insecticides on insects and pathogenic fungi associated with alsike clover roots. J. Econ. Entomol. 54: 543–546.

Leach, C. M., E. A. Dickason, and A. E. Gross. 1963. The relationship of insects, fungi, and nematodes to the deterioration of roots of *Trifolium hybridum* L. Ann. Appl. Biol. 52: 371–385.

Leath, K. T., and R. A. Byers. 1973. Attractiveness of diseased red clover roots to the clover root borer. Phytopathology 63: 428–431.

Leath, K. T., and A. A. Hower. 1993. Interaction of *Fusarium oxysporum* f.sp. *medicaginis* with feeding activity of clover root curculio larvae in alfalfa. Plant Dis. 77: 345–348.

Leibee, G. L., B. C. Pass, and K. V. Yeargan. 1980a. Effect of various temperature-photoperiod regimes on initiation of oviposition in *Sitona hispidulus* (Coleoptera: Curculionidae). J. Kansas Entomol. Soc. 53: 763–769.

Leibee, G. L., B. C. Pass, and K. V. Yeargan. 1980b. Instar determination of clover root curculio, *Sitona hispidulus* (Coleoptera: Curculionidae). J. Kansas Entomol. Soc. 53: 473–475.

Leibee, G. L., B. C. Pass, and K. V. Yeargan. 1981. Seasonal abundance and activity of *Sitona hispidulus* adults in Kentucky. Environ. Entomol. 10: 27–30.

Lindsey, A. A. 1939. Food of the starling in central New York state. Wilson Ornithol. Soc. 51: 176–182.

Loan, C. 1960. A hymenopterous parasite of *Sitona scisifrons* Say (Coleoptera: Curculionidae). Can. J. Zool. 38: 837.

Loan, C. C. 1963. The bionomics of *Sitona scissifrons* (Coleoptera: Curculionidae) and its parasite *Microctonus sitonae* (Hymenoptera: Braconidae). Ann. Entomol. Soc. Am. 56: 600–612.

Loan, C. C. 1965. Status of *Pygostolus falcatus* as a parasite of *Sitona* spp. following releases in Manitoba and Ontario. J. Econ. Entomol. 58: 798–799.

Loan, C., and F. G. Holdaway. 1961a. *Pygostolus falcatus* (Nees) (Hymenoptera, Braconidae), a parasite of *Sitona* species (Coleoptera, Curculionidae). Bull. Entomol. Res. 52: 437–488.

Loan, C., and F. G. Holdaway. 1961b. *Microctonus aethiops* (Nees) auctt. *Perilitus rutilus* (Nees) (Hymenoptera: Braconidae), European parasites of *Sitona* weevils (Coleoptera: Curculionidae). Can. Entomol. 93: 1057–1079.

Loya, L. J., and A. A. Hower. 2002. Population dynamics, persistence, and efficacy of the entomopathogenic nematode *Heterorhabditis bacteriophora* (Oswego strain) in association with the clover root curculio (Coleoptera : Curculionidae) in Pennsylvania. Environ. Entomol. 31: 1240–1250.

Loya, L. J., and A. A. Hower, Jr. 2003. Infectivity and reproductive potential of the Oswego strain of *Heterorhabditis bacteriophora* associated with life stages of the clover root curculio, *Sitona hispidulus*. J. Invertebr. Pathol. 83: 63–72.

Manglitz, G. R., D. M. Anderson, and H. J. Gorz. 1963. Observations on the larval feeding habits of two species of *Sitona* (Coleoptera: Curculionidae) in sweetclover fields. Ann. Entomol. Soc. Am. 56: 831–835.

Markkula, M., and S. Roivainen. 1961. The effect of temperature, food plant, and starvation on the oviposition of some *Sitona* (Coleoptera: Curculionidae) species. Ann. Entomol. Fenn. 27: 30–45.

Marshall, G. E., and D. A. Wilbur. 1934. The clover root curculio (*Sitona hispidula* Fab.) in Kansas. J. Econ. Entomol. 27: 807–814.

Marvaldi, A. E. 1999. Eggs and oviposition habits in Entimini (Coleoptera : Curculionidae). Coleopterists Bull. 53: 115–126.

Maureira, I. J., and T. C. Osborn. 2005. Molecular markers in genetics and breeding: improvement of alfalfa (*Medicago sativa* L.), pp. 139–154. *In* H. Lorz, G. Wenzel (eds.) Molecular marker systems in plant breeding and crop improvement. Springer, Heidelber

McNeill, M. R., C. van Koten, V. M. Cave, D. Chapman, and H. Hodgson. 2016. Does white clover (*Trifolium repens*) abundance in temperate pastures determine *Sitona obsoletus* (Coleoptera: Curculionidae) larval populations? Front. Plant Sci. 7 Article 1397: 1–11.

Melamed-Madjar, V. 1966. Observations on four species of *Sitona* (Coleoptera, Curculionidae) occurring in Israel. Bull. Entomol. Res. 56: 505–514.

Munro, J. A., and R. L. Post. 1948. Parasites to aid in the control of the sweet clover weevil. Science. 108: 609.

Murray, P. J., and R. O. Clements. 1994. Investigations of the host feeding preferences of *Sitona* weevils found commonly on white clover (*Trifolium repens*) in the UK. Entomol. Exp. Appl. 71: 73–79.

Murray, P. J., and D. J. Hatch. 1994. *Sitona* weevils (Coleoptera: Curculionidae) as agents for rapid transfer of nitrogen from white clover (*Trifolium repens* L.) to perennial ryegrass (*Lolium perenne* L.). Ann. Appl. Biol. 125: 29–33.

Murray, P. J., L. A. Dawson, and S. J. Grayston. 2002. Influence of root herbivory on growth response and carbon assimilation by white clover plants. Appl. Soil Ecol. 20: 97–105.

Neal, J. W. J., and R. H. Ratcliffe. 1975. Clover root curculio control with granular Carbofuran as measured by alfalfa regrowth, yield, and root damage. J. Econ. Entomol. 68: 829–831.

Nelson, T. L., M. R. McNeill, C. Van Koten, and S. L. Goldson. 2015. Comparative virulence of *Beauveria bassiana* isolates against clover root weevil (*Sitona obsoletus*). N.Z. Plant Prot. 68: 139–145.

Neumann, G., and E. J. Shields. 2004. Survey for entomopathogenic nematodes and entomopathogenic fungi in alfalfa snout beetle, *Otiorhynchus ligustici* (L.) (Coleoptera: Curculionidae), infested fields in Hungary and in New York state. Great Lakes Entomol. 37: 152–158.

Newton, R. C., and J. H. Graham. 1960. Incidence of root-feeding weevils, root rot, internal breakdown, and virus and their effect on longevity of red clover. J. Econ. Entomol. 53: 865–867.

Ng, Y. S., M. A. Latheef, and B. C. Pass. 1977. Estimation of egg populations of the clover root curculio, *Sitona hispidula* (Coleoptera: Curculionidae), in Kentucky alfalfa and red clover fields. Can. Entomol. 109: 1257–1263.

Nielsen, B. S., and T. S. Jensen. 1993. Spring dispersal of *Sitona lineatus*: the use of aggregation pheromone traps for monitoring. Entomol. Exp. Appl. 66: 21–30.

Nozzolillo, C., J. T. Arnason, F. Campos, N. Donskov, and M. Jurzysta. 1997. Alfalfa leaf saponins and insect resistance. J. Chem. Ecol. 23: 995–1002.

Pacchioli, M. A., and A. A. Hower. 2004. Soil and moisture effects on the dynamics of early instar clover root curculio (Coleoptera: Curculionidae) and biomass of alfalfa root nodules. Environ. Entomol. 33: 119–127.

Pausch, R. D., S. J. Roberts, R. J. Barney, and E. J. Armbrust. 1980. Localized field migration of the adult alfalfa weevil, clover leaf weevil, and clover root curculio (Coleoptera: Curculionidae), and its implication of a fall pest management program. Great Lakes Entomol. 13: 195–200.

Pedersen, M. W., E. L. Sorensen, and M. J. Anderson. 1975. A comparison of pea aphid-resistant and susceptible alfalfas for field performance, saponin concentration, digestibility, and insect resistance. Crop Sci. 15: 254–256.

Pedersen, M. W., D. K. Barnes, E. L. Sorensen, G. D. Griffin, M. W. Nielson, R. R. Hill, F. I. Frosheiser, R. M. Sonoda, C. H. Hanson, O. J. Hunt, R. N. Peaden, J. H. Elgin, T. E. Devine, M. J. Anderson, B. P. Goplen, L. J. Elling, and R. E. Howarth. 1976. Effects of low and high saponin selection in alfalfa on agronomic and pest resistance traits and the inter- relationship of these traits. Crop Sci. 16: 193–199.

Pesho, G. R. 1975. Clover root curculio: estimates of larval injury to alfalfa tap roots. J. Econ. Entomol. 68: 61–65.

Phillips, C. B., and B. I. P. Barratt. 2004. A guide to assist detection of newly arrived *Sitona* species (Coleoptera: Curculionidae) in New Zealand and Australia, pp. 22–33. *In* Proceedings, Proceedings of the 8th Australasian Conference of Grassland Invertebrate Ecology. AgResearch Ltd, Lincoln, NZ..

Phillips, W. G., and L. P. Ditman. 1962. Studies on biology and economic importance of clover root curculio *Sitona hispidula* (Fab.) in Maryland. University of Maryland Agricultural Experiment Station, Baltimore, MD. Bulletin A: pp. 1–21.

Phillips, C. B., S. L. Goldson, L. Reimer, and U. Kuhlmann. 2000. Progress in the search for biological control agents of clover root weevil, *Sitona lepidus* (Coleoptera: Curculionidae). N. Z. J. Agric. Res. 43: 541–547.

Pietola, L. M., and A. J. Smucker. 1995. Fine root dynamics of alfalfa after multiple cuttings and during a late invasion by weeds. Agron. J. 87: 1161–1169.

Poprawski, T. J., M. Marchal, and P. H. Robert. 1985. Comparative susceptibility of *Otiorhynchus sulcatus* and *Sitona lineatus* (Coleoptera: Curculionidae) early stages to five entomopathogenic Hyphomycetes. Environ. Entomol. 14: 7.

Powell, G. S., and W. V. Campbell. 1983. Histological examination of larval clover root curculio (Coleoptera: Curculionidae) damage to ladino white clover. J. Econ. Entomol. 76: 741–743.

Powell, G. S., and W. V. Campbell. 1984. Seasonal aspects of the reproductive biology of *Sitona hispidulus* (Coleoptera: Curculionidae) in North Carolina. Ann. Entomol. Soc. Am. 77: 108–114.

Prescott, H. W., and R. C. Newton. 1963. Flight study of the clover root curculio. J. Econ. Entomol. 56: 368–370.

Price, S. J. 2017. Understanding the biology of clover root curculio and improving their management using biofumigation in alfalfa. M.S. thesis. Utah State University, Logan.

Quinn, M. A., and M. H. Hall. 1992. Compensatory response of a legume root-nodule system to nodule herbivory by *Sitona hispidulus*. Entomol. Exp. Appl. 64: 167–176.

Quinn, M. A., and A. A. Hower. 1985a. Determination of overwintering survivorship and predicting time of eclosion for eggs of *Sitona hispidulus* (Coleoptera : Curculionidae). Environ. Entomol. 14: 850–854.

Quinn, M. A., and A. A. Hower. 1985b. Changes in the spatial distribution of the clover root curculio (Coleoptera: Curculionidae) in alfalfa field soil. Environ. Entomol. 14: 864–867.

Quinn, M. A., and A. A. Hower. 1985c. Isolation of *Beauveria bassiana* (Deuteromycotina Hyphomycetes) from alfalfa field soil and its effect on adult *Sitona hispidulus* (Coleoptera: Curculionidae). Environ. Entomol. 14: 620–623.

Quinn, M. A., and A. A. Hower. 1986a. Multivariate analysis of the population structure of *Sitona hispidulus* (Coleoptera: Curculionidae) in alfalfa field soil. Can. Entomol. 118: 517–524.

Quinn, M. A., and A. A. Hower. 1986b. Effects of root nodules and taproots on survival and abundance of *Sitona hispidulus* (Coleoptera: Curculionidae) on *Medicago sativa*. Ecol. Entomol. 11: 391–400.

Quinn, M. A., and A. A. Hower. 1987. Predation of eggs of *Sitona hispidulus* [Col.: Curculionidae] in alfalfa. Entomophaga. 32: 3–8.

Quinn, M. A., D. F. Bezdicek, L. E. Smart, and J. Martin. 1999. An aggregation pheromone system for monitoring pea leaf weevil (Coleoptera: Curculionidae) in the Pacific Northwest. J. Kansas Entomol. Soc. 72: 315–321.

Ramirez, R. A., and L. R. Spears. 2014. Stem nematode counteracts plant resistance of aphids in alfalfa, *Medicago sativa*. J. Chem. Ecol. 40: 1099–1109.

Rautapää, J., and M. Markkula. 1966. Diapausal aestivation of clover root curculio, *Sitona hispidulus* Fabr. (Col., Curculionidae). Ann. Entomol. Fenn. 32: 146–152.

Rautapää, M., and M. Markkula. 1972. Acclimation of *Sitona hispidulus* Fabr. and *Sitona decipiens* Lindb. (Col., Curculionidae) to falling temperature. Ann. Entomol. Fenn. 38: 51–59.

Reitz, S. 2018. Alfalfa hay – clover root curculio, p. E7. *In* Pacific Northwest insect management handbook management. Oregon State University, Corvallis, OR.

Roberts, S. J., R. D. Pausch, R. J. Barney, and E. J. Armbrust. 1982. Effect of spatial distribution on determining the number of samples required to estimate populations of *Hypera postica*, *Sitona hispidulus*, and *Hypera punctata* for specified probability and accuracy levels. Environ. Entomol. 11: 444–451.

Rockwood, L. P. 1951. Some Hyphomycetous fungi found on insects in the Pacific Northwest. J. Econ. Entomol. 44: 215–217.

Rossi, M., F. L. Goggin, S. B. Milligan, I. Kaloshian, D. E. Ullman, and V. M. Williamson. 1998. The nematode resistance gene Mi of tomato confers resistance against the potato aphid. Proc. Natl. Acad. Sci. U.S.A. 95: 9750–9754.

Rotrekl, J., and J. Cejtchaml. 2008. Control by seed dressing of leaf weevils of the genus *Sitona* (Col: Curculionidae) feeding on sprouting alfalfa. Plant Prot. Sci. 44: 61–67.

Russell, W. E., F. J. Olsen, and J. H. Jones. 1978. Frost heaving in alfalfa establishment on soils with different drainage characteristics. Agron. J. 70: 869–872.

Schauff, M. E. 1984. Taxonomic notes on *Anaphes diana* (Girault), an imported mymarid (Hymenoptera: Mymaridae) egg parasite of *Sitona* weevils (Coleoptera: Curculionidae). Proc. Entomol. Soc. Wash. 86: 214–216.

Seidenglanz, M., J. Rotrekl, I. Smýkalová, J. Poslušná, and P. Kolařík. 2010. Differences between the effects of insecticidal seed and foliar treatments on pea leaf weevils (*Sitona lineatus* L.) in the field pea (*Pisum sativum* L.). Plant Prot. Sci. 46: 25–33.

Seyedbagheri, M. M. 2012. Clover root curculio: the known insect, the un- known damage. Progressive Forage. (https://www.progressiveforage.com/ forage-production/pests-and-diseases/clover-root-curculio-the-known- insect-the-unknown-damage). Last accessed 29 September 2017.

Shields, E. J., A. Testa, G. Neumann, K. L. Flanders, and P. C. Schroeder. 2009. Biological control of alfalfa snout beetle with a multi-species application of locally adapted persistent entomopathogenic nematodes: the first success. Am. Entomol. 55: 250–257.

Shrestha, G., G. V. P. Reddy, and S. T. Jaronski. 2018. Field efficacy of *Bacillus thuringiensis galleriae* strain SDS-502 for the management of alfalfa weevil and its impact on *Bathyplectes* spp. parasitization rate. J. Invertebr. Pathol. 153: 6–11.

St Onge, A., H. A. Cárcamo, and M. L. Evenden. 2018. Evaluation of semiochemical-baited traps for monitoring the Pea Leaf Weevil, *Sitona lineatus* (Coleoptera: Curculionidae) in field Pea Crops. Environ. Entomol. 47: 93–106.

Tan, Y. P., and A. A. Hower. 1991. Development and feeding-behavior of clover root curculio (Coleoptera, Curculionidae) larvae on alfalfa. Environ. Entomol. 20: 1013–1018.

Tava, A., and M. Odoardi. 1996. Saponins from *Medicago* spp.: chemical characterization and biological activity against insects, pp. 97–109. In G. R. Walter and K. Yamasaki (eds.), Saponins Used in Food and Agriculture. Plenum Press, New York.

Thompson, L. S., and C. B. Willis. 1967. Distribution and abundance of *Sitona hispidula* (F.) and the effect of insect injury on root decay of red clover in the maritime provinces. Can. J. Plant Sci. 47: 435–440.

Thompson, L. S., and C. B. Willis. 1971. Forage legumes preferred by the clover root curculio and preferences of the curculio and root lesion nematodes for species of *Trifolium* and *Medicago*. J. Econ. Entomol. 64: 1518–1520.

Tietz, N. 2012. Mysterious alfalfa menace: clover root curculio sneak-attacks estern fields. Hay Forage Grower. 14–15.

Townsend, L. 2012. Clover root curculio in alfalfa. University of Kentucky Cooperative Extension Service, Kentucky. 119.

Turner, E. C. Jr. 1957. Control of the clover root curculio on alfalfa. J. Econ. Entomol. 50: 645–648.

Underhill, G. W., E. C. Turner, Jr., and R. G. Henderson. 1955. Control of the clover root curculio on alfalfa with notes on life history and habits. J. Econ. Entomol. 48: 184–187.

(USDA-NASS) United States Department of Agriculture – National Agricultural Statistics Service. 2018. National Agricultural Statistics Service Quick Stats. (http://quickstats.nass.usda.gov/). Last accessed 12 July 2018.

Vankosky, M. A. 2010. Integrated pest management of *Sitona lineatus* in crops of *Pisum sativum* in western Canada. M.S. thesis. University of Alberta, Edmonton, Alberta.

Vankosky, M. A., H. A. Cárcamo, and L. M. Dosdall. 2011a. Identification of potential natural enemies of the pea leaf weevil, *Sitona lineatus* L. in western Canada. J. Appl. Entomol. 135: 293–301.

Vankosky, M. A., H. A. Cárcamo, R. H. McKenzie, and L. M. Dosdall. 2011b. Integrated management of *Sitona lineatus* with nitrogen fertilizer, rhizobium, and thiamethoxam insecticide. Agron. J. 103: 565–572.

Waters, N. D. 1964. Effects of *Hypera nigrirostris*, *Hylastinus obscurus*, and *Sitona hispidula* populations on red clover in southwestern Idaho. J. Econ. Entomol. 57: 907–910.

Webster, F. M. 1915a. Importance of observations on apparently unimportant insects. Can. Entomol. 47: 69–73.

Webster, F. M. 1915b. Alfalfa attacked by the clover-root curculio, USDA Farm Bull. Bureau Entomol. USDA- Bureau of Entomology, Washington, DC.

Wenninger, E. J., and G. E. Shewmaker. 2014. Clover root curculio in alfalfa: identification, biology, and management. Pacific Northwest Extension. PNW 663: 1–5.

Wiech, K., and M. Jaworska. 1990. Susceptibility of *Sitona* weevils (Col., Curculionidae) to entomogenous nematodes. J. Appl. Entomol. 110: 214–216.

Wildermuth, V. L. 1910. The clover-root curculio. *In* USDA Bureau of Entomology. USDA- Bureau of Entomology, Washington, DC.

Willoughby, B., and H. Eerens. 2006. Managing clover in the presence of the clover root weevil: a summary of current knowledge prepared for New Zealand farmers. New Zealand Landcare Trust, Hamilton, New Zealand.

Willoughby, B., T. Glare, F. Kettlewell, and T. Nelson. 1998. Beauveria *Bassiana* as a potential biocontrol agent against the clover root weevil, *Sitona lepidus*, pp. 9–15. *In* Proceedings, Proceedings of the 51st New Zealand Plant Protection Society Conference, 11–13 August 1998, Hamilton, New Zealand.

Wilson, C. M., and S. A. Barber. 1954. The influence of the sweetclover weevil and soil fertility on sweetclover stands. J. Econ. Entomol. 47: 117–122.

Wolfson, J. L. 1987. Impact of Rhizobium nodules on *Sitona hispidulus*, the clover root curculio. Entomol. Exp. Appl. 43: 237–243.

Wondafrash, M., N. M. Van Dam, and T. O. Tytgat. 2013. Plant systemic induced responses mediate interactions between root parasitic nematodes and aboveground herbivorous insects. Front. Plant Sci. 4: 87

Tables and Figures

Table 1-1. Natural enemies reported attacking *Sitona hispidulus* in both their native and introduced range.

Taxa	Species	Stage attacked	References
Hymenoptera: Braconidae	*Microctonus aethiopoides*	Adult	(Aeschlimann 1980) (Jackson 1922) (Loan and Holdaway 1961b)
	Microctonus Sitonae	Adult	(Loan 1963)
	Perilitus cerealium	Adult	(Jackson 1928a)
	Perilitus rutilus	Adult	(Jackson 1928b) (Loan and Holdaway 1961b)
	Pygostolus falcatus	Adult	(Aeschlimann 1980)
Hymenoptera: Mymaridae	*Anaphes diana* (= *Patasson lameerei*)	Egg	(Aeschlimann 1975, 1977) (Dysart and Bingham 1976)
Diptera: Tachinidae	*Microsoma* (= *Campogaster*) *exigua*	Adult	(Aeschlimann 1980, 1990)
	Strongygaster (= *Hyalomyodes*) *triangulifera*	Adult	(Loan 1963)
Coleoptera: Carabidae	*Agonum cuprienne*	Egg	(Quinn and Hower 1987)
	Agonum placidum	Egg	(Quinn and Hower 1987)
	Amara aenea	Egg	(Quinn and Hower 1987)
	Anisodactylus sanctaecrucis	Egg	(Quinn and Hower 1987)
	Colliuris pensylvanica	Egg	(Quinn and Hower 1987)
	Cyclotrachelus (= *Evarthrus*) *sodalis*	Adult	(Barney et al. 1979) (Barney and Armbrust 1980)
	Harpalus pensylvanicus	Adult	(Barney et al. 1979) (Barney and Armbrust 1980)
	Pterostichus lucublandus	Egg	(Quinn and Hower 1987)
	Pterostichus (= *Abacidus*) *permundus*	Adult	(Barney et al. 1979) (Barney and Armbrust 1980)
Orthoptera: Gryllidae	*Gryllus pennsylvanicus*	Adult	(Barney et al. 1979) (Barney and Armbrust 1980)
Arachnida: Acari	*Allotrombium* sp.	Egg	(Aeschlimann 1980)

Taxa	Species	Stage attacked	References
	Anystis spp.	Egg	(Aeschlimann 1980)
Entomopathogenic Fungi	*Beauveria bassiana*	All stages	(Aeschlimann 1980) (Crow et al. 1968) (Kilpatrick 1961) (Quinn and Hower 1985c) (Rockwood 1951)
	Metarrhizium anisopliae	Larvae	(Kilpatrick 1961)
Entomopathogenic Nematodes	*Heterorhabditis bacteriophora*	All stages	(Jaworska and Wiech 1988) (Loya and Hower 2002, 2003) (Wiech and Jaworska 1990)
	Steinernema bibionis	All stages	(Jaworska and Wiech 1988) (Wiech and Jaworska 1990)
	Steinernema feltiae	All stages	(Jaworska and Wiech 1988) (Wiech and Jaworska 1990)

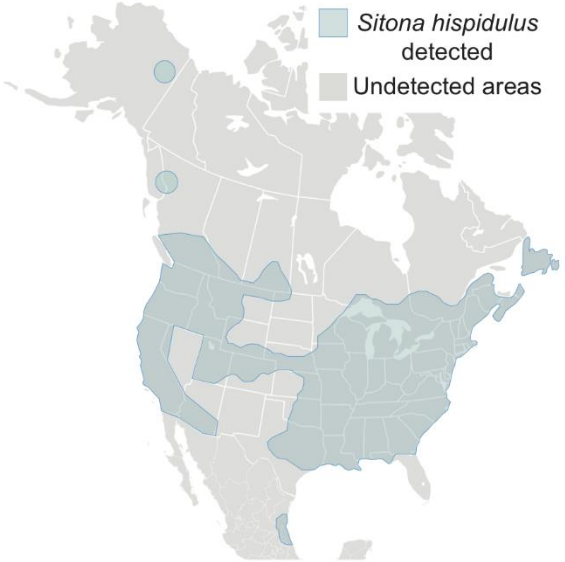

Fig. 1-1. (A) *Sitona hispidulus* is ubiquitous throughout the continental United States and can be found as far north as Alaska and south to Mexico. This map has been adapted from distributions reported in Bright (1994) and Bright and Bouchard (2008).

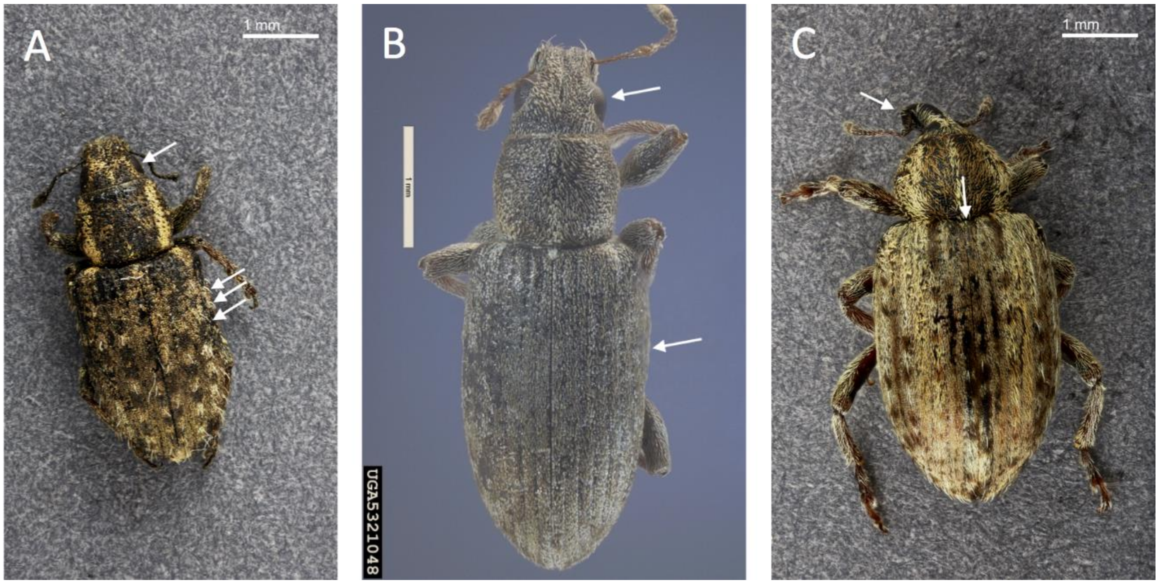

Fig. 1-2. (A) Distribution and hosts of *Sitona hispidulus* can overlap with (B) *Sitona cylindricollis* and (C) *Hypera postica*. *Sitona hispidulus* adults can be distinguished from other curculionids and sitonids occurring in alfalfa and clovers by their checkerboard elytral pattern with long semi-erect white and brown setae, short and stout rostrum, and weakly-convex eyes. Unlike *S. hispidulus*, *H. postica* has a long rostrum and a dark medial stripe on the elytra, while *S. cylindricollis* has convex eyes and lacks semi-erect setae. Photo credit: 2-A and 2-C Kaitlin Rim, Utah State University; 2-B Pest and Diseases Image Library, Bugwood.org

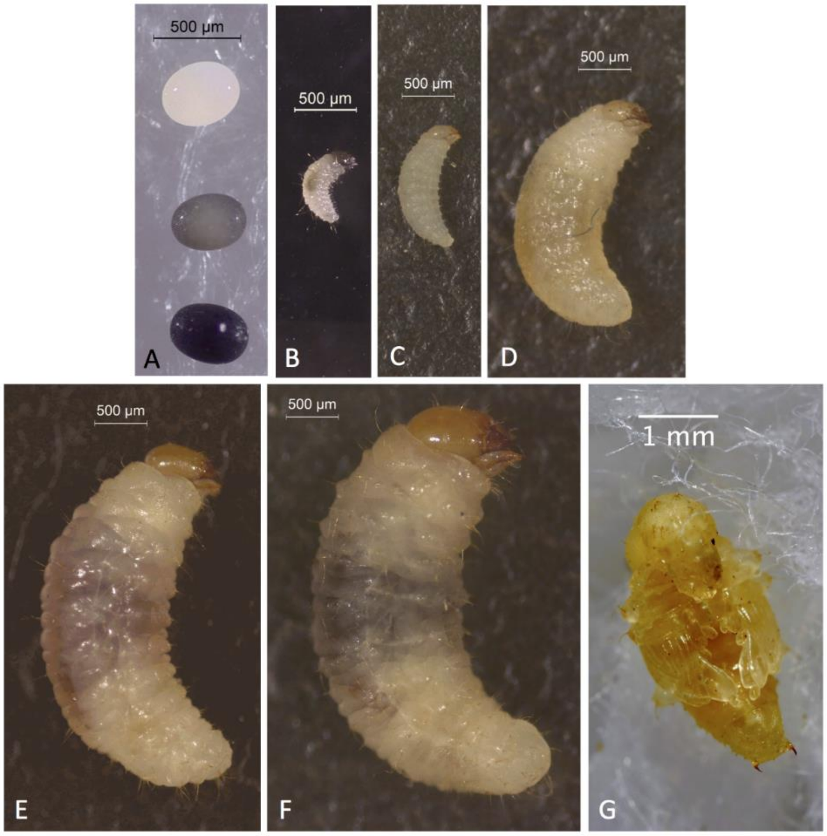

Fig. 1-3. *Sitona hispidulus* has an (A) egg stage that sclerotizes to black over 48 hours, (B-F) five larval stages, and a (F) pupal stage before eclosion. Photo credit: Kaitlin Rim, Utah State University.

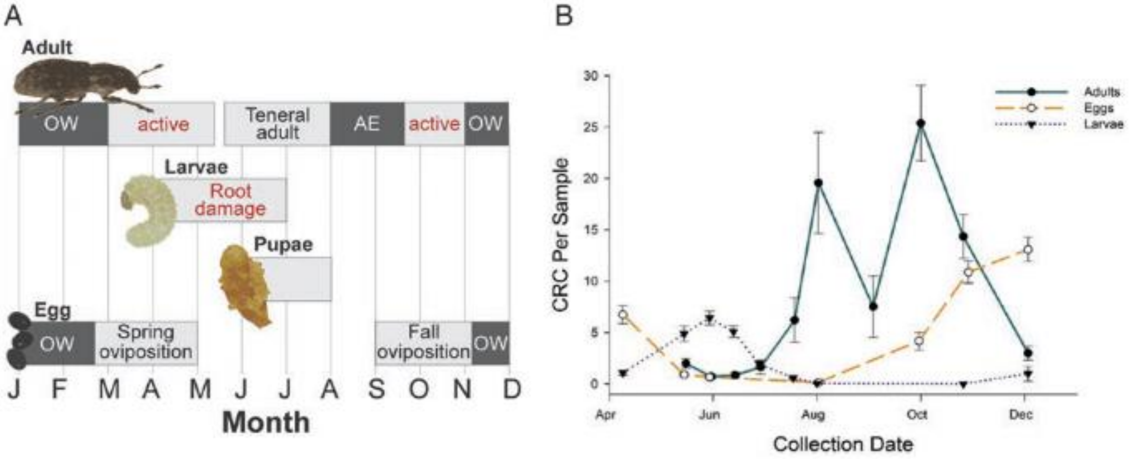

Fig. 1-4. (A) Generalized life cycle of *Sitona hispidulus* throughout the continental U.S. (AE = aestivate, OW = overwintering). Seasonal distribution and life stage abundance of *S. hispidulus* may change slightly depending on the region. For instance, (B) in northern Utah, *S. hispidulus* mainly overwinters as eggs while other areas have reported higher adult overwintering. Values are means ±1 SE (Price 2017). Samples were taken from seven fields (mean size 17.53 hectares) of varying age and management style from April 2 to December 3, 2016. During each sampling event, 4 D-vac samples, 8 egg samples, and 8 soil cores were taken from each field and dissected to measure *S. hispidulus* adults, eggs, and larvae, respectively.

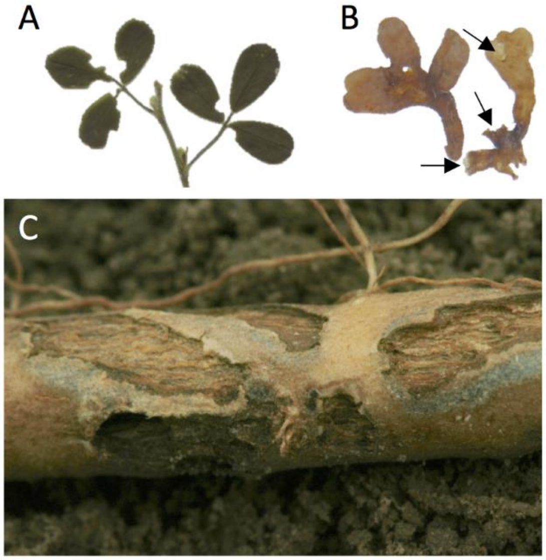

Fig. 1-5. Characteristic *Sitona hispidulus* damage. (A) Adult *S. hispidulus* leaf notching observed on *Medicago sativa* trifoliate leaf. (B) Damaged *M. sativa* root nodules characteristic of *S.hispidulus* first instar feeding (indicated by arrows). (C) Taproot scarring observed on *M. sativa*. Photo credit: Steven Price, Utah State University.

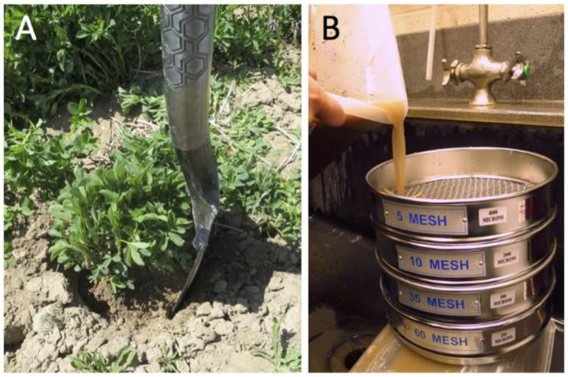

Fig. 1-6. Soil cores are taken by removing root and soil material from around alfalfa crowns (A). Soil is gently broken apart and agitated by hand with water. Supernatant containing soil particles, organic matter, soil mesofauna, and floating *Sitona hispidulus* immatures is poured through a series of mesh sieves (U.S. standard sieve set #5, #10, #35, #60) (B). This process continues until soil is completely disaggregated. *Sitona hispidulus* immatures are typically recovered from #35 and #60 mesh. Photo credit: Kaitlin Rim, Utah State University.

Research Objectives

Historically, CRC research focused on populations in the Midwest and eastern US (e.g. Bigger 1930, Marshall and Wilbur 1934, Herron 1953, Underhill et al. 1955, Roussel et al. 1956, Lau and Filmer 1959, Godfrey and Yeargan 1987, Kalb et al. 1994). Therefore, research has been initiated on the biology and control of CRC populations in the West (Utah, Idaho, and California). As Kalb et al. (1994) stated, “The greatest promise for management…lies with advances in alfalfa breeding and biological control.”

1. Survey and identify potential endemic entomopathogenic biological control agents in Utah alfalfa fields (see Chapter II; formatted according to APA citation style),
2. Evaluate the impacts of biological control agents and chemical control treatments for suppression of CRC under field conditions (see Chapter III; formatted according to guidelines for the journal *Crop Protection*),
3. Investigate the effects of host plant resistance on CRC adult feeding and oviposition as well as larval feeding behavior (see Chapter IV; formatted according to the guidelines for the journal *Annals of the Entomological Society of America*).

Chapter I was a literature review of clover root curculio and is a pre-copyedited, author produced version of an article accepted for publication in the *Journal of Integrated Pest Management* following peer review. The version of record Rim, K, Price, Steven J., Wenninger, Erik J., Long, Rachael, and Ramirez, Ricardo A. 2019. Biology and management of clover root curculio (Coleoptera: Curculionidae). Journal of Integrated Pest Management. 10:1-14 is available online at https://doi.org/10.1093/jipm/pmz020.

CHAPTER II

SURVEY FOR NEMATODE AND FUNGAL PATHOGENS OF CLOVER ROOT CURCULIO IN UTAH ALFALFA SOILS

Abstract

Clover root curculio (CRC; *Sitona hispidulus* F.) feed on clovers and alfalfa throughout North America. Larvae are the most damaging stage, causing reductions in alfalfa and clover stand health due to root feeding activity that may also facilitate secondary plant pathogen infections. Historically, soil-active insecticides and fumigants successfully suppressed CRC populations below damaging levels. Since the regulatory cancellation of these products in the 2000s, incidences of CRC damage have increased and management options targeting larvae are limited. Entomopathogens are important natural enemies of below-ground insects. Further, new technologies facilitate isolation, identification, mass rearing, and field applications of biological control agents. In the current study, we aimed to isolate locally adapted strains of entomopathogenic fungi and nematodes from northern Utah alfalfa by collecting field soil, searching for infected CRC immatures, and subjecting sentinel hosts (*Galleria mellonella* L.) to collected soil. Hosts showing signs of nematode infection were placed in modified white traps while hosts killed by fungi were plated on growth medium. Soil characteristics were also recorded for all field sites. *Beauveria* sp. was recovered from CRC immatures with an overall infection rate of 4.7%. Entomopathogenic nematodes were not recovered during this study. Future surveys should try other entomopathogen isolation techniques and survey seasonally throughout the duration of CRC immature peaks. Locally adapted *Beauveria*

strains may be more efficacious than commercially available strains and should be tested for pathogenicity and virulence towards CRC in the laboratory and field.

Introduction

The clover root curculio (CRC), *Sitona hispidulus* F. (Coleoptera: Curculionidae), has been a pest of leguminous forage crops such as clover (*Trifolium* spp.) and alfalfa (*Medicago sativa* L.) (Fabales: Fabaceae) throughout the continental United States since the early 1900s (Bright, 1994; Webster, 1915; Wildermuth, 1910). Adult CRC feed on foliage during late summer and oviposit at the soil surface typically in the fall and the following spring (Bigger, 1930; Marshall & Wilbur, 1934). After eggs hatch during spring, 1st instars burrow into the soil where they progress through five stages (Leibee, Pass, & Yeargan, 1980; Marshall & Wilbur, 1934). Larvae are the most damaging stage of this pest given that early instar larvae consume nitrogen-fixing root nodules, and mid- to late-instars lacerate lateral roots and feed on the taproot cortex (Bigger, 1930; Manglitz, Anderson, & Gorz, 1963; Marshall & Wilbur, 1934). Not only does CRC larval feeding cause direct plant damage, but larvae may facilitate infections by secondary plant pathogens (e.g., *Fusarium* wilts) (e.g., Leath and Hower 1993). This subsequently results in decreased alfalfa stand life (Gotlieb, Pellett, & Parker, 1987), yield (Godfrey & Yeargan, 1987), and forage quality (Godfrey, Yeargan, & Muntifering, 1987).

Historically, soil-active insecticides and fumigants with long-lasting residual activity were successful in suppressing CRC populations below damaging levels, but their cancellation in the 2000s (EPA, 2009) corresponded with increases in damage from belowground pests including CRC (Tietz, 2012). Several insecticides have been registered in the Intermountain West against CRC adults (e.g., lambda-cyhalothrin, gamma cyhalothrin, chlorantraniliprole, chlorpyrifos) (Reitz, 2018). However, due to adult mobility and ability to evade spray events, the limited knowledge on whether adult reductions suppress subsequent larval numbers, and the potential non-target effects on alfalfa weevil natural enemies in the spring, these are not recommended (Rim et al., 2019b; Wenninger & Shewmaker, 2014). Furthermore, there are currently limited management options targeting CRC immatures, and insecticides are not available to target these stages. Current management practices focus on crop rotation to a non-legume, planting alfalfa in the spring, removing stands at the end of their productive life, and carefully monitoring water and nutrient levels (Rim et al., 2019a; Wenninger & Shewmaker, 2014). More effective, longer-lasting management options that can be easily incorporated into contemporary forage production systems to reduce CRC immature populations and related alfalfa damage need to be investigated.

Entomopathogens are important natural enemies of below-ground insects and have been isolated and applied augmentatively to target many cryptic below-ground pests (e.g., larval stages of Curculionidae, Scarabaeidae, Sciaridae, Noctuidae) (Hazir et al. 2004). Various entomopathogens have been tested against CRC and previously described infecting CRC naturally, but the prevalence of these agents in the field and their role in managing CRC populations in the Intermountain West is not well known. For instance, the most prevalent entomopathogenic fungus infecting all CRC stages is *Beauveria bassiana* (Balsamo) Vuillemin (Aeschlimann, 1980; Crow, Puttler, & Daugherty, 1968; Kilpatrick, 1961; Wildermuth, 1910). Furthermore, entomopathogenic nematodes (EPNs) have also been tested against CRC under laboratory and field conditions. *Heterohabditis bacteriophora* Poinar, *Steinernema feltiae* Filipjev, and *S. bibionis* Steiner can infect CRC from larvae to adults in the laboratory, but late instar CRC support higher *S. feltiae* and *H. bacteriophora* infectivity and reproduction than pupae and adults (Jaworska &

Wiech, 1988; Wiech & Jaworska, 1990). Additionally, *H. bacteriophora* (Oswego strain) is efficacious against larvae and pupae in the field and can persist for multiple years to reduce CRC adult emergence and root damage (Loya & Hower, 2002, 2003).

Upon isolation and identification of important entomopathogens of insect pests, pathogen mass rearing is relatively easy through *in vivo* or *in vitro* methods (Hazir et al., 2004), and many commercial insectaries supply beneficial organisms that can be easily applied with traditional equipment (Shapiro-Ilan, Gouge, Piggott, & Fife, 2006). Entomopathogens can be safer and longer-lasting alternatives to traditional chemical controls and can be compatible with other chemical and biological pesticides (Kaya & Gaugler, 1993).

Despite the many benefits of entomopathogens (e.g., broad host range, rapid kill rate, applicator safety), their use against soil-dwelling pests in alfalfa production is rare. One major reason for low adoptability may be because different species and/or strains of entomopathogens often have different environmental requirements and are sensitive to environmental factors like solar radiation (UV) (e.g., Gaugler & Boush, 1978), temperature (e.g., Kamionek, 1974; Molyneux, 1986), and moisture (e.g., Kamionek, 1974). However, using optimal application strategies (e.g., Shapiro-Ilan et al., 2006) to apply species or strains with environmental adaptations to specific geographic regions and/or greater specificity towards target organisms may increase efficacy in the field. For example, locally adapted *Steinernema* and *Heterorhabditis* spp. were successfully isolated from alfalfa soils in northern New York, and are now a fundamental component of integrated pest management of alfalfa snout beetle (*Otiorhynchus ligustici* (L.)), for which chemical control options are also limited (Cornell University Cooperative

Extension, 2007; Neumann & Shields, 2004; Shields, Testa, Neumann, Flanders, & Schroeder, 2009).

Due to the success of locally adapted entomopathogens isolated in New York against the alfalfa snout beetle, we conducted surveys in northern Utah (Cache Co.) for local endemic entomopathogens. Entomopathogens contributing to CRC immature mortality may have potential for future biological control efforts against CRC immature stages.

Materials & Methods

Field locations and survey methods

Six alfalfa fields in Cache County, Utah, were surveyed on 21 June 2017 (Table 2-1). At each field, 8 soil core samples (28 cm diameter x 30 cm depth) each centered around an alfalfa crown were collected randomly throughout fields (except site #1 where 5 cores were sampled) (total 45 cores). Sampling time corresponded with late-stage larval CRC activity and when we would expect higher incidences of EPN infection. Samples were stored at 5-7 °C until they could be processed (1 mo.). All collected root material was observed for CRC damage, and the following environmental and agronomic conditions were recorded for each field: soil composition (SoilWeb, 2018), time since last irrigation event, time since last cutting, and soil moisture (% volumetric soil water content) (Field Scout Soil Moisture Meter TDR 100).

Isolating Entomopathogens

Soil cores were sifted through #10 mesh (2 mm) for dead and infected CRC immatures (larvae and pupae). Immatures appearing dead or diseased were individually placed onto modified white traps kept at room temperature (22-25 ˚C) (White, 1927).

Briefly, this procedure used a (40 mm diameter) petri dish lid placed inside a larger petri dish bottom (90 mm diameter). Immatures were placed on filter paper (No. 1, 55 mm) (Whatman, Buckinghamshire, UK) positioned on top of the dish lid. Subsequently, the dish bottom was filled with deionized water to the height of the smaller dish lid. Individuals on white traps were monitored for infection by entomopathogenic nematodes (EPNs), fungi, or bacteria every other day for a total of 7 d.

After CRC were removed from processed soil, sentinel waxworm larvae, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) were subjected to soil following the insect baiting technique described by Bedding and Akhurst (1975). This involved processing soil that was cleared of large debris, moistened with deionized water, and packed into a clean plastic container (500 mL). Six waxworm larvae were placed on the surface of the soil, and the container was inverted after being covered with a lid. Dead sentinel hosts (*G. mellonella*) were removed after 48 h, individually placed onto modified white traps, and monitored following the same procedure used for immature CRC.

Rearing and Identifying Entomopathogens

Each isolated nematode strain was reared for multiple generations on *G. mellonella* hosts in ‘infection traps’ described by Stock and Goodrich-Blair (2012) and observed for virulence (short infection time, large number of offspring per host). To do this, an aqueous suspension of infective-juveniles (IJ) were distributed on a piece of filter paper (No. 1, 90 mm) in the inverted lid of a petri dish (90 mm diameter). Ten waxworm larvae were added to the lid and covered with the inverted petri bottom. Each dish was stored inside a plastic bag in the dark at room temperature (22-25 ˚C) to allow for host infection. Infected waxworm cadavers were removed after 3-5 d and placed in modified white traps, where IJs could then be isolated. Nematode isolates were sent to Dr. Patricia Stock at the University of Arizona for identification using molecular techniques while populations were sustained *in vivo* at Utah State University on *G. mellonella*.

Conidia from fungal-infected CRC were transferred to potato dextrose agar with a sterile loop and were allowed to proliferate at room temperature (22-25 ˚C). Fungal growth was checked every two days until sporulation occurred. Fungi were identified with assistance from Dr. Don Roberts at Utah State University.

Data Processing

To calculate infection rates across sites for fungal infections of CRC and nematode infections of *G. mellonella,* the number of infected hosts from each site was divided by the total number of individuals collected or exposed from each site, respectively. Overall infection rates were calculated by dividing the sum of infected hosts from all sites by the total number of hosts over all sites.

Results

Topsoil content of sampled fields was generally silty with low sand and medium clay content (Table 2-1). However, fields varied in time since last cutting (<1 wk to 3 wks) and time since last irrigation event (1 wk to >3 wks) (Table 2-1). The soil moisture content (% volumetric water content) ranged across all field sites, but no field had a volumetric water content of greater than 10% (Table 2-2). Site #1 was the only field that had not been irrigated in over three weeks, and as a result, had the lowest volumetric soil water content (Table 2-2). All soil core samples exhibited root damage associated with CRC larval feeding, and CRC immatures were collected at all sites. A total of 82 CRC immatures (62 larvae, 18 pupae) were collected over all sites and a total of 591 *G. mellonella* were subjected to field soil during this survey (Table 2-2).

Entomopathogenic fungi (all identified as *Beauveria* spp.) were recovered from CRC immatures in two of the six fields sampled, with an overall infection rate of 4.7% (Table 2-2; Fig. 2-1A). None of the collected CRC were infected with entomopathogenic nematodes, however 11.2% of sentinel waxworm hosts had nematode emergence (Table 2-2; Fig. 2-1B). Nematode samples sent to the University of Arizona were identified as free-living, non-entomopathogenic rhabditids.

Discussion

Although found in low numbers, the entomopathogenic fungus, *Beauveria* spp., appears to be the most common entomopathogen found infecting CRC larvae and pupae in northern Utah. Previous studies on CRC infection by *B. bassiana* have focused on adults, and report mortality ranging from 0 to 23% (Aeschlimann, 1980; Crow et al., 1968; Quinn & Hower, 1985; Rockwood, 1951). However, entomopathogenic fungi, specifically *Beauveria* spp., have been consistently reported as the primary natural enemy of *Sitona* immatures (Aeschlimann, 1980; Jackson, 1922; Kilpatrick, 1961), although natural infection rates were often low and variable. For instance, in Italy and France, *B. bassiana* infection caused 2.7% and 31.1% *Sitona* immature mortality, respectively (Aeschlimann, 1980). Further, in New Hampshire white clover (*Trifolium repens* L.), Kilpatrick (1961) reported a total 4.6% of field-collected *Sitona* immatures died from *B. bassiana* infection. This infection rate was similar to the overall infection rate reported in the current study.

Surveys for *Sitona* pathogens have also reported rare (<1%) sitonid mortality from *Metarhizium anisopliae* and *Aspergillus* spp. (Aeschlimann, 1980; Kilpatrick, 1961). Although these species were not isolated from CRC or *G. mellonella* hosts during this particular study, strains of *Metarhizium* (6 strains) and *Aspergillus* (1 strain) have been previously isolated by Dr. DW Roberts from Cache Co., UT soils (compared to 16 strains of *Beauveria*) (USDA-ARS, 2019). *Beauveria* and *Metarhizium* are widespread throughout North America and Europe; however, many abiotic factors and farm management techniques may influence presence and prevalence of naturally occurring fungi populations. For example, most entomopathogens are sensitive to disturbance events, but it has been suggested that *M. anisopliae* is more common in agricultural habitats with higher pesticide applications (reduced host availability) and frequent disturbance events (e.g., tilling) (Hummel et al., 2009), while *B. bassiana* is more common in natural or undisturbed habitats (e.g., forests and uncultivated land) (Bidochka, Kasperski, & Wild, 2002; Quesada-Moraga, Navas-Cortés, Maranhao, Ortiz-Urquiza, & Santiago-Álvarez, 2007; Tarasco, De Bievre, Papierok, Poliseno, & Triggiani, 1997). This suggests that *Beauveria* may be well supported in alfalfa fields due to perennial plantings (4-6 years) with little to no tillage and a high prevalence of insect hosts (e.g., CRC, alfalfa weevil). Additionally, multivariate analyses by Quesada-Moraga et al. (2007) found that predictive variables for *B. bassiana* occurrence included higher clay content (>10% clay), higher pH, higher altitude (≥ 400 m), and lower latitude (< 40°). Although we did not measure soil pH during this study, the measured soil and environmental conditions lie within the ranges for the *B. bassiana* predictive factors reported in Quesada-Moraga et al. (2007). However, to further understand these abiotic relationships in Utah and potentially isolate different genera of fungi, future studies should survey for CRC immatures over a broader period of time and environmental conditions (April-July), survey fields with different management types, and perform more complete analyses of soil samples (e.g., pH, organic matter).

Other surveys for entomopathogenic fungi in clover or alfalfa have similarly found *Beauveria* as the most prevalent fungal pathogen. For example, Steenberg et al. (1995) found most adult and immature predatory beetles in alfalfa naturally infected by *B. bassiana* and few infected by *M. anisopliae*. British surveys for adult *Sitona obsoletus* natural enemies in clover reported *B. bassiana* as the most dominant fungal pathogen (34 strains), while only one strain of *M. anisopliae* was collected (Goldson, McNeill, Gerard, et al., 2004). Multiple studies have isolated and subsequently tested *B. bassiana* found naturally infecting curculionid pests in alfalfa or clover for efficacy and virulence against these pests (e.g., Ellingboe et al., 1957; Quinn & Hower, 1985; Willoughby et al., 1998; Yucel et al., 2018). Results suggested that naturally-occurring strains caused > 50% mortality after one week and/or performed equally as well as commercial strains. To expand upon this research, isolated fungal strains should be tested against CRC at varying temperatures and conidial concentrations for their efficacy under various conditions.

Despite reported *Sitona* larval infections by *Hexamermis* sp. in Europe (Aeschlimann, 1980), we did not observe natural infection by this group in northern Utah alfalfa fields during our survey. Research has shown that nematode movement, activity, and survival decreases as soil clay content increases (Georgis & Poinar, 1983; Molyneux & Bedding, 1984) and as moisture decreases (Kung, Gaugler, & Kaya, 1991). We posit the small pore spaces and poor oxygenation in clay and silty clay loam soils collected in this study interfered with host finding and successful EPN isolation. Additionally, EPN survival and pathogenicity was typically reduced as soil moisture decreases to the levels recorded in this survey (Kung et al., 1991; Molyneux & Bedding, 1984), and at moistures less than 4%, EPNs may enter a state of inactivity (anhydrobiosis) to reduce desiccation (Crowe & Madin, 1975), thus, they may not be detected with the baiting method at these low moistures. Interestingly, an endemic population of *H. bacteriophora* was previously isolated from Brigham City (Box Elder Co., UT) soil infested with plum curculio (*Contrachelus nenuphar* (Herbst)) (Alston et al., 2005; Kim, 2007; Kim & Alston, 2008). This result was likely because the soils in Brigham City, UT associated with fruit tree hosts were sandy loam (>50% sand, 6-10% clay) with moistures ranging from 31-70% (Kim, 2007). These represent optimal conditions for nematode establishment and persistence (Kaya & Gaugler, 1993). Further surveys for EPN strains endemic to northern Utah should focus on soils with similar conditions.

There may also be other pathogens not found in this study contributing to natural CRC population control, and future surveys for potential biological control agents of CRC should search for other pathogens such as bacteria or viruses in addition to fungi and nematodes. Improved methods that may also enhance CRC pathogen isolation include direct extraction of pathogens from field soil (e.g., Saunders & All, 1982), and sampling seasonally throughout the duration of CRC immature peaks (late April-June). We posit that the locally occurring *Beauveria* sp. strain isolated during this study may potentially be augmentatively applied in CRC management programs. For example, a few locally-adapted strains of *B. bassiana* found infecting *Sitona obsoletus* in Europe are being formulated into a granular fungal biopesticide to manage newly invasive (<10 years) *S. obsoletus* populations in New Zealand (Goldson, McNeill, Proffitt, et al., 2004; Nelson, McNeill, Van Koten, & Goldson, 2015; Willoughby et al., 1998). Similar formulations may be efficacious against CRC in Utah, yet future studies need to focus on laboratory and field testing for pathogenicity and virulence towards CRC.

Acknowledgments

Drs. Donald Roberts and Patricia Stock provided valuable advice and assistance throughout this survey. Special thanks to Steven Price for collecting soil core samples. We are particularly thankful for the assistance and support given by Morgan Christman, Desireé Wickwar, and Gunbharpur Gill both personally and professionally throughout this study. Advice and corrections to earlier versions of this manuscript given by Dr. Julian Golec were greatly appreciated.

References

Aeschlimann, J. P. (1980). The *Sitona* [Col.: Curculionidae] species occuring on *Medicago* and their natural enemies in the Mediterranean region. *Entomophaga*, *25*(2), 139–153. https://doi.org/https://doi.org/10.1007/BF02374316

Alston, D. G., Rangel, D. E. N., Lacey, L. A., Golez, H. G., Jeong, J. K., & Roberts, D. W. (2005). Evaluation of novel fungal and nematode isolates for control of *Conotrachelus nenuphar* (Coleoptera: Curculionidae) larvae. *Biological Control*, *35*(2), 163–171. https://doi.org/10.1016/j.biocontrol.2005.06.011

Bedding, R. A., & Akhurst, R. J. (1975). A simple technique for the detection of insect paristic rhabditid nematodes in soil. *Nematologica*, *21*(1), 109–110. https://doi.org/10.1163/187529275X00419

Bidochka, M. J., Kasperski, J. E., & Wild, G. A. M. (2002). Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Canadian Journal of Botany*, *76*(7), 1198–1204. https://doi.org/10.1139/cjb-76-7-1198

Bigger, J. H. (1930). Notes on the life history of the clover root curculio, *Sitona hispidula* Fab., in central Illinois. *Journal of Economic Entomology*, *23*(2), 334–342. https://doi.org/https://doi.org/10.1093/jee/23.2.334

Bright, D. E. (1994). Revision of the genus *Sitona* (Coleoptera : Curculionidae) of North America. *Annals of the Entomological Society of America*, *87*(3), 277–306. https://doi.org/https://doi.org/10.1093/aesa/87.3.277

Cornell University Cooperative Extension. (2007). *Management of Alfalfa Snout Beetle*. Retrieved from www.nnyagdev.org/wpcontent/uploads/2012/01/Shields_ASBPamphlet_FINAL.pdf

Crow, W. R., Puttler, B., & Daugherty, D. M. (1968). *Beauveria bassiana* infecting adult clover root curculios in Missouri. *Journal of Economic Entomology*, *61*(2), 576–577. https://doi.org/https://doi.org/10.1093/jee/61.2.576

Crowe, J. H., & Madin, K. A. C. (1975). Anhydrobiosis in nematodes: Evaporative water loss and survival. *Journal of Experimental Zoology*, *193*(3), 323–333.

Ellingboe, A. H., Kernkamp, M. F., & Haws, B. A. (1957). Sweetclover weevil parasitized by *Beauveria bassiana* (Bals.) Vuill. in Minnesota. *Journal of Economic Entomology*, *50*(2), 173–174. https://doi.org/10.1142/9789814494922_0048

EPA. (2009). Carbofuran cancellation process. Retrieved July 3, 2018, from https://archive.epa.gov/pesticides/reregistration/web/html/carbofuran_noic.html

Gaugler, R., & Boush, G. M. (1978). Effects of ultraviolet radiation and sunlight on the entomogenous nematode, *Neoaplectana carpocapsae*. *Journal of Invertebrate Pathology*, *32*(3), 291–296. https://doi.org/10.1016/0022-2011(78)90191-X

Georgis, R., & Poinar, G. O. (1983). Effect of soil texture on the distribution and infectivity of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae). *Journal of Nematology*, *15*(2), 308–311.

Godfrey, L. D., Yeargan, K. V., & Muntifering, R. B. (1987). Digestibility, protein content, and nutrient yields of alfalfa stressed by selected early season insect pests and diseases. *Journal of Economic Entomology*, *80*(1), 257–262. https://doi.org/https://doi.org/10.1093/jee/80.1.257

Godfrey, L. D., & Yeargan, K. V. (1987). Effects and interactions of early season pests on alfalfa yield in Kentucky. *Journal of Economic Entomology*, *80*(1), 248–256.

https://doi.org/https://doi.org/10.1093/jee/80.1.248

Goldson, S. L., McNeill, M. R., Gerard, P. J., Proffitt, J. R., Phillips, C. B., Cane, R. P., & Murray, P. J. (2004). British-based search for natural enemies of the clover root weevil, *Sitona lepidus* in Europe. *New Zealand Journal of Zoology*, *31*(3), 233–240. https://doi.org/10.1080/03014223.2004.9518375

Gotlieb, A., Pellett, N., & Parker, B. (1987). *Sitona*/*Fusarium*/Cold hardiness interaction in Alfalfa. *Phytopathology*, *77*, 1615.

Hazir, S., Kaya, H. K., Stock, S. P., & Keskin, N. (2004). Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests. *Turk J Biol*, *27*(2003), 181–202. Retrieved from http://dergipark.gov.tr/tbtkbiology/issue/11733/140060

Hummel, R. L., Walgenbach, J. F., Barbercheck, M. E., Kennedy, G. G., Hoyt, G. D., & Arellano, C. (2009). Effects of production practices on soil-borne entomopathogens in western North Carolina vegetable systems. *Environmental Entomology*, *31*(1), 84–91. https://doi.org/10.1603/0046-225x-31.1.84

Jackson, D. J. (1922). Bionomica of weevils of the genus *Sitona* injurious to leguminous crops in Britain. *Annals of Applied Biology*, *9*(2), 93–115. https://doi.org/10.1111/j.1744-7348.1922.tb05941.x

Jaworska, M., & Wiech, K. (1988). Susceptibility of the clover root weevil, *Sitona hispidulus* F. (Coleoptera, Curculionidae) to *Steinernema feltiae*, *Steinernema bibionis*, and *Heterorhabditis bacteriophora*. *Journal of Applied Entomology*, Vol. 106, pp. 372–376.

Kamionek, M. (1974). The survival of invasive larvae of *Neoaplectana carpocapsae*

Weiser in a waterless environment under various conditions of temperature and humidity. *Zeszyty Problemowe Postępów Nauk Rolniczych*, *154*, 409–412.

Kaya, H. K., & Gaugler, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology*, *38*, 181–206.

Kilpatrick, R. A. (1961). Fungi associated with the larvae of *Sitona* spp. *Phytopathology*, *51*(9), 640–641.

Kim, H.-G. (2007). *Effects of environmental conditions and insect life stages on performance of entomopathogenic nematodes for suppression of plum curculio, Conotrachelus nenuphar (Coleoptera: Curculionidae), in northern Utah*. Utah State University.

Kim, H. G., & Alston, D. G. (2008). Potential of two entomopathogenic nematodes for suppression of plum curculio (*Conotrachelus nenuphar*, Coleoptera: Curculionidae) life stages in northern climates. *Environmental Entomology*, *37*(5), 1272–1279. https://doi.org/10.1603/0046-225X(2008)37

Kung, S.-P., Gaugler, R., & Kaya, H. K. (1991). Effects of soil temperature, moisture, and relative humidity on entomopathogenic nematode persistence. *Journal of Invertebrate Pathology*, *57*(2), 242–249. https://doi.org/10.1016/0022-2011(91)90123-8

Leath, K. T., & Hower, A. A. (1993). Interaction of *Fusarium oxysporum* f.sp. *medicaginis* with feeding activity of clover root curculio larvae in alfalfa. *Plant Disease*, *77*(8), 345–348.

Leibee, G. L., Pass, B. C., & Yeargan, K. V. (1980). Instar determination of clover root curculio, *Sitona hispidulus* (Coleoptera: Curculionidae). *Journal of the Kansas*

Entomological Society, *53*(3), 473–475.

Loya, L J, & Hower, A. A. (2002). Population dynamics, persistence, and efficacy of the entomopathogenic nematode *Heterorhabditis bacteriophora* (Oswego strain) in association with the clover root curculio (Coleoptera : Curculionidae) in Pennsylvania. *Environmental Entomology*, *31*(6), 1240–1250. https://doi.org/10.1603/0046-225X-31.6.1240

Loya, Lane J., & Hower, A. A. (2003). Infectivity and reproductive potential of the Oswego strain of *Heterorhabditis bacteriophora* associated with life stages of the clover root curculio, *Sitona hispidulus*. *Journal of Invertebrate Pathology*, *83*(1), 63–72. https://doi.org/10.1016/S0022-2011(03)00044-2

Manglitz, G. R., Anderson, D. M., & Gorz, H. J. (1963). Observations on the larval feeding habits of two species of *Sitona* (Coleoptera: Curculionidae) in sweetclover fields. *Annals of the Entomological Society of America*, *56*(6), 831–835. https://doi.org/10.1093/aesa/56.6.831

Marshall, G. E., & Wilbur, D. A. (1934). The clover root curculio (*Sitona hispidula* Fab.) in Kansas. *Journal of Economic Entomology*, *27*(4), 807–814. https://doi.org/https://doi.org/10.1093/jee/27.4.807

Molyneux, A. S. (1986). *Heterorhabditis* spp. and *Steinernema* (= *Neoaplectana*) spp.: Temperature, and aspects of behavior and infectivity. *Experimental Parasitology*, *62*, 169–180.

Molyneux, A. S., & Bedding, R. A. (1984). Influence of soil texture and moisture on the infectivity of *Heterorhabditis* sp. and *Steinernema glaseri* for larvae of the sheep blowfly, Lucilia cuprina. *Nematologica*, *30*(3), 358–365.

Nelson, T. L., McNeill, M. R., Van Koten, C., & Goldson, S. L. (2015). Comparative virulence of *Beauveria bassiana* isolates against clover root weevil (*Sitona obsoletus*). *New Zealand Plant Protection*, *68*, 139–145.

Neumann, G., & Shields, E. J. (2004). Survey for entomopathogenic nematodes and entomopathogenic fungi in alfalfa snout beetle, *Otiorhynchus ligustici* (L.) (Coleoptera: Curculionidae), infested fields in Hungary and in New York state. *The Great Lakes Entomologist*, *37*(3–4), 152–158.

Quesada-Moraga, E., Navas-Cortés, J. A., Maranhao, E. A. A., Ortiz-Urquiza, A., & Santiago-Álvarez, C. (2007). Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. *Mycological Research*, *111*(8), 947–966. https://doi.org/10.1016/j.mycres.2007.06.006

Quinn, M. A., & Hower, A. A. (1985). Isolation of *Beauveria bassiana* (Deuteromycotina Hyphomycetes) from alfalfa field soil and its effect on adult *Sitona hispidulus* (Coleoptera Curculionidae). *Environmental Entomology*, *14*, 620–623. https://doi.org/https://doi.org/10.1093/ee/14.5.620

Reitz, S. (2018). Alfalfa hay - Clover root curculio. In *Pacific Northwest Insect Management Handbook* (p. E7). Retrieved from https://pnwhandbooks.org/insect/hay-pasture/alfalfa/alfalfa-hay-clover-root-curculio

Rim, K., Price, S. J., & Ramirez, R. A. (2019a). Clover root curculio (Sitona hispidulus F.). In *Utah State University Extension*. Retrieved from https://digitalcommons.usu.edu/extension_curall/1949

Rim, K., Price, S. J., Wenninger, E. J., Long, R., & Ramirez, R. A. (2019b). Biology and management of clover root curculio (Coleoptera: Curculionidae). *Journal of*

Integrated Pest Management, 10(1), 1–14. https://doi.org/10.1093/jipm/pmz020

Rockwood, L. P. (1951). *Some Hyphomycetous Fungi Found on Insects in the Pacific Northwest*. *44*(2), 215–217.

Saunders, M. C., & All, J. N. (1982). Laboratory extraction methods and field detection of entomophilic rhabditoid nematodes from soil. *Environmental Entomology, 11*(6), 1164–1165. https://doi.org/10.1093/ee/11.6.1164

Shapiro-Ilan, D. I., Gouge, D. H., Piggott, S. J., & Fife, J. P. (2006). Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biological Control*, *38*(1), 124–133. https://doi.org/10.1016/j.biocontrol.2005.09.005

Shields, E. J., Testa, A., Neumann, G., Flanders, K. L., & Schroeder, P. C. (2009). Biological control of alfalfa snout beetle with a multi-species application of locally adapted persistent entomopathogenic nematodes: The first success. *American Entomologist*, *55*(4), 250–257.

Steenberg, T., Langer, V., & Esbjerg, P. (1995). Entomopathogenic fungi in predatory beetles (Col.: Carabidae and Staphylinidae) from agricultural fields. *Entomophaga*, *40*(1), 77–85. https://doi.org/10.1007/BF02372683

Stock, S. P., & Goodrich-Blair, H. (2012). Nematode parasites, pathogens and associates of insects and invertebrates of economic importance. In L. A. Lacey (Ed.), *Manual of Techniques in Invertebrate Pathology* (Second, pp. 373–426). https://doi.org/10.1016/B978-0-12-386899-2.00012-9

Tarasco, E., De Bievre, C., Papierok, B., Poliseno, M., & Triggiani, O. (1997). Occurrence of entomopathogenic fungi in soils in southern Italy. *Entomologica, 15*, 157–166.

Tietz, N. (2012, March). Mysterious alfalfa menace: Clover root curculio sneak-attacks Western fields. *Hay and Forage Grower*, 14–15.

USDA-ARS. (2019). ARS collection of entomopathogenic fungal cultures database: Utah. Retrieved from http://arsef.fpsnl.cornell.edu/4DACTION/W_List_Tables/param

Webster, F. M. (1915). Alfalfa attacked by the clover-root curculio. In *USDA Farm Bulletin* (649th ed.). Bureau of Entomology.

Wenninger, E. J., & Shewmaker, G. E. (2014). Clover root curculio in alfalfa: Identification, biology, and management. *Pacific Northwest Extension*, *PNW 663*, 1–5. Retrieved from www.extension.uidaho.edu/forage/Fact Sheets/clover root curculio.pdf

White, G. F. (1927). A method for obtaining infective nematode larvae from cultures. *Science*, *66*(1709), 302–303. Retrieved from http://www.jstor.org/stable/1651653

Wiech, K., & Jaworska, M. (1990). Susceptibility of *Sitona* weevils (Col., Curculionidae) to entomogenous nematodes. *Journal of Applied Entomology*, *110*, 214–216.

Wildermuth, V. L. (1910). The clover-root curculio. In *USDA Bureau of Entomology* (85th ed.).

Willoughby, B., Glare, T., Kettlewell, F., & Nelson, T. (1998). *Beauveria Bassiana* as a potential biocontrol agent against the clover root weevil, *Sitona lepidus*. *Proceedings of the NZ Plant Protection Conference*, 9–15.

Yucel, B., Gencer, D., Demir, I., Gozuacik, C., & Demirbag, Z. (2018). Determination of fungal pathogens of *Hypera postica* (Gyllenhall) (Coleoptera: Curculionidae):

isolation, characterization, and susceptibility. *Egyptian Journal of Biological Pest Control*, *28*(1). https://doi.org/10.1186/s41938-018-0043-2

Tables and Figures

Table 2-1: Field collection site location and characteristic information. NA = field information not available

Site Number	Location	Coordinates	Field Age (yrs)	Topsoil Composition[1]	Last cutting prior to collection	Last irrigation event prior to collection
1	Trenton, UT	41.880803, -111.949908	4	Fine silty loam (22.5% clay, 9.5% sand)	< 1 wk	> 3 wk
2	Richmond, UT	41.890729, -111.840814	5	Silty clay loam (32.5% clay, 19.7% sand)	2 wk	2 wk
3	Richmond, UT	41.883600, -111.828262	3	Fine silty loam (12.5% clay, 30.9% sand)	2.5 wk	2.5 wk
4	Wellsville, UT	41.662046, -111.919956	4	Fine silty (22.5% clay, 33.3% sand)	3 wk	1 wk
5	College Ward, UT	41.659281, -111.898328	NA	Fine silty (22.5% clay, 33.3% sand)	3 wk	2 wk
6	College Ward, UT	41.652032, -111.901998	6	Fine silty (22.5% clay, 33.3% sand)	3 wk	1 wk

[1] Soil information obtained from SoilWeb (2018).

Table 2-2: Average soil moisture (± SEM) of soil cores, immature CRC (larvae and pupae) abundance, and fungal infection rate across and over all sites. Total number of waxworms exposed to collected soil and nematode infection rate across and over all sites.

		CRC			**Sentinel Waxworms**		
Site Number	**Average soil moisture (VWC* %)**	**Total immatures (n)**	***Beauveria* spp. Infected (n)**	**Infection rate (%)**	**Hosts exposed (n)**	**Nematode infected (n)**	**Infection rate (%)**
1	5.85 ± 0.86	1	0	0	66	0	0
2	6.66 ± 0.23	19	0	0	96	1	1.0
3	6.66 ± 0.83	26	1	3.8	141	14	9.9
4	8.86 ± 0.76	11	0	0	96	13	13.5
5	6.40 ± 0.92	19	0	0	96	12	12.5
6	9.69 ± 0.93	10	3	30.0	96	13	13.5
		Overall CRC infection rate:		**4.7**	**Overall waxworm infection rate:**		**11.15**

***** Volumetric Soil Water Content = VWC

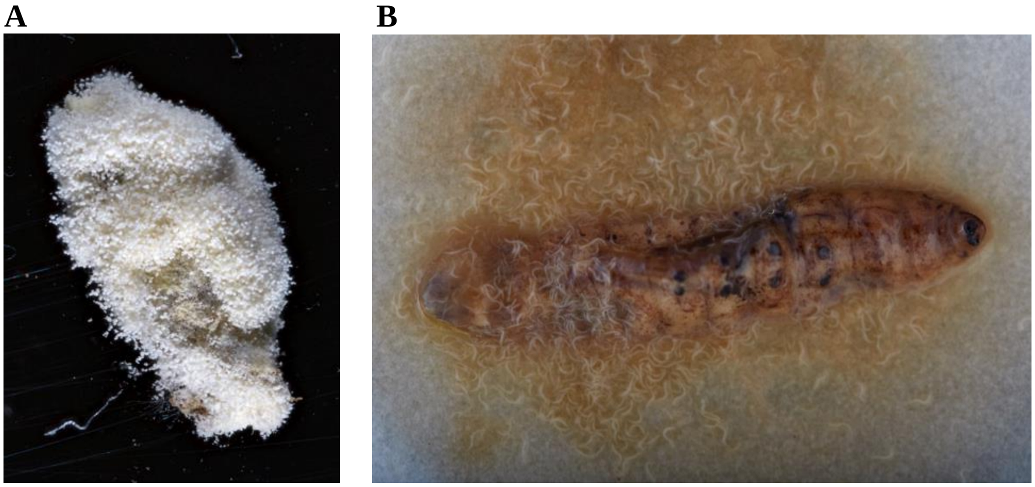

Figure 2-1: (A) Clover root curculio pupa with *Beauveria* sp. fungal growth, and (B) *Galleria mellonella* larva with rhabditid nematodes.

CHAPTER III

EVALUATION OF CHEMICAL AND BIOLOGICAL INSECTICIDES FOR MANAGEMENT OF THE CLOVER ROOT CURCULIO IN INTERMOUNTAIN WEST ALFALFA (*Medicago sativa)*

Abstract

Clover root curculio (CRC[1]) (*Sitona hispidulus* F.) larvae feeding belowground on alfalfa (*Medicago sativa* L.) roots negatively impacts plant and stand health and may lead to economic losses. Contemporary research on management of CRC larvae is limited, and increased damage resulting from the cancellation of broad-spectrum insecticides substantiates the need for evaluating modern formulations of biological and systemic insecticides. Further, without economic thresholds and predictive degree-day models, it is difficult to determine insecticide application times. However, recent research has revealed seasonal timing of the larval peak in the Intermountain West. Therefore, we tested applications of *Bacillus thuringiensis* spp. *galleriae,* the entomopathogenic nematode *Steinernema riobrave,* the entomopathogenic fungus *Beauveria bassiana*, and a systemic insecticide, flupyradifurone, in a randomized complete block design for efficacy against resident CRC immatures across two alfalfa field seasons (2018-2019) in Kimberly, ID. Treatments were applied at two phenological times, before larval peak or during larval peak. Soil core samples were taken 4-wk post-application to determine the number of CRC pupae and larvae, larval head capsule width as a measure of development, and plant damage. We found that biological and systemic insecticides did

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[1] **Abbreviations:** Clover root curculio = CRC, entomopathogenic nematode = EPN, infective juvenille = IJ

not reduce resident CRC populations or affect larval development compared to untreated controls. Root damage was also similar across control and insecticide treatments. Further, application timing did not improve insecticide efficacy. Given the diverse insecticide types evaluated, differing mechanisms may exist for their lack of success against CRC. For instance, possible reduced penetration of *Bacillus* in soil strata, in part from prohibitive soil characteristics reducing exposure and necessary ingestion to be effective; and low flupyradifurone translocation to the root zone. The challenges for successful use of biological insecticides in field settings is well documented. Yet, it is also important to note the difficulties in evaluating CRC suppression, in part, because of the variation in resident populations and their cryptic nature.

Keywords: *Sitona hispidulus,* entomopathogens, *Steinernema riobrave*, *Beauveria bassiana*, *Bacillus thuringiensis* spp. *galleriae*, flupyradifurone

1. Introduction

Incidences of clover root curculio (CRC) (*Sitona hispidulus* F.) damage and presence in forage production were first recorded in New Jersey, United States in 1875 (Wildermuth, 1910). Due to a research focus on major above ground alfalfa pests (e.g., alfalfa weevil, armyworm, and aphids) and the non-target control of CRC through broad-spectrum insecticide applications targeting these aboveground pests (e.g., Hower et al., 1995), CRC remained a minor pest throughout the 1900s. Coincident with the federal ban on carbofuran (EPA, 2009), CRC has become a major pest throughout the U.S. Typically, adults feed inconsequentially on alfalfa (*Medicago* sativa L.) and clover (*Trifolium* spp.) leaves while the larval stage causes significant damage to host root systems (Rim et al., 2019b). Destructive root damage caused by larvae is associated with reduced stand establishment (Godfrey et al., 1986), disruption of nutrient and water uptake (Jewett, 1934; Tan and Hower, 1991), increased secondary plant pathogen infection (Graham and Newton, 1960, 1959), decreased winter plant survival (Underhill et al., 1955), and reduced forage quality and yield (Godfrey and Yeargan, 1989). Current integrated pest management programs are focused on cultural control through crop rotation, irrigation and nutrient management programs, and altered planting dates (Rim et al., 2019a, 2019b; Wenninger and Shewmaker, 2014). Moreover, chemical control of CRC is difficult as economic thresholds have not been determined, susceptible life stages are associated with cryptic habitats, there are no registered insecticides for larvae, and applications of registered chemistries against adults are not recommended (Rim et al., 2019a; Wenninger and Shewmaker, 2014).

Historically, applications of persistent insecticides with soil activity to target alfalfa weevil (*Hypera postica* (Gylenhall)) may have suppressed CRC populations (Hower et al., 1995). However, insecticide trials targeting CRC specifically yielded variable results, and even when larval populations were reduced, yield and stand longevity were often not improved (Dickason et al., 1968; Dunn et al., 1964; James et al., 1980; Neal and Ratcliffe, 1975; Phillips and Ditman, 1962). Further, chlorinated hydrocarbon applications yielded inconsistent results in their efficacy against CRC depending on field characteristics, CRC phenology, and application timing (Dickason et al., 1958; Underhill et al., 1955). Regardless, most of the insecticides tested in these studies (e.g., chlorinated hydrocarbons, carbamates, organophosphates) have since been cancelled by the Environmental Protection Agency (National Pesticide Information Center, 2015). Moreover, different regions of the U.S. may differ in CRC phenology, and some areas with mild winters may have increased larval overwintering (Quinn and Hower, 1985a). Timing applications to target larvae are difficult due to these phenological discrepancies and the lack of predictive degree-day models. However, seasonal data exists for CRC populations in northern Utah and can inform timing of applications targeting susceptible life stages (Price, 2017). Due to the cancellation of insecticides, the paucity of recent literature on CRC, and the historical focus on the eastern U.S. populations, contemporary research into management practices, especially in the western U.S., is crucial. Therefore, a renewed evaluation of chemical and biological insecticides for CRC larval suppression is needed.

Although CRC larvae are the most susceptible and most damaging stage, targeting this belowground stage can be challenging. However, systemic insecticides play an important role in integrated pest management programs for soil pests (e.g., wireworms, root worms) and a variety of other crop pests. For instance, imidacloprid and flupyradifurone seed treatments for soybean demonstrated that these insecticides can be translocated to root material (Stamm et al., 2016). Although neonicotinoid field pea seed-treatments did not reduce *Sitona lineatus* L. (Coleoptera: Curculionidae) larval numbers and nodule damage, overall root vigor and plant health improved through increased nodulation, nodule size, and N-fixing ability (Seidenglanz et al., 2010; Vankosky et al., 2011). In addition to seed treatments, soil or foliar applied systemic insecticides can translocate from non-target tissues to target tissues rapidly. For instance, soil applications of dinotefuran and flupyradifurone reduce aboveground pest densities and leaf damage in two weeks or less (Palumbo, 2012; Stansly and Kostyk, 2016). Neonicotinoid sprays significantly reduce belowground tuber damage from wireworms (Elateridae) in potato (Kuhar and Alvarez, 2008), and are efficacious preventative treatments for managing other cryptic pests such as early-instar billbug larvae feeding in turf stems (Reynolds and Brandenburg, 2015). However, neonicotinoids have come under scrutiny for their purported negative environmental and ecological effects (e.g., Goulson, 2013; Hallmann et al., 2014; Woodcock et al., 2017). Further, no neonicotinoids are registered as foliar sprays in alfalfa, and seed treatments to target insect pests, are not common practice (Bachmann et al., 2019). Therefore, other potential systemics, namely flupyradifurone, which is considered a reduced risk pesticide (EPA, 2018) and currently registered for foliar application against hemipteran alfalfa pests (Bachmann et al., 2019), may benefit CRC management.

Likewise, integrated pest management of other cryptic belowground pests like CRC have found success using biological insecticides such as entomopathogenic bacteria, nematodes, and fungi (e.g., Glare et al., 2004; Hazir et al., 2004; Shields and Testa, 2017). In addition, if shown to be effective reduced-risk products, biological insecticides can be easier to register and can be applied with similar equipment to synthetic products (Shapiro-Ilan et al., 2006). *Bacillus thuringiensis* (*Bt*), for example, has increased in importance since genes from its endotoxins are components of some transgenic crops (Lacey et al., 2015). In alfalfa, nodules colonized by symbiotic *Rhizobium* bacteria engineered with *Bacillus thuringiensis tenebrionis* Berliner *cry*III endotoxin genes had a 26% reduction in damage from early-instar CRC and *S. lineatus* L. (Bezdicek et al., 1994). However, the use of genetically engineered alfalfa has been difficult to adopt due to the potential for cross-pollination and gene transfer to non-engineered alfalfa or native congeners (Amand and Peaden, 2000; Jenczewski et al., 1999). While *Bt* is not a standard application in forage, applications of *Bt* to crops in other systems have been successful. For example, granular formulations of *Bt galleriae* reduced the number of immature rice water weevil (*Lissorhoptrus oryzophilus* Kushel), a weevil that damages rice roots, stunting growth and reducing yield in California (Aghaee and Godfrey, 2015). Therefore, *Bt* applications may provide some management for CRC immatures.

Heterorhabditis spp. and *Steinernema* spp. nematodes, have offered success against weevil pests such as black vine weevil (*Otiorhynchus sulcatus* (F.)) (Bedding and Miller, 1981), root weevils on citrus (*Pachnaeus litus* (Germar) and *Diaprepes abreviatus* (L.)) (Bullock et al., 1999: McCoy et al., 2000), plum curculio (*Conotrachalus nenuphar*

Herbst) (Kim and Alston, 2008), and the pea weevil (*S. lineatus* L.) (Jaworska, 1998) that can cause considerable damage to crop root systems. The use of locally-adapted entomopathogenic nematodes (EPNs) has provided persistent and effective long-term control of the alfalfa snout beetle (*Otiorhynchus ligustici* (L.)), another belowground weevil pest of alfalfa (Cornell University Cooperative Extension, 2007; Neumann and Shields, 2004; Shields et al., 2009). Further, field research in Pennsylvania alfalfa demonstrated a locally adapted EPN strain, *H. bacteriophora* (Oswego strain), lead to significant reductions in CRC adult emergence and root damage three years after EPN application (Loya and Hower, 2002). However, the most prevalent natural enemy of CRC larvae and adults is an entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin (Aeschlimann, 1980; Crow et al., 1968; Jackson, 1922; Kilpatrick, 1961; Wildermuth, 1910). Typically, *B. bassiana* natural infection rates in the field are low (2-5% infected) (e.g., Aeschlimann, 1980; Kilpatrick, 1961), and infectivity varies between fungal strain and *Sitona* spp. stage (e.g., Poprawski et al., 1985). Though, studies have investigated CRC susceptibility to *B. bassiana* in the laboratory (e.g., Quinn and Hower, 1985), few studies have investigated CRC biological control through direct applications of *B. bassiana* in the field.

There are some challenges associated with the use of biological insecticides in the field. Some agents are sensitive to environmental factors such as soil texture and moisture, pH, temperature and ultraviolet radiation, and interspecific competition (e.g., Gaugler and Boush, 1978; Kung et al., 1991, 1990). As a result, application methods may be more complex or may need to be adjusted to increase efficacy and prevent degradation of microbial agents in the field. Efficacy can also vary between species and concentrations may need to be higher in field settings to overcome the negative effects of biotic and abiotic factors or to target pests that may evade infection by tunneling into roots (Georgis, 1990). Further, the semi-arid conditions in the Intermountain West may require applications of species that can succeed under drought conditions. For instance, the nematode *Steinernema riobrave* is known to have higher temperature thresholds and be desiccation-tolerant (Somvanshi et al., 2008; Yaari et al., 2016). These along with an increased specificity towards beetles and weevils (e.g. Bullock et al. 1999; Cabanillas 2003; Shapiro-Ilan et al. 2004) makes *S. riobrave* a promising biological agent for potential CRC larval management in the Intermountain West. When microbial agents selected for their specificity to certain host groups and environmental range are applied adhering to label instructions and accounting for potential pitfalls, they may provide effective and persistent belowground pest suppression (e.g., Shields et al., 2009).

Chemical insecticides registered for CRC adult management are not economically feasible due in part to non-target effects, and there are no registered insecticides for management of insects belowground in alfalfa (Rinehold, 2017; Wenninger and Shewmaker, 2014). Therefore, this study aims to evaluate the efficacy of three microbial agents – *Bt galleriae*, *S. riobrave*, and *B. bassiana* – as well as a systemic chemical insecticide (flupyradifurone) for CRC larval management. Further, we investigated the impact of application timing based on larval phenological peaks on insecticide efficacy in 2018. This study assessed the utility of microbial, biological, and chemical control programs applied at different time points during CRC larval phenology for larval suppression and reduction of taproot damage in commercial alfalfa fields.

2. Materials and Methods

2.1 Field Characteristics and Experimental Design

Two field experiments were conducted at University of Idaho's Kimberly Research and Extension Center (Kimberly, ID), one each in 2018 and 2019 field seasons. In 2018, the experiment was applied to a 4-year-old alfalfa stand established in 2014. The native soil in this field was silty clay loam (Table 3-S1). Each of six replicated blocks (randomized complete block design (RCBD)) consisted of eight 2.9 × 3.0 m plots with a 3.0 m buffer between plots (experimental unit). Plots were randomly assigned a treatment time combined with one of three treatments or an untreated control (2 application times × 4 treatments = 8 plots/block, N = 48). Treatments included the biological insecticides *S. riobrave* (Sierra Biological Inc., Lyndonville, NY) and *Bt-galleriae* (grubGONE!, Phyllom Bioproducts, Oakland, CA), and the systemic insecticide, flupyradifurone (Sivanto™, Bayer, Leverkusen, Germany). The two treatment application times were determined from northern Utah CRC phenology data (Price, 2017; Rim et al., 2019b) with the first application applied to early-instars before the larval peak at the start of the season (April 25, 2018), and the second applied later to target late-instars during CRC larval peak (June 21, 2018) (Table 3-S2).

In 2019, a 6-year-old field (established in 2013) was selected due to its higher CRC densities than the field used in 2018. The native soil in this field was silt loam (Table 3-S1). Experimental units were the same as previously described. However, in 2019, there were five treatments consisting of the biological insecticides *S. riobrave*, *Bt-galleriae*, and *B. bassiana* (BotaniGard® ES, Laverlam International Corp., Butte, MT), the systemic insecticide flupyradifurone, and an untreated control. Similarly, each of the five treatments were established within a RCBD replicated 5 times (n = 25). Given irrigation needs for treatments were not accessible for an early application, a single application on May 24, 2019 aimed to target CRC larvae during larval peak similar to the second application in 2018 (Table 3-S2).

2.2 Insecticide application details

Guided by application recommendations for EPN applications (Georgis, 1990; Shapiro-Ilan et al., 2006), treatments were applied in the morning (08:00-09:00) using a single wheel push type sprayer (Bellspray, Inc. R&D Sprayers) with a 2.29 m boom with 46 cm nozzle spacing. Treatments were applied at the following concentrations: *S. riobrave* concentration of 2.5×10^9 infective juveniles (IJs)/ha (Georgis, 1990; Shapiro-Ilan et al., 2006), a granular formulation of *Bt-galleriae* at the label rate of 112 kg/ha (Hubble-Wirgler and Sutherland, 2017; Redmond et al., 2019), *B. bassiana* at the label rate of 4.9×10^{13} viable spores/ha, and flupyradifurone at the label rate of 205 g ai/ha. The sprayer was equipped with high capacity cone nozzles (TeeJet TXA8001VK Hollow Cone Tip, Ceramic) operated at 241 kPa for *S. riobrave*, *B. bassiana*, and chemical insecticide application (Georgis, 1990; Shapiro-Ilan et al., 2006). Screens were removed from the sprayer for EPN treatments to reduce nematode mortality (Hazir et al., 2004). The label rate of *Bt-galleriae* was hand-applied to each plot by shaking granules through the punctured lid of a glass jar. Fields were overhead irrigated 1.27 cm after each application event in both 2018 and 2019 to ensure adequate biological survival based on the label and previous research (Georgis, 1990; Shapiro-Ilan et al., 2006). However, for the first application timing in 2018 (April 25), *S. riobrave* and *Bt galleriae* plots were hand irrigated post-application with spray equipment (11.4 L H_2O/plot) given that overhead irrigation was not accessible.

2.3 Data collection

2.3.1 Effect of Treatments on CRC Immatures

At approximately 4-weeks post-application, CRC larval and pupal populations in each plot were measured by haphazardly taking four soil cores (subsamples approximately 10 cm diam. × 25 cm depth) using a drain spade. Using modified methods from Lau and Filmer (1959) and Leibee et al. (1980), soil cores were processed by washing samples through a series of sieves (U.S. standard set #5, #10, #35, #60) separating CRC immatures from soil particulate. Head capsule width of each larva was measured with an ocular micrometer under a stereomicroscope (Leica MZ6, Leica Microsystems, Wetzlar, Germany) to evaluate insecticide affects by instar.

2.3.2 Effect of Treatments on Alfalfa Taproot Damage

Roots from each subsample in 2018 were reserved to quantify CRC-associated feeding damage using methods from Price (2017). First, lateral roots were removed from the taproot, and the taproot was then sliced longitudinally so the inner cortex could be removed which aids in the root sample lying flat for root scarring calculations. To calculate the percent of damaged area, the flattened root was scanned (Canon CanoScan LiDE 60) with a metric ruler for scale. Within season CRC root scars (≤ 1 year old damage) were digitally traced using Photoshop. New damage was determined by the damaged area being lighter in coloration when compared to the outer cortex, a distinct ridge or edge as the feeding boundary, or the presence of loose fibers from fresh feeding in the damaged area. Root scarring area was subsequently analyzed using ImageJ software (ImageJ 1.49f; http://rsbweb.nih.gov/ij/). Root damage data from 2019 was not quantified because new damage was difficult to quantify from the prior 6 years of accumulative CRC larval damage (61.0 ± 15.2 % existing root damage).

2.3.3 *Steinernema* spp. Persistence in Soil

To verify successful nematode applications, nematode presence was confirmed by subjecting greater wax moth (*Galleria mellonella* L.) larvae to four soil samples taken (depth of 10 cm, 4 subsamples per plot) haphazardly from both nematode and control treatments at 1, 4, and 8 weeks post-application using the insect baiting technique described by Bedding and Akhurst (1975). Briefly, soil was cleared of large debris such as plant material or stones, moistened with deionized water, and placed in a clean plastic container (500 mL, 11.5 cm diameter × 7.5 cm height). Five greater wax moth larvae were placed on the surface of the soil, and the container was inverted after being covered with a lid. Dead sentinel hosts (*G. mellonella*) were removed after 48 h and observed for signs of EPN infection. Insects infected with *Steinernema* spp. adopted a grey coloration characteristic of the symbiotic bacteria *Xenorhabdus* associated with this genus of EPNs (Boemare et al., 1996).

2.4 Data Analysis

Data from the 2018 and 2019 trials were analyzed separately using R statistical software (RStudio).

2.4.1 Effect of Treatments on CRC Immatures

In 2018, CRC immature counts (larvae and pupae combined) were analyzed within a two-way Analysis of Variance (ANOVA) with four levels of treatment (two biological insecticides, *S. riobrave*, *Bt-galleriae*; one systemic insecticide, flupyradifurone; and an untreated control) and two levels of application time (before larval peak and during larval peak). In 2019, a single application was applied therefore a one-way ANOVA was performed to compare CRC immature counts and larval head capsule widths for the 5 treatments that included *S. riobrave*, *Bt-galleriae*, *B. bassiana*, flupyradifurone, and control. When significant differences were detected via ANOVA, the post-hoc Tukey's HSD test was used to separate significantly different mean values.

2.4.2 Effect of Treatments on Alfalfa Taproot Damage

The proportion of root area consumed in 2018 was transformed ($\sin^{-1}\sqrt{\frac{area\ consumed\ (mm^2)}{Total\ root\ area\ (mm^2)}}$) for normality and homoscedasticity and was similarly analyzed by two-way ANOVA with block as a random effect. When significant differences were detected via ANOVA, the post-hoc Tukey's HSD test was used to separate significantly different mean values.

2.4.3 *Steinernema* spp. Persistence in Soil

For each of the three application times, two in 2018 and one in 2019, a repeated measures ANOVA was used to compare untreated control plots and *S. riobrave* treated plots at 1, 4, and 8 weeks post-application. When significant differences were detected via ANOVA, the post-hoc Tukey's HSD test was used to separate significantly different mean values.

3. Results

3.1 Effect of Treatments on CRC Immatures

Overall in 2018, there was no significant difference in the number of immature CRC (larvae + pupae) collected among treatments (F = 1.29, df = 3, 32, *P* = 0.295). Although there were 6-8 times more CRC immatures collected after the first application timing compared to CRC collected after the second application timing (F = 42.23, df = 1, 32, *P* <0.001) (Table 3-1), insecticide treatments did not improve CRC suppression for either timing (treatment × time interaction: F = 0.33, df = 3, 32, *P* = 0.801). Further, we found there were no significant differences in the effects of insecticides on CRC larval development (head capsule widths) compared to the control (F = 0.82, df = 3, 23, *P* =0.496) (Fig 3-1). Similarly, head capsule widths of larvae collected after the second application timing were 1.75 times larger than larvae collected after the first application timing (F = 82.51, df = 1, 23, *P* <0.001), but insecticide treatment efficacy was not impacted by increased larval size during the second application timing (treatment × time interaction: F = 0.69, df = 3, 23, *P* =0.570) (Fig 3-1).

For the 2019 season similarly, CRC was not significantly suppressed by any of the insecticide applications compared to the control (F = 1.65, df = 4, 16, *P* = 0.211) (Table 3-1). Further, larval head capsule widths did not significantly differ between insecticide treatments and the control (F = 2.82, df = 4, 15, *P* = 0.063) (Fig 3-2).

Table 3-1. Effect of spray treatments on *Sitona hispidulus* number of immatures in Kimberly, ID, for 2018 and 2019. Means (± SE) presented did not differ significantly (P = 0.05) among treatments; NT = not tested

	Number of Immatures *			Larval Head Capsule Width		
	2018		2019	2018		2019
Treatment	1st App.	2nd App.		1st App.	2nd App.	
Control	4.13 ± 1.01	0.54 ± 0.15	3.05 ± 0.69	0.46 ± 0.08	0.77 ± 0.04	0.63 ± 0.08
Flupyradifurone	4.25 ± 1.27	0.67 ± 0.33	5.30 ± 1.13	0.37 ± 0.02	0.78 ± 0.03	0.77 ± 0.01
Bt-galleriae	3.38 ± 0.59	0.50 ± 0.31	5.20 ± 0.87	0.42 ± 0.05	0.77 ± 0.03	0.80 ± 0.02
S. riobrave	2.75 ± 0.50	0.42 ±0.17	5.80 ± 1.01	0.44 ± 0.03	0.71 ± 0.06	0.76 ± 0.02
B. bassiana	NT	NT	4.90 ± 0.59	NT	NT	0.74 ± 0.02

* larvae and pupae combined

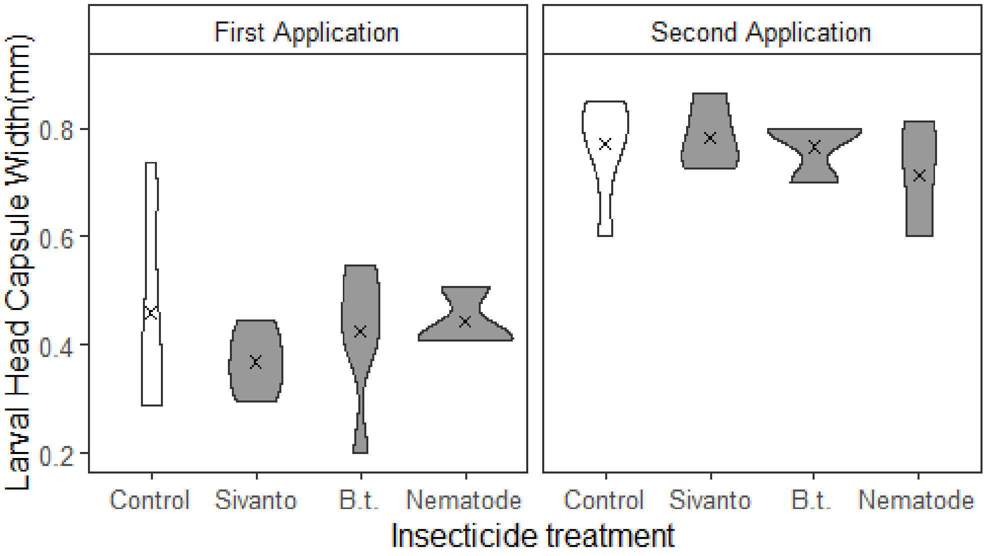

Figure 3-1. Larval head capsule widths for *Sitona hispidulus* larvae collected from control and insecticide treated plots in Kimberly, ID alfalfa for 2018. The width of the shaded area represents the proportion of data located there. Means (indicated by ×) presented did not differ significantly ($P = 0.05$) among treatments.

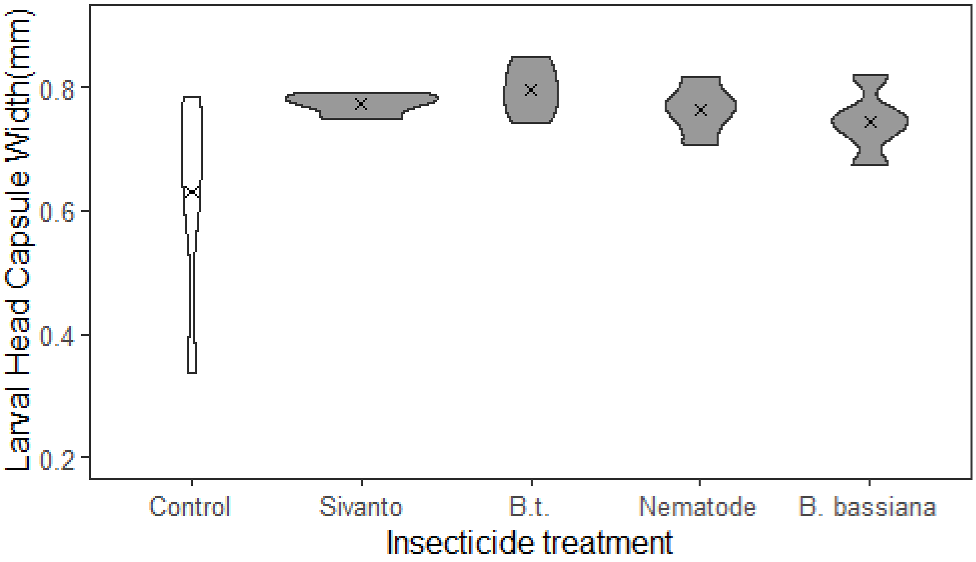

Figure 3-2. Larval head capsule widths for *Sitona hispidulus* larvae collected from control and insecticide treated plots in Kimberly, ID alfalfa for 2019. The width of the shaded area represents the proportion of data located there. Means (indicated by ×) presented did not differ significantly ($P = 0.05$) among treatments.

Table 3-2. Mean (±SE) proportion of alfalfa root area consumed by *Sitona hispidulus* larvae in Kimberly, ID, 2018. Means presented did not differ significantly (*P* = 0.05) among treatments.

Treatment	1st Application	2nd Application
Control	0.09 ± 0.02	0.09 ± 0.01
Flupyradifurone	0.09 ± 0.01	0.10 ± 0.02
Bt-galleriae	0.08 ± 0.02	0.13 ± 0.02
S. riobrave	0.09 ± 0.02	0.08 ± 0.01

3.2 Effect of Treatments on Alfalfa Taproot Damage

With no differences in CRC immature (larvae + pupae) suppression occurring after insecticide applications in 2018, taproot damage was also equal among treatments and the control (F = 0.31, df = 3, 32, *P* = 0.818) (Table 3-2). Further, the proportion of taproot damage did not differ between application times (F = 9.61, df = 1, 32, *P* = 0.334) (Table 3-2).

3.3 *Steinernema* spp. Persistence in Soil

After the first application in 2018, the average proportion of *Steinernema* spp. infected greater wax moths in *S. riobrave* treated plots was not significantly different from the infection in control (F = 1.40, df = 1,10, *P* = 0.264) regardless of time post-application (F = 1.21, df = 2, 10, *P* = 0.339) (Fig 3-3A). However, after the second 2018 application, there was a significant interaction between treatment (*S. riobrave* treated and control) and time post-application (1, 4, or 8 weeks) (treatment × post-application time interaction: F = 6.79, df = 2, 25, *P* = 0.004). Post hoc tests indicated the proportion of wax moths infected by *Steinernema* spp. in *S. riobrave* treated plots at 4 weeks post-application was significantly higher (*P* < 0.001) than infection in *S. riobrave* treated plots at 1- and 8-weeks post-application. Further, only at 4-weeks post-application was infection in *S. riobrave* treated plots significantly higher (P < 0.001) than infection in control plots (Fig 3-3B). The proportion of greater wax moth infection in control plots remained the same over time (P = 1.00). Similarly in 2019, the proportion of *Steinernema* spp. infected greater wax moths was significantly affected by treatment and time (treatment × post-application time interaction: F = 41.03, df = 1, 22, P <0.001). Wax moth larval infection in *S. riobrave* treated plots was highest 1-week after application (P < 0.001) and steadily declined over time. Infection in *S. riobrave* treated plots was significantly higher than infection in control plots at 1- and 4-weeks post-application (P <0.001), but at 8-weeks post-application, infection was similar (P = 1.00) between treatment and control (Fig 3-3C). Similarly, wax moth infection in control plots did not change significantly over time (P = 1.00).

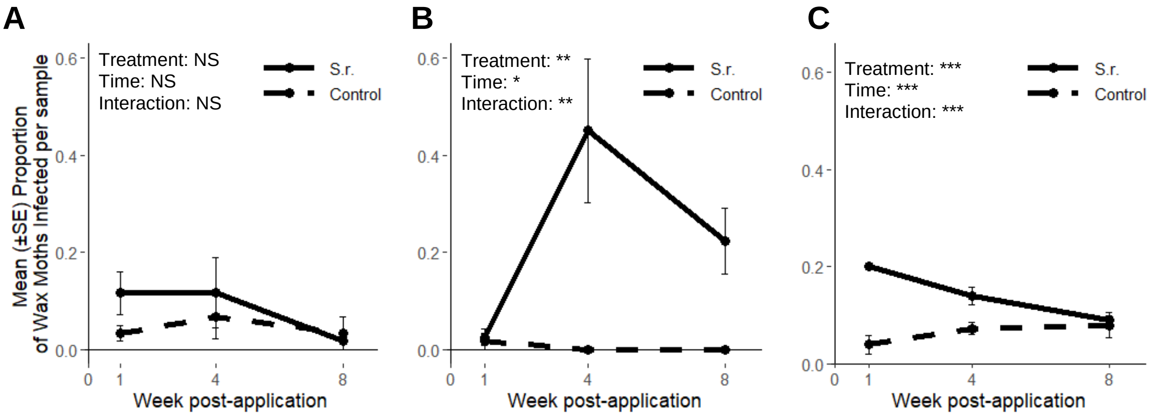

Figure 3-3. Mean (±SE) proportion of wax moths infected by *Steinernema* spp. in *Steinernema riobrave* treated and non-treated (control) plots over time (1, 4, and 8 weeks post-application) for the first application (A) and second application (B) in 2018, and the single application in 2019 (C). Results from repeated measures ANOVA are shown inside the graphs. NS (P > 0.05) * (P < 0.05) **(P < 0.01) *** (P < 0.001). S.r. = *S. riobrave*

4. Discussion

Contemporary research on CRC larval management is limited and increases in damage resulting from the cancellation of broad-spectrum insecticides with soil-activity substantiates the need for evaluating biological and systemic insecticides. In the present study, resident CRC populations were observed in all plots in both 2018 and 2019, and root damage occurred on all collected plants. Insecticides applied during this study, biological or synthetic, did not affect the total number of immatures collected or improve the resulting feeding damage compared to the untreated control. Given the diversity of insecticide types and modes of action it was surprising that our evaluation did not result in any one product suppressing CRC. Yet, CRC larval management with insecticides has historically been challenging. For instance, the efficacy of spring chlorinated hydrocarbon applications in reducing belowground larval damage ranged from not effective or moderately effective (Dunn et al., 1964; Waters, 1964) to highly effective (Forsythe and Gyrisco, 1962; Turner Jr., 1957) even with significant reductions in aboveground adult populations (Dunn et al., 1964; Forsythe and Gyrisco, 1962). Despite our results, we can glean important information and consider the many factors that may explain them. These include but are not limited to reduced systemic translocation to roots and reduced biological agent establishment, soil penetration, or host finding as a result of soil characteristics.

Management of belowground pests like CRC often presents unique challenges, due, in part, to complications with ease of access for monitoring, measuring damage, and targeting with insecticides. For example, although *S. lineatus* larvae belowground cause significant damage to pea roots, monitoring and chemical control focuses on adults that are easy to access aboveground, even though there may be little to no direct impacts on immature stages belowground or significant increases in yield (Cárcamo et al., 2018). Furthermore, difficulties in laboratory rearing forces researchers to rely on field-collected organisms or resident populations for laboratory and field experiments. For example, in historic CRC rearing methods (Baker and Byers, 1977; Byers, 1995; Newton, 1958) survival was often too low (< 17% survival) to result in large numbers of larvae for experiments. There are no effective methods for CRC rearing, and field-collected larvae are difficult to obtain in high quantities to screen insecticidal products against larvae in the laboratory. Thus, field testing has been the primary mode of insecticide research for CRC. However, additional challenges occur when relying on resident field populations for testing, such as low pest densities or patchy distributions. This is indeed the case with CRC as populations have aggregated field distributions (Ng et al., 1977; Quinn and Hower, 1985), and an average of 3.0 ± 0.3 immatures (larvae + pupae) were collected across all samples in this study. Given these challenges, it is imperative to test products over multiple years, fields, pest densities, and times.

The systemic insecticide treatment, flupyradifurone, failed to reduce numbers of CRC immatures in both years. Similarly, Barratt (1985) found no differences in *Sitona discoideus* Gyll. (Coleoptera: Curculionidae) larval density between plots treated with systemic insecticides, oxamyl and fenamiphos, and control plots. This was hypothesized to result from insufficient insecticide translocation and inadequate application concentrations (Barratt, 1985). Research is limited on flupyradifurone translocation rates and activity belowground following foliar sprays, but translocation to root material from seed treatments has been recorded by Stamm et al. (2016). Still, flupyradifurone translocation to leaf material has been demonstrated to be rapid (as early as 4 days) and persistent (24 days) in greenhouse-grown tomato after soil drenches (Nauen et al., 2014). Further, flupyradifurone soil applications at maximum yearly rates (409 g ai/ha) were moderately efficacious in decreasing belowground cabbage maggot (*Delia radicum* L.) numbers and feeding after 1 and 3 days (Joseph and Zarate, 2015). Due to the two applications in 2018, we tested a rate at half of the maximum yearly rate (205 g ai/ha) for each application. Although post-application irrigation was intended to wash active ingredients into the root zone, the reduced efficacy belowground was still likely a result of xylem transport of flupyradifurone in the direction of transpiration upward to the shoots (Nauen et al., 2014). Therefore, applications of flupyradifurone, despite application rate or application technique (foliar vs. soil drench), may not translocate to root material at adequate concentrations to target belowground pests. Indeed, the past success of carbofuran in CRC management may be a result of the downward transportation of active ingredients through phloem (Kleier, 1994).

The nematode application rate informed by recommendations (Georgis, 1990; Shapiro-Ilan et al., 2006) and used here allowed for nematode establishment as we recovered nematode-infected greater wax moth larvae. However, CRC immature populations were not impacted by this treatment. We posit prohibitive soil characteristics (e.g., high clay content) and environmental factors (e.g., heat, UV, low moisture) in field settings may require higher application rates to offset potential reduced nematode movement and survival (Georgis and Poinar, 1983; Kung et al., 1991, 1990) or patchy CRC distributions (Ng et al., 1977; Quinn and Hower, 1985). For instance, while similar rates of 2.5 bil *H. bacteriophora* IJs/ha provided >70% CRC control in the laboratory

(Loya and Hower, 2003), only field applications of 7 bil IJs/ha and 15 bil IJs/ha decreased CRC populations and damage in the field (Loya and Hower, 2002). Moreover, nematodes only persisted for multiple years in field soil when applied at 15 bil IJs/ha (Loya and Hower, 2002). *Steinernema riobrave* effectively manages other belowground weevil pests in the field, namely plum curculio (*Conotrachelus nenuphar* (Herbst); Coleoptera: Curculionidae) (Shapiro-Ilan et al., 2004) and *Diaprepes abbreviatus* L.(Coleoptera: Curculionidae) (McCoy et al., 2000), and is especially well-adapted for environments with higher ambient temperatures. But even in these systems, field application rates for successful control exceed 10 bil IJs per ha (McCoy et al., 2000; Shapiro-Ilan et al., 2004). Field application rates higher than the 2.5 bil IJs/ha recommended by Georgis (1990) and Shapiro-Ilan et al. (2006) may be necessary to observe significant reductions in CRC populations and damage, as was observed in Loya and Hower (2002). However, the economic feasibility of these types of applications should also be evaluated.

Furthermore, while pre-application soil moisture levels were adequate (>14% VWC, Table 3-S2) for *Steinernema* infectivity at all application times and years (Grant and Villani, 2009; Molyneux and Bedding, 1984), plots were individually hand-watered after the first application in 2018 due to unavailability of overhead irrigation at application time. This delayed and reduced volume of post-application irrigation was reflected in the low *Steinernema* spp. wax moth larvae infection observed after the first application in 2018. The increased time required to hand-water each plot in 2018 may have increased exposure of *S. riobrave* to harmful ultraviolet light and desiccation (Georgis, 1990; Kaya and Gaugler, 1993), further reducing nematode survival and soil penetration. However, even with adequate irrigation (1.27 cm) during the second application in 2018 and in 2019, CRC populations were not affected.

Similar to this study, granular *Bt-galleriae* (grubGONE!) applied at label rates, the same product and rate tested herein, failed to reduce Scarabaeidae larval populations in field studies (*Popillia japonica* Newman and *Cyclocephala hirta* Le Conte) at several sites and application timings (Hubble-Wirgler and Sutherland, 2017; Redmond et al., 2019). The challenges described in these studies may correspond to issues in efficacy in our study as we hypothesize that *Bt-galleriae* did not establish in the soil or could not adequately penetrate the soil to be ingested by susceptible stages. Early stage larval CRC (1st-3rd instar) may additionally be less likely to ingest *Bt* as they are frequently concealed inside nodules, feeding, and protected by the hollowed-out nodule (Danthanarayana, 1969). *Bt* spore survival depends on a variety of soil metrics including soil pH, moisture, nutrients, and the presence of resident microorganisms (West et al., 1985). Soil moisture and pH in 2018 and 2019 fields were adequate for *Bt* survival (West et al., 1985), however, nutrient levels important for microbes and levels of indigenous microorganisms were not assessed in this study. It is possible that resident microorganisms competed with *Bt* for nutrients, thus, reducing survival and establishment of *Bt* and its crystalline protein that provides insecticidal activity (West et al., 1984).

Importantly, *Bt* soil penetration depends on soil texture. For example, Saxena et al. (2002) found that in clay amended soils (12% clay) only 16% of *Bt* crystalline protein leached through 15 cm deep soil columns, and that *Manduca sexta* L. (Lepidoptera: Sphingidae) larval mortality from leachates was reduced by nearly half in this soil type compared to non-amended soil. In 2018 and 2019 fields, native soil was silty clay loam and silt loam, respectively, with clay contents exceeding 12% and potentially decreasing *Bt* penetration. Additionally, soil penetration of *Bt* under field settings has been reported to range from <1 to 6 cm deep even when irrigation is adequate (Akiba, 1991). Considering CRC larval feeding is concentrated in the upper 25 cm of alfalfa taproots (Dickason et al., 1968) and that *Bt* must be ingested for successful infection, *Bt* applications to soil surfaces, especially in heavy clay soils, may fail to reach larvae feeding deeper in the soil profile.

Most studies on microbial management of CRC with fungi focuses on adults (e.g., Crow et al., 1968; Quinn and Hower, 1985b) and none, to our knowledge, have tested *B. bassiana* in the field to manage CRC adult or larval populations. Nelson et al. (2004) found that regardless of application rate, a *B. bassiana* spore establishment of >10^4 spores/g of soil was important for efficacy against *S. lepidus* larvae belowground. Further, spore suspensions, like the suspension used in the current study, had higher (10^4 – 10^5 spores/g of soil) and more uniform spore establishment in the soil than granular applications (Nelson et al., 2004). However, suspensions were applied to the soil surface in Nelson et al. (2004), while in the current study, the suspension was applied to tall alfalfa. Although post-application irrigation was intended to wash *B. bassiana* spores off leaves to the soil surface, we did not measure spore levels to ensure adequate levels of spores established (>10^4 spores/g of soil) in the alfalfa root zone for effective CRC immature management. Ideally, field applications of *B. bassiana* to target CRC immatures should be applied directly to the soil surface or when alfalfa is short (<10 cm) early in the season or immediately after harvest. However, early season larval densities may be low (e.g., Table 3-S2, larvae at first application 2018) when alfalfa is short early in the season, and traditional first harvests in Idaho and northern Utah occur after most larval damage has occurred (mid- to late June). Thus, our application focused on ensuring sufficient numbers of larval hosts were available for pathogen infection, but also that larval reductions at this time might still decrease root damage. Because *B. bassiana* is the most common natural enemy of CRC, future studies on augmentative biocontrol should continue to evaluate *B. bassiana* applications but may determine the appropriate foliar rate for adequate spore establishment and persistence in the soil after irrigation, investigate other formulations (granular), or test different application techniques (chemigation, soil drench).

Although larval head capsule widths did not significantly differ among treatments at any application, we hypothesize that the reduced variation observed in insecticide treated plots may be a result of particular instars being more susceptible to certain products, especially in regard to the biological insecticides. For example, in the laboratory, *H. bacteriophora* Poinar, *Steinernema feltiae* Filipjev, and *Steinernema bibionis* Steiner infect and reproduce in all CRC stages, but later instars are the most susceptible stage (Jaworska and Wiech, 1988; Wiech and Jaworska, 1990). Additionally, Loya and Hower (2003) found that *H. bacteriophora* could not reproduce in first instars. Furthermore, we hypothesize that 1st and 2nd instars feeding inside nodules and protected by the nodule epidermis are less likely to encounter and ingest *Bt* that is in the soil medium. Larval head capsule widths after the first application in 2018 were generally smaller overall, but larvae for untreated control plots varied widely from 2nd to 4th instar, while widths for insecticide treatments grouped around 2nd and 3rd instars. Similarly, head widths in the control plots at the second application time in 2018 and the 2019 applications varied across 3rd to 5th instars, but head capsule widths in insecticide treated plots were mostly restricted to 5th instars. Further studies in the laboratory and field investigating the interaction of CRC stage and feeding habits as they relate to susceptibility and biological agent reproduction are needed.

Lastly in 2018, the proportion of root area consumed was not different between insecticide treatments and the untreated control. Because CRC taproot damage is cumulative (Dickason et al., 1968), there are sometimes difficulties identifying “new damage” (≤ 1 year old damage) from old damage, especially in older stands. Although damage estimations taken using these methods were an appropriate metric for assessing taproot damage, it is equally important to note that mainly 4th and 5th instars are consuming taproot material (Dintenfass and Brown, 1986). Alternative methods to quantify damage to alfalfa roots such as counting damaged nodules and rootlets (Price, 2017) may provide more complete information on larval feeding damage from a wider range of larval stages (1st-3rd instars) within a season.

Studying belowground pests like CRC can be difficult as rearing, monitoring, measuring damage, and targeting with pest control programs is complicated by cryptic habits and soil interactions. Further barriers arise in CRC damage evaluations in that damage builds up quickly over time in perennial alfalfa systems. Resident pest populations are also challenging to evaluate because of potentially low population densities and aggregated CRC population distributions, resulting in data that is often highly variable and skewed towards zero. Given these constraints, evaluating insecticide field applications for belowground pests can be a logistical challenge requiring a considerable amount of time. Indeed, we did not observe differences among the tested insecticides. While this result can be attributed to the initial challenges in studying belowground pests, inadequate suppression may also be related to several factors specific to the unique biological insecticides used and new systemic product available for alfalfa. Using the most appropriate application rates for success in other systems, other factors related to local conditions may have affected the biological products. While a degree day model is needed, it is also clear that applications in our study were made at an appropriate time since larvae were present during applications. Future studies may continue to test insecticides for CRC larval management, but, as with the congener *S. lineatus*, it may be more feasible to focus insecticide evaluations on determining action thresholds and spray regimes for adult CRC in various regions, evaluating the future impacts on CRC larvae and damage over time.

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References

Aeschlimann, J.P., 1980. The *Sitona* [Col.: Curculionidae] species occuring on *Medicago* and their natural enemies in the Mediterranean region. Entomophaga 25, 139–153. https://doi.org/https://doi.org/10.1007/BF02374316

Aghaee, M.A., Godfrey, L.D., 2015. The efficacy of *Bacillus thuringiensis* spp. *galleriae* against rice water weevil (Coleoptera: Curculionidae) for integrated pest management in California rice. J. Econ. Entomol. 108, 45–52. https://doi.org/10.1093/jee/tou024

Akiba, Y., 1991. Assessment of rainwater-mediated dispersion of field-sprayed *Bacillus thuringiensis* in the soil. Appl. Entomol. Zool. 26, 477–483.

Alston, D.G., Rangel, D.E.N., Lacey, L.A., Golez, H.G., Jeong, J.K., Roberts, D.W., 2005. Evaluation of novel fungal and nematode isolates for control of *Conotrachelus nenuphar* (Coleoptera: Curculionidae) larvae. Biol. Control 35, 163–171. https://doi.org/10.1016/j.biocontrol.2005.06.011

Amand, P.C.S., Peaden, D.Z.S.R.N., 2000. Risk of alfalfa transgene dissemination and scale-dependent effects. Theor. Appl. Genet. 101, 107–114.

Bachmann, A., Johnson, P.O., Vos, D., Alms, J., Wrage, L.J., Varenhorst, A.J., Rozeboom, P., Wagner, P.M., Byamukama, E., Yabwalo, D., Strunk, C., 2019. 2019 South Dakota pest management guide: Alfalfa & oilseeds. South Dakota State Univeristy Extension.

Baker, P.B., Byers, R.A., 1977. A laboratory technique for rearing the clover root curculio. Meisheimer. Entomol. Ser. 8–10.

Barratt, B.P., 1985. An attempt to control *Sitona discoideus* larvae with systemicinsecticides, in: Proceedings of the 38th New Zealand Weed and Pest Control Conference. Rotorua, NZ, pp. 35–37.

Bedding, R.A., Akhurst, R.J., 1975. A simple technique for the detection of insect paristic rhabditid nematodes in soil. Nematologica 21, 109–110. https://doi.org/10.1163/187529275X00419

Bedding, R.A., Miller, L.A., 1981. Use of a nematode, *Heterorhabditis heliothidis*, to control black vine weevil, *Otiorhynchus sulcatus*, in potted plants. Ann. Appl. Biol. 99, 211–216. https://doi.org/10.1111/j.1744-7348.1981.tb04788.x

Bezdicek, D.F., Quinn, M.A., Forse, L., Heron, D., Kahn, M.L., 1994. Insecticidal activity and competitiveness of *Rhizobium* spp. containing the *Bacillus thuringiensis* subsp. *tenebrionis* δ-endotoxin gene (*cryIII*) in legume nodules. Soil Biol. Biochem. 26, 1637–1646. https://doi.org/10.1016/0038-0717(94)90316-6

Boemare, N., Laumond, C., Mauleon, H., 1996. The entomopathogenic nematode-bacterium complex: Biology, life cycle and vertebrate safety. Biocontrol Sci. Technol. 6, 333–346. https://doi.org/10.1080/09583159631316

Brownbridge, M., Costa, S., Jaronski, S.T., 2001. Effects of in vitro passage of *Beauveria bassiana* on virulence to *Bemisia argentifolii*. J. Invertebr. Pathol. 77, 280–283. https://doi.org/10.1006/jipa.2001.5020

Bullock, R.C., Pelosi, R.R., Killer, E.E., 1999. Management of citrus root weevils (Coleoptera : Curculionidae) on Florida citrus with soil- applied entomopathogenic nematodes (Nematoda : Rhabditida) Author(s): R . C . Bullock , R . R . Pelosi and E . E . Killer Published by: Florida Entomologic. Florida Entomol. 82, 1–7. https://doi.org/10.2307/3495831

Byers, R.A., 1995. Factors affecting rearing of clover root curculio (Coleoptera: Curculionidae) in cone containers. J. Econ. Entomol. 88, 407–414. https://doi.org/https://doi.org/10.1093/jee/88.2.407

Cabanillas, H.E., 2003. Susceptibility of the boll weevil to *Steinernema riobrave* and other entomopathogenic nematodes. J. Invertebr. Pathol. 82, 188–197. https://doi.org/10.1016/S0022-2011(03)00016-8

Cárcamo, H.A., Vankosky, M.A., Wijerathna, A., Olfert, O.O., Meers, S.B., Evenden, M.L., 2018. Progress toward integrated pest management of pea leaf weevil: A review. Ann. Entomol. Soc. Am. 111, 144–153. https://doi.org/10.1093/aesa/say007

Cornell University Cooperative Extension, 2007. Management of alfalfa snout beetle.

Crow, W R, Puttler, B., Daugherty, D.M., 1968. *Beauveria bassiana* infecting adult clover root curculios in Missouri. J. Econ. Entomol. 61, 576–577. https://doi.org/https://doi.org/10.1093/jee/61.2.576

Danthanarayana, W., 1969. Population dynamics of the weevil *Sitona regensteinensis* (Hbst .) on broom. J. Anim. Ecol. 38, 1–18.

Dickason, E.A., Leach, C.M., Gross, A.E., 1968. Clover root curculio injury and vascular decay of alfalfa roots. J. Econ. Entomol. 61, 1163–1168. https://doi.org/https://doi.org/10.1093/jee/61.5.1163

Dickason, E.A., Leach, C.M., Gross, A.E., 1958. Control of the clover root curculio. J. Econ. Entomol. 51, 554–555. https://doi.org/https://doi.org/10.1093/jee/51.4.554

Dintenfass, L.P., Brown, G.C., 1986. Feeding rate of larval clover root curculio, *Sitona hispidulus* (Coleoptera: Curculionidae), on alfalfa taproots. J. Econ. Entomol. 79, 506–510. https://doi.org/https://doi.org/10.1093/jee/79.2.506

Dunn, G.M., Kilpatrick, R.A., Chow, H.S., 1964. Effects of dieldrin and methyl bromide on *Sitona* larvae and root rot of white clover. Crop Sci. 4, 276–279.

EPA, 2018. Reduced risk and organophosphate alternative decisions for conventional pesticides [WWW Document]. URL https://www.epa.gov/pesticide-registration/reduced-risk-and-organophosphate-alternative-decisions-conventional (accessed 12.10.19).

EPA, 2009. Carbofuran cancellation process [WWW Document]. URL https://archive.epa.gov/pesticides/reregistration/web/html/carbofuran_noic.html (accessed 7.3.18).

Forsythe, H.Y., Gyrisco, G.G., 1962. Evaluating the control of the cover root curculio larva on alfalfa. J. Econ. Entomol. 55, 906–908. https://doi.org/https://doi.org/10.1093/jee/55.6.906

Gaugler, R., Boush, G.M., 1978. Effects of ultraviolet radiation and sunlight on the entomogenous nematode, *Neoaplectana carpocapsae.* J. Invertebr. Pathol. 32, 291–296. https://doi.org/10.1016/0022-2011(78)90191-X

Georgis, R., 1990. Formulation and application technology, in: Gaugler, R., Kaya, H.K. (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press Inc., Boca Raton, FL, pp. 173–191.

Georgis, R., Poinar, G.O., 1983. Effect of soil texture on the distribution and infectivity of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae). J. Nematol. 15, 308–311.

Georgis, R., Poinar, G.O.J., 1984. Greenhouse control of the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) by Heterorhabditid and

Steinernematid nematodes. Environ. Entomol. 13, 1138–1140.

Glare, T., Willoughby, B., Nelson, T.L., Wilson, D.J., Eden, T.M., 2004. Towards a fungal biopesticide for clover root weevil control, In: Proceedings of the 8th Australasian Conference on Grassland Invertebrate Ecology. pp. 112-117.

Godfrey, L.D., Legg, D.E., Yeargan, K. V, 1986. Effects of soil-borne organisms on spring alfalfa establishment in an alfalfa rotation system. J. Econ. Entomol. 79, 1055–1063.

Godfrey, L.D., Yeargan, K. V, 1989. Effects of clover root curculio , alfalfa weevil (Coleoptera : Curculionidae), and soil-borne fungi on alfalfa stand density and longevity in Kentucky. J. Econ. Entomol. 82, 1749–1756. https://doi.org/https://doi.org/10.1093/jee/82.6.1749

Goulson, D., 2013. An overview of the environmental risks posed by neonicotinoid insecticides. J. Appl. Ecol. 50, 977–987. https://doi.org/10.1111/1365-2664.12111

Graham, J.H., Newton, R.C., 1960. Relationship between injury by the clover root curculio and incidence of *Fusarium* root rot in Ladino white clover. Plant Dis. Report. 44, 534–535.

Graham, J.H., Newton, R.C., 1959. Relationship between root feeding insects and incidence of crown and root rot in red clover. Plant Dis. Report. 43, 1114–1116.

Grant, J.A., Villani, M.G., 2009. Soil moisture effects on entomopathogenic nematodes. Environ. Entomol. 32, 80–87. https://doi.org/10.1603/0046-225x-32.1.80

Hallmann, C.A., Foppen, R.P.B., Van Turnhout, C.A.M., De Kroon, H., Jongejans, E., 2014. Declines in insectivorous birds are associated with high neonicotinoid concentrations. Nature 511, 341–343. https://doi.org/10.1038/nature13531

Hazir, S., Kaya, H.K., Stock, S.P., Keskin, N., 2004. Entomopathogenic nematodes (*Steinernematidae* and *Heterorhabditidae*) for biological control of soil pests. Turk J Biol 27, 181–202.

Hower, A.A., Quinn, M.A., Alexander, S.D., Leath, K.T., 1995. Productivity and persistence of alfalfa in response to clover root curculio (Coleoptera: Curculionidae) injury in Pennsylvania. J. Econ. Entomol. 88, 1433–1440. https://doi.org/https://doi.org/10.1093/jee/88.5.1433

Hubble-Wirgler, C.W., Sutherland, A.M., 2017. Evaluation of a granular formulation of *Bacillus thuringiensis galleriae* for use against white grubs within municipal turf, 2016. Arthropod Manag. Tests 42. https://doi.org/10.1093/amt/tsx119

Jackson, D.J., 1922. Bionomica of weevils of the genus *Sitona* injurious to leguminous crops in Britain. Ann. Appl. Biol. 9, 93–115. https://doi.org/10.1111/j.1744-7348.1922.tb05941.x

James, J.R., Lucas, L.T., Chamblee, D.S., Campbell, W.V., 1980. Influence of fungicide and insecticide applications on persistence of ladino clover. Agon. J. 72, 781–784.

Jaworska, M., 1998. The laboratory preference of annual legumes by pea weevil *Sitona lineatus* L. (Col., curculionidae) and their effect on susceptibility of weevils to entomogenous nematodes. J. Invertebr. Pathol. 71, 248–50. https://doi.org/10.1006/jipa.1997.4708

Jaworska, M., Ropek, D., 1994. Influence of host-plant on the susceptibility of *Sitona lineatus* L. (col.,curculionidae) to *Steinernema carpocapsae* Weiser. J. Invertebr. Pathol.

Jaworska, M., Wiech, K., 1988. Susceptibility of the clover root weevil, *Sitona hispidulus*

F. (Coleoptera, Curculionidae) to *Steinernema feltiae*, *Steinernema bibionis*, and *Heterorhabditis bacteriophora*. J. Appl. Entomol.

Jenczewski, E., Prosperi, J.M., Ronfort, J., 1999. Evidence for gene flow between wild and cultivated *Medicago sativa* (Leguminosae) based on allozyme markers and quantitative traits. Am. J. Bot. 86, 677–687. https://doi.org/10.2307/2656577

Jewett, H.H., 1934. The clover root curculio. Kentucky Agric. Exp. Stn. Circ. 42, 13–23.

Joseph, S. V., Zarate, J., 2015. Comparing efficacy of insecticides against cabbage maggot (Diptera: Anthomyiidae) in the laboratory. Crop Prot. 77, 148–156. https://doi.org/10.1016/j.cropro.2015.07.022

Kakouli-Duarte, T., Labuschagne, L., Hague, N.G.M., 1997. Biological control of the black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with entomopathogenic nematodes (Nematoda: Rhabditida). Ann. Appl. Biol. 131, 11–27. https://doi.org/DOI: 10.1111/j.1744-7348.1997.tb05393.x

Kaya, H.K., Gaugler, R., 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. 38, 181–206.

Kilpatrick, R.A., 1961. Fungi associated with the larvae of *Sitona* spp. Phytopathology 51, 640–641.

Kim, H.G., Alston, D.G., 2008. Potential of two entomopathogenic nematodes for suppression of plum curculio (*Conotrachelus nenuphar*, Coleoptera: Curculionidae) life stages in northern climates. Environ. Entomol. 37, 1272–1279. https://doi.org/10.1603/0046-225X(2008)37

Kleier, D.A., 1994. Phloem mobility of xenobiotics. V.* Structural requirements for phloem-systemic pesticides. Pestic. Sci. 42, 1–11.

https://doi.org/10/1002/ps.2780420102

Kuhar, T.P., Alvarez, J.M., 2008. Timing of injury and efficacy of soil-applied insecticides against wireworms on potato in Virginia. Crop Prot. 27, 792–798. https://doi.org/10.1016/j.cropro.2007.11.011

Kung, S.-P., Gaugler, R., Kaya, H.K., 1991. Effects of soil temperature, moisture, and relative humidity on entomopathogenic nematode persistence. J. Invertebr. Pathol. 57, 242–249. https://doi.org/10.1016/0022-2011(91)90123-8

Kung, S.-P., Gaugler, R.R., Kaya, H.K., 1990. Influence of soil pH and oxygen on persistence of Steinernema spp. J. Nematol. 22, 440–445.

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009

Lau, N.E., Filmer, R.S., 1959. Injury of clover root curculios to red clover in New Jersey. J. Econ. Entomol. 52, 1155–1156. https://doi.org/https://doi.org/10.1093/jee/52.6.1155

Leibee, G.L., Pass, B.C., Yeargan, K. V., 1980. Instar determination of clover root curculio, *Sitona hispidulus* (Coleoptera: Curculionidae). J. Kansas Entomol. Soc. 53, 473–475.

Loya, L.J., Hower, A.A., 2003. Infectivity and reproductive potential of the Oswego strain of *Heterorhabditis bacteriophora* associated with life stages of the clover root curculio, *Sitona hispidulus*. J. Invertebr. Pathol. 83, 63–72. https://doi.org/10.1016/S0022-2011(03)00044-2

Loya, L.J., Hower, A.A., 2002. Population dynamics, persistence, and efficacy of the entomopathogenic nematode Heterorhabditis bacteriophora (Oswego strain) in association with the clover root curculio (Coleoptera : Curculionidae) in Pennsylvania. Environ. Entomol. 31, 1240–1250. https://doi.org/10.1603/0046-225X-31.6.1240

McCoy, Clayton W, Shapiro, D.I., Duncan, L.W., Nguyen, K., 2000. Entomopathogenic nematodes and other natural enemies as mortality factors for larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae). Biol. Control 19, 182–190. https://doi.org/10.1006/bcon.2000.0852

Molyneux, A.S., Bedding, R.A., 1984. Influence of Soil Texture and Moisture on the Infectivity of Heterorhabditis sp. D1 and Steinernema glaseri for larvae of the sheep blowfly, Lucilia cuprina. Nematologica 30, 358–365.

National Pesticide Information Center, 2015. NPIC product research online [WWW Document]. URL http://npic.orst.edu/NPRO/ (accessed 9.28.19).

Nauen, R., Jeschke, P., Velten, R., Beck, M.E., Ebbinghaus-Kintscher, U., Thielert, W., Wölfel, K., Haas, M., Kunz, K., Raupach, G., 2014. Flupyradifurone: A brief profile of a new butenolide insecticide. Pest Manag. Sci. 71, 850–862. https://doi.org/10.1002/ps.3932

Neal, J.W.J., Ratcliffe, R.H., 1975. Clover root curculio control with granular carbofuran as measured by alfalfa regrowth, yield, and root damage. J. Econ. Entomol. 68, 829–831. https://doi.org/https://doi.org/10.1093/jee/68.6.829

Nelson, T.L., McNeill, M.R., Van Koten, C., Goldson, S.L., 2015. Comparative virulence of *Beauveria bassiana* isolates against clover root weevil (*Sitona obsoletus*). New Zeal. Plant Prot. 68, 139–145.

Nelson, T.L., Willoughby, B.E., Wilson, D.J., Eden, T., 2004. Establishing the fungus *Beauveria bassiana* in pasture for clover root weevil (*Sitona Lepidus*) Control. New Zeal. Plant Prot. 57, 314–318.

Neumann, G., Shields, E.J., 2004. Survey for entomopathogenic nematodes and entomopathogenic fungi in alfalfa snout beetle, *Otiorhynchus ligustici* (L.) (Coleoptera: Curculionidae), Infested fields in Hungary and in New York State. Gt. Lakes Entomol. 37, 152–158.

Newton, R.C., 1958. Rearing *Sitona hispidula* larvae for various research uses. J. Econ. Entomol. 51, 917–918.

Ng, Y.S., Latheef, M.A., Pass, B.C., 1977. Estimation of egg populations of the clover root curculio, *Sitona hispidula* (Coleoptera: Curculionidae), in Kentucky alfalfa and red clover fields. Can. Entomol. 109, 1257–1263.

Palumbo, J.C., 2012. Soil-surface applied systemic insecticides for control of sweetpotato whitefly in broccoli, 2011. Arthropod Manag. Tests 37, 4182. https://doi.org/10.4182/amt.2012.E12

Phillips, W.G., Ditman, L.P., 1962. Studies on biology and economic importance of clover root curculio *Sitona hispidula* (Fab.) in Maryland. Univ. Maryl. Agric. Exp. Stn. Bulletin A, 1–21.

Poprawski, T.J., Marchal, M., Robert, P.H., 1985. Comparative susceptibility of *Otiorhynchus sulcatus* and *Sitona lineatus* (Coleoptera: Curculionidae) early stages to five entomopathogenic Hyphomycetes. Environ. Entomol. 14, 7.

Price, S.J., 2017. Understanding the biology of clover root curculio and improving their management using biofumigation in alfalfa. Utah State University.

Quinn, M.A., Hower, 1985. Changes in the spatial distribution of the clover root curculio (Coleoptera: Curculionidae) in alfalfa field soil. Environ. Entomol. 14, 864–867. https://doi.org/https://doi.org/10.1093/ee/14.6.864

Quinn, M. A., Hower, A.A., 1985a. Determination of overwintering survivorship and predicting time of eclosion for eggs of *Sitona hispidulus* (Coleoptera: Curculionidae). Environ. Entomol. 14, 850–854. https://doi.org/10.1093/ee/14.6.850

Quinn, M. A., Hower, A.A., 1985b. Isolation of *Beauveria bassiana* (Deuteromycotina Hyphomycetes) from Alfalfa Field Soil and Its Effect on Adult Sitona hispidulus (Coleoptera Curculionidae). Environ. Entomol. 14, 620–623. https://doi.org/https://doi.org/10.1093/ee/14.5.620

Redmond, C.T., Wallis, L., Geis, M., Williamson, R.C., Potter, D.A., 2019. Strengths and limitations of *Bacillus thuringiensis galleriae* for managing Japanese beetle (*Popillia japonica*) adults and grubs with caveats for cross-order activity to monarch butterfly (*Danaus plexippus*) larvae. Pest Manag. Sci. https://doi.org/10.1002/ps.5532

Reynolds, D.S., Brandenburg, R.L., 2015. Hunting billbug (*Sphenophorus venatus vestitus*) response to insecticide application in warm- season turfgrass and implications for management. Crop. Forage Turfgrass Manag. 1, 1–7. https://doi.org/10.2134/CFTM2014.0059

Rim, K., Price, S.J., Ramirez, R.A., 2019a. Clover root curculio (*Sitona hispidulus* F.), Utah State University Extension.

Rim, K., Price, S.J., Wenninger, E.J., Long, R., Ramirez, R.A., 2019b. Biology and management of clover root curculio (Coleoptera: Curculionidae). J. Integr. Pest

Manag. 10, 1–14. https://doi.org/10.1093/jipm/pmz020

Rinehold, J., 2017. Alfalfa hay pests [WWW Document]. Pacific Northwest Ext. URL https://pnwhandbooks.org/insect/hay-pasture/alfalfa (accessed 2.27.18).

Saxena, D., Flores, S., Stotzky, G., 2002. Vertical movement in soil of insecticidal *Cry1Ab* protein from *Bacillus thuringiensis*. Soil Biol. Biochem. 34, 111–120. https://doi.org/10.1016/S0038-0717(01)00193-6

Seidenglanz, M., Rotrekl, J., Smýkalová, I., Poslušná, J., Kolařík, P., 2010. Differences Between the effects of insecticidal seed and foliar treatments on pea leaf weevils (*Sitona lineatus* L.) in the field pea (*Pisum sativum* L.). Plant Prot. Sci. 46, 25–33.

Shapiro-Ilan, D.I., Gouge, D.H., Piggott, S.J., Fife, J.P., 2006. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. Biol. Control 38, 124–133. https://doi.org/10.1016/j.biocontrol.2005.09.005

Shapiro-Ilan, D.I., Mizell, R.F., Cottrell, T.E., Horton, D.L., 2004. Measuring field efficacy of *Steinernema feltiae* and *Steinernema riobrave* for suppression of plum curculio, *Conotrachelus nenuphar*, larvae. Biol. Control 30, 496–503. https://doi.org/10.1016/j.biocontrol.2003.12.006

Shields, E.J., Testa, A., Neumann, G., Flanders, K.L., Schroeder, P.C., 2009. Biological control of alfalfa snout beetle with a multi-species application of locally adapted persistent entomopathogenic nematodes: The first success. Am. Entomol. 55, 250–257.

Somvanshi, V.S., Koltai, H., Glazer, I., 2008. Expression of different desiccation-tolerance related genes in various species of entomopathogenic nematodes. Mol.

Biochem. Parasitol. 158, 65–71. https://doi.org/10.1016/j.molbiopara.2007.11.012

Stamm, M.D., Heng-Moss, T.M., Baxendale, F.P., Siegfried, B.D., Blankenship, E.E., Nauen, R., 2016. Uptake and translocation of imidacloprid, clothianidin and flupyradifurone in seed-treated soybeans. Pest Manag. Sci. 72, 1099–1109. https://doi.org/10.1002/ps.4152

Stansly, P.A., Kostyk, B.C., 2016. Soil and foliar applications of systemic insecticides for control of sweetpotato whitefly on zucchini squash, 2015. Arthropod Manag. Tests 41, 1–2. https://doi.org/10.1093/amt/tsw062

Tan, Y.P., Hower, A.A., 1991. Development and feeding-behavior of clover root curculio (Coleoptera, Curculionidae) larvae on alfalfa. Environ. Entomol. 20, 1013–1018. https://doi.org/10.1093/ee/20.4.1013

Turner Jr., E.C., 1957. Control of the clover root curculio on alfalfa. J. Econ. Entomol. 50, 645–648. https://doi.org/https://doi.org/10.1093/jee/50.5.645

Underhill, G.W., Turner Jr., E.C., Henderson, R.G., 1955. Control of the clover root curculio on alfalfa with notes on life history and habits. J. Econ. Entomol. 48, 184–187. https://doi.org/10.1093/jee/48.2.184

Vankosky, M.A., Cárcamo, H.A., McKenzie, R.H., Dosdall, L.M., 2011. Integrated management of *Sitona lineatus* with nitrogen fertilizer, *Rhizobium,* and thiamethoxam insecticide. Agron. J. 103, 565–572. https://doi.org/10.2134/agronj2010.0314

Waters, N.D., 1964. Effects of *Hypera nigrirostris*, *Hylastinus obscurus,* and *Sitona hispidula* populations on red clover in southwestern Idaho. J. Econ. Entomol. 57, 907–910. https://doi.org/https://doi.org/10.1093/jee/57.6.907

Wenninger, E.J., Shewmaker, G.E., 2014. Clover root curculio in alfalfa: Identification, biology, and management. Pacific Northwest Ext. PNW 663, 1–5.

West, A.W., Burges, H.D., Dixon, T.J., Wyborn, C.H., 1985. Survival of *Bacillus thuringiensis* and *Bacillus cereus* spore inocula in soil: Effects of pH, moisture, nutrient availability and indigenous microorganisms. Soil Biol. Biochem. 17, 657–665. https://doi.org/10.1016/0038-0717(85)90043-4

West, A.W., Burges, H.D., White, R.J., Wyborn, C.H., 1984. Persistence of bacillus thuringiensis parasporal crystal insecticidal activity in soil. J. Invertebr. Pathol. 44, 128–133. https://doi.org/10.1016/0022-2011(84)90002-8

Wiech, K., Jaworska, M., 1990. Susceptibility of *Sitona* weevils (Col., Curculionidae) to entomogenous nematodes. J. Appl. Entomol. 110, 214–216.

Wildermuth, V.L., 1910. The clover-root curculio, in: USDA Bureau of Entomology.

Willmott, D., Hart, A., Long, S., Edmondson, R., Richardson, P., 2002. Use of a cold-active entomopathogenic nematode *Steinernema kraussei* to control overwintering larvae of the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) in outdoor strawberry plants. Nematology 4, 925–932. https://doi.org/10.1163/156854102321122548

Willoughby, B., Glare, T., Kettlewell, F., Nelson, T., 1998. *Beauveria Bassiana* as a potential biocontrol agent against the clover root weevil, *Sitona lepidus*, in: Proceedings of the NZ Plant Protection Conference. pp. 9–15.

Woodcock, B.A., Bullock, J.M., Shore, R.F., Heard, M.S., Pereira, M.G., Redhead, J., Ridding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Hulmes, L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S., Pywell, R.F.,

2017. Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science (80-.). 356, 1393–1395. https://doi.org/10.1126/science.aaa1190

Yaari, M., Doron-Faigenboim, A., Koltai, H., Salame, L., Glazer, I., 2016. Transcriptome analysis of stress tolerance in entomopathogenic nematodes of the genus *Steinernema*. Int. J. Parasitol. 46, 83–95. https://doi.org/10.1016/j.ijpara.2015.08.011

Supplemental Tables

Table 3-S1. Field characteristics were determined by taking ten soil samples 25-30 cm deep haphazardly throughout the field, combining samples in a clean container until thoroughly mixed, and sending 300 g of soil to be analyzed at Utah State University Analytical Laboratories.

Field Season	Age (yr)	Soil Texture	pH	Salinity (dS/m)	Phosphorus (mg/kg)	Potassium (mg/kg)
2018	4	Silty Clay Loam	7.6	0.70	12.9	208
2019	6	Silt Loam	7.7	0.58	12.0	218

Table 3-S2. Pre-application measurements were taken before each application in both field seasons. Field soil moisture (% volumetric water content (%VWC)) was measured thrice from each plot throughout the field using a soil moisture meter (Field Scout TDR100, Spectrum Technologies Inc., Aurora, IL) with 7.62 cm probes. Additionally, 5 soil core samples were taken haphazardly throughout the field to determine initial CRC immature density and stage. Mean (±SE) soil moisture, mean (±SE) number of *Sitona hispidulus* eggs, hatched eggs, and larvae, and mean (±SE) head capsule width were calculated from these data. NA = not applicable

	Date	**Soil Moisture Content (%VWC)**	**Eggs**	**Hatched Eggs**	**Larvae**	**Mean head capsule width (mm)**
2018						
1st Application	April 25	27.89 ± 0.47	19.6 ± 5.36	6.2 ± 2.58	0.0 ± 0.0	NA
2nd Application	June 21	53.36 ± 0.67	2.2 ± 1.60	-	1.88 ± 1.03	0.88 ± 0.04
2019						
Application	May 24	42.23 ± 0.50	14.9 ± 2.96	21.2 ± 1.07	3.6 ± 0.93	0.38 ± 0.05

CHAPTER IV

EVALUATION OF ALFALFA (FABALES: FABACEAE) CULTIVARS FOR RESISTANCE TO CLOVER ROOT CURCULIO

Abstract

Since the cancellation of broad-spectrum soil-active insecticides in the 2000s, clover root curculio (*Sitona hispiduls* F.) (CRC) larval root damage has increased in alfalfa (*Medicago sativa* L.) production. Few studies have evaluated contemporary cultivars for potential resistance to CRC. Therefore, we first investigated potential cross-resistance between commercial root-pathogen resistant varieties (root-knot nematode and *Phytopthora* root rot) and CRC. Here we tested three varieties, a variety susceptible to root-pathogens (control), and one variety each with moderate and high root-pathogen resistance. Next, we tested 5 developmental alfalfa lines against larvae where two were CRC-susceptible (control) lines, and three were resistant to CRC. A soil-less arena was used to observe nodule feeding and development (head capsule width) of larvae. Further, one CRC-resistant line paired with its genetically similar CRC-susceptible line were tested against CRC adults. Leaf area consumption and oviposition were estimated for plants exposed to CRC adults under greenhouse conditions. Regardless of resistant variety or alfalfa line, adult feeding and oviposition were similar within each trial compared to susceptible controls. Further, results from cross-resistance trials demonstrated there were no differences in larval nodule consumption or development, suggesting root-pathogen resistance may not influence resistance towards CRC larvae. Although larval development was similar across susceptible and resistant lines, one CRC-resistant line displayed overall increased nodulation, and thus, had a significantly lower proportion of nodules consumed by larvae. Further research is needed to understand if nodulation is an advantageous adaptation, as nodule growth may increase alfalfa resilience towards larvae, but also increase resources for neonates.

Keywords**:** feeding behavior, oviposition, belowground herbivore, host-plant resistance, conferred resistance

Introduction

Clover root curculio (CRC) (*Sitona hispidulus* F.) (Coleoptera: Curculionidae) is an alfalfa and clover (Fabales: Fabaceae) pest that negatively affects alfalfa plant health and yield. Adults feed aboveground on leaves, yet typically do not cause economic losses (Rim et al. 2019). Conversely, immature CRC feed on roots belowground and are recognized as the damaging stage (Rim et al. 2019). Larval feeding can result in reduced stand life and density, plant stunting, yield loss, and decreased plant overwintering survival (e.g., Godfrey and Yeargan 1987, Godfrey et al. 1987, Gotlieb et al. 1987). Furthermore, larval feeding damage increases plant susceptibility to *Fusarium* (Hypocreales: Nectriaceae) infections which can exacerbate these problems (Graham and Newton 1960, Hill et al. 1969, Leath and Hower 1993). Since the cancellation of broad-spectrum soil-active insecticides (e.g., carbofuran) and fumigants commonly used against alfalfa weevil (*Hyera postica* Gyllenhal, Coleoptera: Curculionidae) (EPA 2009, Tietz 2012), there have been notable increases in CRC populations and associated root damage (Tietz 2012). Furthermore, broad-spectrum insecticides registered for CRC adult management may have detrimental non-target effects, and current management practices are limited in their ability to manage cryptic CRC larvae (Wenninger and Shewmaker 2014, Rim et al. 2019). Thus, developing integrated pest management tactics is critically needed.

The development of tolerant or resistant host plants has been integral to successful integrated pest management programs in cropping systems. For example, the development of solid-stemmed wheat in the 1940s significantly reduced wheat stem sawfly (*Cephus cinctus* Norton, Hymenoptera: Cephidae) infestations (Platt et al. 1948) and continue to be used in spring wheat production (Morrill et al. 1994). Fortunately, over 100 alfalfa varieties are commercially available with varying resistance to a multitude of alfalfa pests (NAFA 2017). However, development of resistant alfalfa varieties primarily targets hemipteran pests like aphids and leafhoppers, or plant pathogens like nematodes and bacterial wilts. Presently, no commercial alfalfa cultivars are available with resistance to chewing insects such as alfalfa weevil or CRC, in part due to the high genetic variability in alfalfa (Maureira and Osborn 2005), and also considering that mechanisms underlying alfalfa resistance to chewing insects (e.g., saponins) may potentially interfere with palatability (e.g., bloat in livestock; Fay and Dale 1993).

Historic evaluations for CRC resistance identified potentially resistant lines (e.g., Lahontan), but have yet to identify resistance mechanisms (e.g. Byers and Kendall 1982, Byers et al. 1996). However, research on congeners may shed some light on host plant characteristics that may confer resistance to CRC. For instance, as leaf thickness increased for pea plants, herbivory from adult *Sitona lineatus* L. (Coleoptera: Curculionidae) decreased (Havlíčková 1980). Additionally, sweetclover root disks impregnated with nitrates, arabinose, ascorbic acid, glucuronic acid, or mannitol, all isolated from healthy plants, deterred *Sitona cylindricollis* Fåhraeus (Coleoptera: Curculionidae) feeding (Akeson et al. 1970).

Cross-resistance is a component of resistant varieties, whereby resistance to one pest results in resistance to other pests through a common resistance mechanism. Cross-resistance has been reported between different pest groups and niches, including aphids and plant pathogenic nematodes (e.g., Kaloshian et al. 1995, Rossi et al. 1998, Vos et al.

1998, Ramirez and Spears 2014). Although this interaction is well-studied in tomato, cross-resistance between two alfalfa foliar pests from different phyla has been recorded. Here, varieties with resistance to stem nematode effectively decreased aphid populations, regardless of aphid resistance (Ramirez and Spears 2014). In fact, plant-mediated interactions between different pests and cross-resistance research has routinely focused on foliar pest complexes or the impact of root herbivory on foliar pests (Bezemer et al. 2003, Soler et al. 2005, Van Dam et al. 2005). Given these interactions and observations of resistance toward a multitude of pest types, sources of resistance may already exist in commercially available varieties when cross-resistance is considered. Given the lack of studies on belowground pest complexes and potential cross-resistance, it is necessary to investigate existing commercial alfalfa varieties with resistance to belowground plant pathogens for cross-resistance towards CRC larvae.

Research is ongoing at the Cornell Forage Breeding Program (Ithaca, New York, USA) to develop CRC-resistant alfalfa lines (Neally et al. 2006, Crawford et al. 2018). Thus far, this project has developed and released an alfalfa snout beetle (*Otiorhychus lingustici* L., Coleoptera: Curculionidae) (ASB) resistant line (Seedway 9558 SBR) (Crawford et al. 2014, Ramanujan 2014). This weevil causes significant root damage to alfalfa in northern New York, similar to damage caused by CRC larvae (York et al. 1971). Additionally, in initial field screenings of experimental CRC-resistant and susceptible lines, resistant lines showed higher crown and root biomass and lower root damage ratings in response to larval feeding (Crawford et al. 2018). However, only plant biomass and damage ratings were collected during this study; thus, further research is needed to understand the effects of these developmental lines by assessing impacts to the target herbivore, CRC.

Here, a laboratory no-choice test was used to evaluate (1) commercial alfalfa varieties from Alforex Seeds (Woodland, CA) resistant to root-knot nematode and other root pathogens for potential cross-resistance to CRC and, (2) developmental alfalfa lines resistant and susceptible to CRC from Cornell University's Forage Breeding laboratory. We measured larval nodule feeding and development of early-instar CRC larvae using a soil-less method. Additionally, adult feeding on aboveground leaf tissue and oviposition on these alfalfa lines and varieties were assessed. We hypothesized that CRC-resistant lines would exhibit reduced adult feeding and oviposition aboveground, and decreased nodule feeding and CRC larval development (head capsule widths) belowground. Lastly, should root-knot nematode resistance be conferred to CRC, we similarly hypothesized to observe reduced nodule feeding, slower larval development, and decreased adult oviposition behavior.

Materials and Methods

Cross-Resistance Alfalfa Variety Trial.

To examine conferred resistance between root pests and CRC, commercial varieties PGI459 and PGI437 (Alforex Seeds, Woodland, CA) were selected from the 2017 National Alfalfa and Forage Alliance alfalfa variety ratings for their high resistance to northern root-knot nematode (RKN, *Meloidogyne hapla*, Tylenchida: Heteroderidae). Further PGI437 and PGI459 were additionally rated resistant and highly resistant to *Phytopthora* (Peronosporales: Peronosporaceae) root rot, respectively. Varieties with 'high resistance' to pests are quantified as having more than half (50%) of plants resisting pest damage while ‘resistant’ varieties resist pests and damage in 31-50% of plants (NAFA 2017). The check variety, Ranger, was selected due to its purported susceptibility to both CRC and northern root-knot nematode (Pedersen et al. 1976, Griffin and Elgin 1977, Griffin 1980). The commercial varieties PGI437 and PGI459 were compared to the check variety, Ranger. Both CRC adults and larvae were tested against PGI437, PGI459, and Ranger to examine cross-resistance between root pathogens and CRC.

CRC-Resistant Alfalfa Line Trial.

Three developmental CRC-resistant (NY1713, NY1720, and NY1719) and two CRC-susceptible (NY1718 and NY1717) non-commercial lines from Cornell University were tested. Developmental lines were derived from Oneida Ultra (NY1720 and NY1717) (Viands et al. 2004a) or Seedway 9558 (NY1713 and NY1718) (Viands et al. 2004b) parental alfalfa populations. The line NY1719, a cross between both CRC-resistant lines NY1713 and NY1720, was also tested. To investigate CRC-resistance in these developmental lines, all five lines were tested against CRC larvae with the CRC-susceptible lines, NY1718 and NY1717, serving as positive controls. Further, the CRC-resistant line NY1713 paired with its CRC-susceptible line, NY1718, was tested against CRC adults.

Collection of clover root curculio.

Clover root curculio eggs were collected from wild populations located at Greenville Research Farm (Logan, Cache Co., UT) whereby a hand trowel was used to collect soil to a depth of 2.5 cm (~ 300 mL of soil per sample) around alfalfa crowns (Rim et al. 2019) during October of 2017 and 2018. Soil samples were processed similarly to the methods described by Rim et al. (2019). Briefly, soil was washed through a series of sieves (U.S. standard set #5, #10, #35, #60), particulate matter from the #60 sieve was examined under a stereomicroscope, and mature CRC eggs were placed on a moistened filter paper within a parafilm-sealed petri dish. Eggs were stored in the refrigerator (5-7°C) until use in larval trials (1-4 wk after collection) where neonate larvae emerged from eggs (see *Larval Evaluations* section).

Adult CRC were field collected from August to October of 2018 and 2019 using a handheld vacuum mulcher (Echo ES-250, Lake Zurich, IL) modified into a sampling device. Clover root curculio adults recovered from vacuum samples were placed in a bug dorm (BioQuip Products Inc., Compton, California, USA) with a moistened cotton roll (Patterson Companies, Saint Paul, MN) and alfalfa bouquets replenished every 3-4 d. Clover root curculio adults are not sexually dimorphic; therefore, we observed copulation or mate guarding behavior for sex determination and collected adult pairs in a 9-dram vial with a moist cotton wick. Adults were subsequently stored at 5-7˚C for 1-2 wks and cotton wicks were moistened *ad libitum* until their use (see *Adult Evaluations* section)

Larval Evaluations.

Plants used in the larval study were grown hydroponically at optimal temperature for alfalfa growth (23-25˚C) (Teuber et al. 1988) inside an incubator (#136LLVL Percival, Perry, IA) under constant environmental conditions (23-25˚C, 14L:10D, 40%RH) for 2-4 months until use (Fig. 4-1). To standardize root nodule numbers at the initiation of the larval feeding experiment, nodules were randomly excised using a knife (X-ACTO, Elmer’s Products Inc., High point, NC) so that only 4 – 6 nodules remained at the start of the experiment. We used a completely random design to assign treatments (uninfested control or CRC infested) with one egg placed adjacent to the crown of each

CRC infested plant (~ 1cm deep). Uninfested controls were used to ensure no other factors were contributing to root material loss and/or growth during the experimental period. Each plant and egg, secured between germination paper, were rolled into transparency film for stability. We established 15 replications (both uninfested control and CRC infested) of each alfalfa variety or line within each respective trial and held them in the same incubator conditions as described above for plants. Eggs were monitored every 24 hr until first-instar larvae emerged, after which feeding continued on the plant undisturbed for one week. At the end of one week, surviving larvae were carefully removed, their head capsule width (mm) measured, and nodules observed for damage. Data were also recorded for the number of additional nodules that developed during the experiment.

Adult Evaluations.

Three to five plants were seeded in a 15.25 cm diameter × 20.32 cm tall pot (experimental unit) filled with Sungro #3 Propagation Mix under greenhouse conditions (23°C, 14L:10D, 37% RH) until plants were approximately 30 cm in height. One of the germinated plants was randomly selected from each pot (20 replications for each line and variety) to be used in the experiment and all others were removed. To standardize the number of leaves, leaves were removed until there were only five trifoliate leaves per plant. Transparent enclosures (cages) were constructed by rolling transparency film into a ~5cm diameter by 27.94 cm-long tube with a rubber band secured mesh bag over one end (top of the cage). Similar to the methods in Connin et al. (1966), plaster of Paris (DAP Products, Baltimore, MD) mixed as directed was poured over the freshly watered soil, creating a flat, white surface to aid in CRC egg collection. The uncovered bottom of each cage was centered around each plant stem and pushed into the unset plaster, creating a plaster floor inside the cage. The plaster set for 30 minutes before CRC pairs were added. Plants were exposed to a mated pair for 4 d and beetle survival was recorded every 24 hr throughout the study. Replications from adult evaluations where one or both beetles died or escaped prior to the 4-d experimental period were excluded. Surviving beetles were then removed and placed in a 70% ethanol solution for sex confirmation via dissection, and eggs were counted within each cage. When CRC dissections revealed both beetles to be male, this replication was also removed from oviposition data. To estimate adult feeding damage (leaf area consumed and total leaf area), stems were cut at the base and shoot material was reserved. Additionally, negative controls were used to observe for indiscriminate oviposition behavior by placing CRC pairs into cages with a moistened cotton roll without plants.

To calculate leaf areas, first, freshly collected shoot material were carefully placed and spread out on a flatbed scanner to obtain a digital image (Canon CanoScan LiDE 60, Canon, Ōta, Tokyo, Japan). A ruler was included to determine scale. Using Photoshop (Adobe, San Jose, CA), feeding damage was filled with a contrasting color (black) and the rest of the plant material was removed from the photo so that only damaged area was shown. Similarly, damaged area and remaining leaf area were filled to calculate total leaf area. These images were then analyzed with ImageJ (ImageJ 1.49f; http://rsbweb.nih.gov/ij/) by calculating the damaged and overall surface area of each of the 5 trifoliate leaves.

Data Analysis.

All data were analyzed using R software (RStudio).

Cross-Resistance Alfalfa Variety Trial.

Commercial alfalfa varieties were evaluated to investigate cross-resistance to CRC by selecting commercial varieties (PGI459 and PGI437) with varied resistance to root pathogens, analyzing larval and adult responses relative to a susceptible control variety (Ranger). To compare the total number of nodules consumed on the alfalfa varieties during larval evaluations, data were analyzed using generalized linear model (GLM) with the $\log_{10}$-link and Poisson distribution error. To compare group means, Tukey tests (95% confidence index) were applied to the GLM using the MultComp package. The proportion of nodules consumed ($\sin^{-1}\sqrt{\frac{\#\ nodules\ consumed}{Total\ \#\ nodules}}$) and larval head capsule width (mm) were compared among treatments using independent Kruskal-Wallis rank sum test for uneven sample sizes and non-normal data.

Replications from adult evaluations where one or both beetles died prior to the 4-d experimental period were excluded leaving 18, 18, and 19 replications for PGI437, PGI459, and Ranger, respectively. A two-way ANOVA for normal and homoscedastic data was performed on the proportion of leaf area consumed ($\sin^{-1}\sqrt{\frac{area\ consumed\ (mm^2)}{leaf\ area\ (mm^2)}}$) for each variety by trifoliate leaf position (1 being the basal most trifoliate leaf and 5 the apical trifoliate leaf). Then, the overall proportion of leaf area consumed was estimated by combining total leaf area and consumed area data for the five trifoliate leaves in each replicate ($\sum_{n=1}^{5} area\ (mm^2)$). Overall proportion of leaf area consumed ($\sin^{-1}\sqrt{\frac{areas\ consumed\ overall\ \ (mm^2)}{leaf\ area\ overall\ (mm^2)}}$) was analyzed by a one-way ANOVA. Tukey’s HSD post hoc tests followed ANOVAs to separate significant differences in adult feeding among varieties.

The total number of eggs laid (oviposition) during the experimental period (4 days) was compared among alfalfa treatments (no-plant negative control, Ranger, PGI437, and PGI459) using a GLM with the $\log_{10}$-link and quasi-Poisson distribution error. Following the GLM analysis, Tukey HSD with a 95% confidence index was performed using the MultComp package for multiple comparisons.

<u>CRC-Resistant Alfalfa Line Trial.</u>

Developmental alfalfa lines were evaluated for resistance (NY1720, NY1713, and NY1719) and susceptibility (NY1717 and NY1718) to CRC larvae. Count data were analyzed using GLM with the $\log_{10}$-link and Poisson distribution error to compare the total number of nodules consumed on alfalfa lines, and the quasi-Poisson distribution error for the number of nodules grown between treatments (uninfested control and CRC-infested) for each line with an average of ≥ 1 grown nodules. To compare group means, Tukey tests (95% confidence index) for multiple comparisons were applied to GLMs using the MultComp package. Independent Kruskal-Wallis rank sum tests for non-normal data were used to analyze the proportion of nodules consumed ($\sin^{-1}\sqrt{\frac{\#\ nodules\ consumed}{Total\ \#\ nodules}}$) and larval head capsule width (mm) data. When Kruskal-Wallis resulted in statistical significance ($p \leq 0.05$) pairwise comparisons using Wilcoxon rank sum test with Bonferroni correction were performed.

Replications from adult evaluations where one or both beetles died prior to the 4-d experimental period were excluded leaving 17 replications for each line tested (NY1718 and NY1713). Similar to the cross-resistance trial, a two-way ANOVA for normal and homoscedastic data was performed on the proportion of leaf area consumed for each line by trifoliate leaf position. The overall proportion of leaf area consumed was then analyzed by a one-way ANOVA. Tukey's HSD post hoc tests followed ANOVAs to separate significant differences in adult feeding among lines.

Dissections for one replication revealed two females, thus, the total number of eggs laid (oviposition) for this replication was divided by two to adjust to one gravid female. Oviposition during the experimental period (4 d) was compared among CRC-susceptible line, NY1718, and CRC-resistant line, NY1713, using a GLM with the $\log_{10}$-link and quasi-Poisson distribution error. Following the GLM analysis, Tukey HSD with a 95% confidence index was performed using the MultComp package for multiple comparisons.

Results

Cross-Resistance Alfalfa Variety Trial.

Clover root curculio larvae fed on all varieties and there were no significant differences between Ranger, the control variety, and the root pathogen-resistant commercial varieties in the total number of nodules consumed by larvae after 7 days (GLM: F = 1.269, $P = 0.281$) (Table 4-1). Similarly, the proportion of nodules consumed was not significantly different among Ranger (control) and the two root-pathogen resistant varieties (Kruskal-Wallis: $\chi^2 = 4.573$, df = 2, $P = 0.102$) (Table 4-1). Further, there was no effect of variety (Ranger, PGI437, and PGI459) on larval head capsule widths. (Kruskal-Wallis: $\chi^2 = 3.319$, df = 2, $P = 0.190$) (Table 4-1).

Across all commercial varieties on average, the proportion of leaf area consumed by adults was significantly greater on the apical trifoliate leaf compared to the four basal trifoliate leaves (ANOVA leaf position: F = 7.483, df = 4, 259, $P < 0.001$). Adults consumed nearly 2 times the leaf area on the uppermost trifoliate leaf (the 5th trifoliate leaf from the bottom) compared to the other leaves (Fig. 4-2). There was no significant interaction effect between leaf position and variety (ANOVA variety × leaf position: F = 0.940, df = 8, 259, $P = 0.4842$). Similarly, the overall proportion of leaf area consumed (all leaves on a single plant combined) was similar among Ranger, PGI437, and PGI459 (ANOVA: F = 1.103, df = 2, 52, $P = 0.339$) (Table 4-2). Oviposition (total eggs) was significantly higher on all varieties compared to the no-plant control (GLM: F = 13.892, $P < 0.001$), where on average, females caged with plants laid 14.28 ± 2.39 eggs and those without plants deposited 0.61 ± 1.16 eggs (Fig. 4-3). Although seemingly more eggs were deposited on PGI459 (19.22 ± 5.42 eggs) with high resistance to both RKN and *Phytopthora* root rot, compared to the susceptible control, Ranger, and PGI437, with moderate resistance to root pathogens (11.30 ± 3.34 and 12.80 ± 3.65 eggs, respectively), differences were not significant ($P = 0.39$ and 0.59, respectively) (Fig. 4-3).

CRC-Resistant Alfalfa Line Trial.

The total number of nodules consumed by larvae did not differ among developmental alfalfa lines (GLM: F = 1.447, $P = 0.216$) (Table 4-3). However, the proportion of nodules consumed on NY1713, the Seedway 9558-derived CRC-resistant line, was approximately 3 times less than NY1720, the Oneida Ultra-derived CRC-resistant line (Kruskal-Wallis: $\chi^2 = 12.992$, df = 4, $P = 0.011$) (Table 4-3). Only the NY1713 line grew more than one nodule on average (4.17 ± 1.12 nodules) over the 7-day experimental period compared to the other Cornell developmental lines (0.21 ± 0.12 nodules). Yet, NY1713 nodules grew similarly on the uninfested control (4.53 ± 1.94 nodules) and the CRC-infested treatment (3.8 ± 1.21 nodules) (GLM: F = 0.107, P =

0.747). Larval head capsule widths did not differ among the developmental lines (Kruskal-Wallis: $\chi^2 = 7.051$, df = 4, *P* = 0.133).

In adult evaluations with CRC-resistant NY1713 and CRC-susceptible NY1718 lines, there was a marginal significant difference in the proportion of leaf area consumed by trifoliate leaf number (ANOVA leaf position: F = 2.421, df = 4, 160, *P* =0.051). On average, adults consumed 1.5 times the leaf area on the uppermost trifoliate leaf (the 5th trifoliate leaf from the bottom) compared to other leaves (Fig. 4-4). There was no significant interaction between leaf position and variety (ANOVA variety × leaf position: F = 0.330, df = 8, 160, *P* = 0.858). Similarly, the overall proportion of leaf area consumed (all leaves on a single plant combined) was similar among lines (ANOVA: F =0.015, df = 1, 32, *P* = 0.912) (Table 4-4). Oviposition (total eggs) was not significantly different between NY1718 and NY1713 (GLM: F = 0.032, *P* = 0.860) (Table 4-4).

Discussion

No differences in larval development (head capsule widths) or in nodule consumption were found between root pathogen-resistant commercial varieties and Ranger, suggesting that, alfalfa bred for resistance to root pathogens may not confer resistance to larval CRC. In a study by Byers and Kendall (1982), the authors similarly found no difference in CRC larval growth (head capsule width and body length) on the *Phytophthora* root rot resistant variety, KS77, compared to a susceptible control. Although our study, and that of Byers and Kendall (1982), showed no conferred resistance, there is a paucity of information on cross-resistance between root pests and the mechanisms of resistance to root pests. Further, even when resistance mechanisms are known, they may differ between varieties (Postnikova et al. 2015). For instance, root-knot nematode resistance has been hypothesized to be a result of programmed cell death around the entry site (Williamson and Kumar 2006) or prevention of nematode entrance into root systems (Potenza et al. 2001, Dhandaydham et al. 2008). The mechanisms of resistance to root pathogens in PGI437 and PGI459 have not been evaluated, but it is possible that resistance mechanisms may differ between these varieties even though they target the same pest or pest group.

However, pathogens and chewing insects affect plants in very different ways, thus, mechanisms likely differ. Kafle et al. (2017) found herbivore identity or feeding guild to be a key factor in influencing plant defense traits. Studies in tomato have also indicated that induced responses to sucking insects differ from those targeting chewing insects (e.g., Stout et al. 1998, Rodriguez-Saona et al. 2010). Therefore, it is possible that defense mechanisms and resistance towards CRC larval feeding are independent of those towards root pathogens.

Interestingly, the cross-resistance and developmental line trial both showed CRC removed greater proportions of leaf area from the apical leaf, the youngest trifoliate leaf on experimental plants (5th leaf), compared to the older, more basal, trifoliate leaves on the plant. This same trend was observed by Price (2017) in a caged field study where beetles caged on uppermost leaves consumed more leaf area (mm^2) than those caged on lower leaves. The observed increase in leaf area consumption on young leaves over mature leaves was consistent with the results in Coley (1980). It is currently unknown what influences adult CRC feeding, yet for the curculionid *S. cylindricollis,* adult diet preference was influenced by changes in concentrations of feeding stimulants (e.g., sucrose) and deterrents (e.g., ascorbic acid, mannitol) as leaves mature (Akeson et al.

1970). Further, as leaves age and mechanical defenses are altered like leaf toughness (Lucas et al. 2000) or leaf thickness (Havlíčková 1980) this may negatively affect or deter feeding. Glandular trichomes on alfalfa are another physical defense shown to reduce potato leafhopper (*Empoasca fabae* Harris, Hemiptera: Cicadellidae) in addition to damage-related plant stunting and yield loss in alfalfa (Sulc et al. 2001). Researchers hypothesize glandular trichomes physically entrap leafhopper nymphs in exudate (Ranger and Hower 2001) or that volatile production differs on plants with glandular trichomes making them less attractive to potato leafhoppers (Ranger et al. 2005). Further, erect glandular trichomes on *Medicago disciformis* DC. and *Medicago scutellata* (L.) Miller resulted in 100% mortality of alfalfa weevil larvae aboveground (Shade et al. 1975, Kreitner and Sorensen 1979). Although we did not assess these particular defenses, it is likely that a combination of chemical and physical defenses results in adult CRC preference towards younger leaves. These kinds of defenses should not be discounted in future studies on CRC adult feeding and oviposition as they have the potential to influence adult behavior.

Despite differences observed for CRC feeding on different trifoliate leaves, there were no differences in the overall proportion of leaf area consumed among any of the root-pathogen resistant varieties or the resistant Cornell developmental line and controls. To standardize plants for the adult trials, plants were pruned until only 5 trifoliate leaves remained. These methods may have resulted in host plant priming, where previously damaged plants have faster responses and may respond more aggressively to future damage or stress (Conrath et al. 2006, Frost et al. 2008). It may be possible that leaf removal in adult evaluations, as well as nodule removal in larval evaluations, induced defenses or primed plants for CRC feeding. However, plants can distinguish between mechanical damage and insect feeding as influenced by insect oral or ovipositional secretions (exogenous plant defense elicitors) (Eder and Cosio 1994). For instance, *Spodoptera exigua* (Lepidoptera: Noctuidae) herbivory and their salivary factor, glucose oxidase, decreased terpenoid biosynthesis gene expression in *Medicago truncatula* Gaertn while mechanical wounding alone had no effect on these same pathways (Bede et al. 2006). Considering the plant's ability to differentiate between mechanical damage and insect herbivory and given that nearly all plants were pruned for experiments, the results presented herein would likely be similar if plants were not pruned for standardization.

Aboveground adult CRC feeding did not differ between root pathogen resistant varieties and the susceptible control, suggesting that pathogen resistance belowground may not influence aboveground resistance to chewing insects. Research on the influence of belowground pathogen resistance to aboveground chewing insects is limited, but in tomato, the *Mi* gene that provides resistance to root-knot nematodes was found to confer resistance to the potato aphid (*Macrosiphum euphorbiae* (Thomas), Hemiptera: Aphididae) and whiteflies (*Bemisia tabaci* (Gennadius), Hemiptera: Aleyrodidae), both piercing-sucking insects damaging aboveground tissues (Vos et al. 1998, Nombela et al. 2003). In this case, although the same gene was conferring resistance, the mechanisms of resistance to each pest was hypothesized to be different (Nombela et al. 2003). Other studies on plant-mediated interactions indicate belowground pathogen infection can decrease subsequent aboveground defense compounds and nutrition, which has positive effects on aboveground chewing herbivore feeding and growth (e.g., Kaplan et al. 2008, Kafle et al. 2017). However, we did not observe increases in aboveground feeding on root pathogen resistant varieties compared to the control either. It is possible the resistance mechanisms against root pathogens in the commercial alfalfa varieties tested here may not be effective against aboveground chewing herbivory, and different resistance pathways may be necessary to target aboveground chewing pests.

Although feeding did not differ between varieties, females displayed discriminate oviposition behavior. Significantly more eggs were laid when alfalfa was present compared to the arenas without a plant. Despite being aboveground, maternal *Sitona lepidus* Gyll. (Coleoptera: Curculionidae) can discriminate between nodulated and non-nodulated white clover plants (Johnson et al. 2006) and will significantly increase oviposition when caged on a host-plant (white clover) compared to those caged with the non-host, *Lolium perenne* L. (Poales: Poaceae) (Mowat and Clawson 1996). However, females in this study did not significantly alter oviposition among pathogen-resistant varieties and the susceptible control. Research on oviposition preferences for aboveground insects with belowground offspring is limited and it is currently unknown what influences CRC oviposition, but a complex of cues may be involved. Chemical attractants and deterrents can have complex interactions in influencing host plant decisions for oviposition, especially when pests are specialists like CRC. For example, maternal *Plutella xylostella* L. (Lepidoptera: Plutellidae), a specialist on Brassicaceae (*Barbarea* spp.), prefer to oviposit on resistant plants and smaller/younger leaves with higher concentrations of saponins and glucosinolates, both deterrents and defense compounds that are important for host-plant recognition (Badenes-Perez et al. 2014). Further research is needed to identify cues or attractants for maternal CRC.

Nevertheless, increased oviposition may not result in subsequent increases in CRC larval populations, feeding, or development. It is important to consider that aboveground damage from adult CRC can alter resources and defenses belowground, impacting subsequent oviposition behavior or larval offspring. For instance, maternal *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae) aboveground feeding decreased root biomass by approximately 30% and subsequently reduced offspring growth and survival (Clark et al. 2011). Clover root curculio larval survival and development depends on nutrient-rich belowground food sources, like nodules (Gerard 2001); reductions in belowground resources resulting from aboveground herbivory may be detrimental to offspring. Furthermore, density dependent larval mortality of *Sitona* spp. occurs when the number of eggs exceed the available resources (e.g., total number of nodules available) (Aeschlimann 1979, Quinn and Hower 1986a, Gerard 2001). Therefore, increased oviposition may be potentially detrimental to neonate nodule finding and survival if the total number of nodules available remains the same or even decreases as a result of maternal feeding.

Indeed, development of chewing insect resistant alfalfa is underway, specifically directing efforts towards belowground pest management such as alfalfa snout beetle and CRC (Crawford et al. 2012, 2018). Thus, understanding the potential mechanisms behind resistance to CRC in the Cornell University developmental alfalfa lines is critical and may inform future advances in chewing-insect resistance. However, like the findings for root pathogen resistant varieties, purported CRC-resistant alfalfa lines did not reduce larval feeding and development. Larvae consumed a similar number of nodules and had similar head capsule widths on the CRC-susceptible and CRC-resistant developmental alfalfa lines. However, Crawford et al. (2018) showed that percent CRC resistance, calculated from ratings for little to no root damage, was higher in CRC-resistant lines than CRC-susceptible lines. Yet, experimental methods herein differed from those of Crawford et al. (2018). Dissimilarly, we measured early-instar nodule feeding in a soil-less system for 7 days, whereas Crawford et al. (2018) did not survey CRC larvae and instead, rated overall taproot damage in the field over 2 years. The soil-less arenas in our study allowed for easy and simplified observations of plant-herbivore interactions in belowground systems. This does not reflect the myriad of biotic and abiotic factors contributing to herbivore feeding, growth, and survival under field settings. For example, Hackell and Gerard (2004) hypothesized that the inability of *S. lepidus* larvae to feed on clover nodules in petri dishes was due to nodule odors flooding the experimental arena and a lack of contact stimuli. Contrastingly, in a field system, nodule odors are dispersed in the soil around nodules in gradients, and the larval cuticle is in contact with the soil medium (Hackell and Gerard 2004). The methods we employed in the larval experiments were sufficient for rapid screening of potentially resistant alfalfa lines, but further research in systems with soil are important to a well-rounded understanding of CRC larval feeding, development, and survival.

Although we did not observe differences in the total number of nodules consumed or larval development, the proportion of nodules consumed lower on CRC-resistant line NY1713 compared to NY1720, also a CRC-resistant line. The significantly lower proportion of nodules consumed by larvae on NY1713 was because this line grew nodules during the 7-day experimental period. We hypothesized this may have been a compensatory response to larval feeding as evidenced by Quinn and Hall (1992), where compensatory growth of alfalfa nodules occurred after nodules were removed by CRC or when mechanically removed. However, NY1713 nodule growth was similar between the uninfested control and the CRC-infested treatment. This suggests compensatory nodule growth did not occur in this CRC-resistant line, but that NY1713 tended to have increased nodulation overall.

Enhanced nodulation may benefit host plants damaged by CRC by decreasing nitrogen stress and potentially increasing tolerance to CRC larval feeding. The negative impacts of larval feeding on nitrogen-fixing nodules and subsequent plant stress is well-known and has been recorded for other sitonids. For example, high populations of *S. lepidus* larvae terminated the nitrogen-fixing abilities of white clover plants (Gerard 2001), and *Sitona discoideus* Gyll. (Coleoptera: Curculionidae) larval feeding disrupted nitrogen-fixation and increased nitrogen stress in field-grown alfalfa (Goldson et al. 1988). These decreases in nitrogen-fixation resulted in reduced aboveground nitrogen, yield, and decreased stem regrowth after harvest (Goldson et al. 1987, 1988). Further, Vankosky et al. (2011) found that although the number of *S. lineatus* damaged nodules did not change in inoculant and thiamethoxam treated field pea, plants displayed increased nodulation, higher numbers of large multilobed nodules, and increased nitrogen fixation compared to controls. If CRC larvae consume the same number of nodules on average (~2 nodules/larva/week), the observed increased nodulation in N1713 may offset the nitrogen stress and disruption of nitrogen-fixation due to regular nodule loss from CRC larval feeding. However, further studies on nitrogen-fixation capacity related to CRC larval feeding on NY1713 is needed to test this hypothesis.

Yet, the increased nodulation observed on NY1713 may potentially increase CRC larval survival and may not necessarily result in subsequent plant yield increases. Although CRC and most *Sitona* early instars are not obligate nodule feeders, research indicates that just the presence of nodules on alfalfa roots increases larval survival by 3-14 times when compared to larval survival on non-nodulated roots (Byers and Kendall 1982, Wolfson 1987). Further, CRC and *Sitona* larval development (head capsule width and body length) was enhanced on nodulated roots (Byers and Kendall 1982, Wolfson 1987, Gerard 2001, Hackell and Gerard 2004). Although NY1713 had a higher number of nodules, we did not observe increased head capsule widths for CRC larvae on NY1713 compared to the other developmental lines. Additionally, *S. lepidus* larvae are attracted to nodules by the volatile protein asparagine (Snapp and Vance 1986), a precursor and potential host-finding and feeding stimulant for larvae (Hackell and Gerard 2004). Increased nodulation may increase volatile emission and, thus, increase host-plant or nodule finding. Lastly, first-instar *Sitona* mortality was high (95-99%) (Aeschlimann 1979) and was posited to be a result of interspecific competition or failed host-plant and nodule finding (Quinn and Hower 1986a, 1986b). Thus, the increased nodulation observed on NY1713 may potentially increase host-plant and nodule-finding as well as CRC larval survival under field conditions.

Like adult evaluations for root pathogen resistant trials, aboveground adult CRC feeding did not differ between the CRC-resistant line, NY1713, and its paired susceptible line, NY1718, suggesting that purported resistance and susceptibility of these lines to field populations of CRC larvae may not necessarily affect aboveground tissues and CRC adult feeding. When developing alfalfa varieties with resistance towards chewing insect pests, it is important to increase resistance without sacrificing nutrition and palatability for livestock feeding on foliage. For example, high saponin content in alfalfa was associated with increased CRC resistance (Pedersen et al. 1975, 1976), but saponins have negative effects on animal metabolism and are associated with bloat (increased gas pressure in the rumen) in livestock (Sen et al. 1998). For these reasons, developing alfalfa with resistance to CRC may be particularly difficult. Therefore, it could be beneficial that resistance or tolerance for these CRC-resistant lines is concentrated in belowground tissues.

Oviposition behavior was also similar among NY1713 and NY1718. Like the results for root pathogen resistant alfalfa trials, maternal CRC may be able to detect that belowground resources exist (nodules); however, female CRC in this study may not have the ability to detect the increased number of nodules found on NY1713. Johnson et al. (2006) posited that maternal congener *S. lepidus* received cues through host shoot and root volatiles as well as chemical and physical cues from the soil. We hypothesized that if female CRC were able to adjust oviposition based on the concentration of belowground resources (higher numbers of nodules), this ability may have been hindered in the current study since plaster covered the soil surface and may have disrupted detection of root volatiles and soil cues.

Overall, CRC larvae were able to feed on nodules and develop on all eight alfalfa cultivars tested. Further, antibiosis was not observed with larvae or adults on root pathogen-resistant alfalfa varieties or CRC-resistant developmental lines, suggesting resistance to root pathogens may not be conferred to CRC. The CRC-resistant developmental lines are still under development at Cornell University, thus, upcoming breeding cycles of these lines may yield different or stronger results. Future studies should focus on evaluating alfalfa cultivars with resistance to other pests as well as investigate the interactions between enhanced nodulation, CRC larval survival, and nitrogen stress to determine if nodule growth is a beneficial physiological adaptation that increases alfalfa resilience towards CRC larvae. Lastly, bioassay procedures to observe belowground insect pests are lacking (Hunter 2001). Therefore, the methods stated here to observe larvae is a novel way to screen host plant cultivars and observe belowground plant-herbivore interactions without expensive equipment or the extensive time commitment that usually accompanies soil sorting methods.

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References

Aeschlimann, J. P. 1979. Sampling methods and construction of life tables for *Sitona humeralis* populations (Col ., Curculionidae) in Mediterranean climatic areas. J. Appl. Ecol. 16: 405–415.

Akeson, W. R., F. A. Haskins, H. J. Gorz, and G. R. Manglitz. **1970**. Feeding response of the sweetclover weevil to various sugars and related compounds. J. Econ. Entomol. 63: 1079–1080.

Badenes-Perez, F. R., J. Gershenzon, and D. G. Heckel. **2014**. Insect attraction versus plant defense: Young leaves high in glucosinolates stimulate oviposition by a specialist herbivore despite poor larval survival due to high saponin content. PLoS One. 9: 39–42.

Bede, J. C., R. O. Musser, G. W. Felton, and K. L. Korth. **2006**. Caterpillar herbivory and salivary enzymes decrease transcript levels of *Medicago truncatula* genes encoding early enzymes in terpenoid biosynthesis. Plant Mol. Biol. 60: 519–531.

Bezemer, T., R. Wagenaar, N. M. Van Dam, and F. L. Wäckers. **2003**. Interactions between above- and belowground insect herbivores as mediated by the plant defense system. Nord. Soc. Oikos. 101: 555–562.

Byers, R. A., and W. A. Kendall. **1982**. Effects of plant genotypes and root nodulation on growth and survival of *Sitona* spp. larvae. Environ. Entomol. 11: 440–443.

Byers, R. A., W. A. Kendall, R. N. Peaden, and D. W. Viands. **1996**. Field and laboratory selection of *Medicago* plant introductions for resistance to the clover root curculio (Coleoptera: Curculionidae). J. Econ. Entomol. 89: 1033–1039.

Clark, K. E., S. E. Hartley, and S. N. Johnson. **2011**. Does mother know best? The preference-performance hypothesis and parent-offspring conflict in aboveground-belowground herbivore life cycles. Ecol. Entomol. 36: 117–124.

Coley, P. D. 1980. Effects of leaf age and plant life history patterns on herbivory. Nature. 284: 545–546.

Connin, R. V, D. L. Cobb, M. S. Gomulinski, and J. C. Arnsman. **1966**. Plaster of Paris as an aid in rearing insects pupating. J. Econ. Entomol. 59: 1530–1531.

Conrath, U., G. J. M. Beckers, V. Flors, P. García-Agustín, G. Jakab, F. Mauch, M. A. Newman, C. M. J. Pieterse, B. Poinssot, M. J. Pozo, A. Pugin, U. Schaffrath, J. Ton, D. Wendehenne, L. Zimmerli, and B. Mauch-Mani. **2006**. Priming: Getting ready for battle. Mol. Plant-Microbe Interact. 19: 1062–1071.

Crawford, J., R. Deubler, J. Schiller, R. Crawford, J. Hansen, and D. Viands. **2018**. Progress from selection in alfalfa breeding for resistance to the root feeding pest clover root curculio. *In* 2018 Jt. Conf. NAAIC, Trifolium, Grass Breeders. Logan, UT.

Crawford, J., J. L. Hansen, E. J. Shields, A. Testa, and D. R. Viands. **2012**. Breeding for resistance to alfalfa snout beetle. *In* 2012 Jt. Conf. NAAIC, Trifolium, Grass Breeders, Ithaca, NY.

Crawford, J., J. L. Hansen, E. J. Shields, A. Testa, and D. R. Viands. **2014**. Breeding for resistance to alfalfa snout beetle. *In* 2014 Jt. Conf. NAAIC, Trifolium, Grass Breeders. Lethbridge, Alberta Canada.

Van Dam, N. M., C. E. Raaijmakers, and W. H. Van Der Putten. **2005**. Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. Entomol. Exp. Appl. 115: 161–170.

Dhandaydham, M., L. Charles, H. Zhu, J. L. Starr, T. Huguet, D. R. Cook, J. M. Prosperi, and C. Opperman. **2008**. Characterization of root-knot nematode resistance in *Medicago truncatula*. J. Nematol. 40: 46–54.

Eder, J., and E. G. Cosio. **1994**. Elicitors of plant defense responses. Int. Rev. Cytol. 148: 1–36.

EPA. **2009**. Carbofuran cancellation process. (https://archive.epa.gov/pesticides/reregistration/web/html/carbofuran_noic.html).

Fay, M. F., and P. J. Dale. **1993**. Condensed tannins in *Trifolium* species and their significance for taxonomy and plant breeding. Genet. Resour. Crop Evol. 40: 7–13.

Frost, C. J., M. C. Mescher, J. E. Carlson, and C. M. De Moraes. **2008**. Plant defense priming against herbivores: Getting ready for a different battle. Plant Physiol. 146: 818–824.

Gerard, P. J. 2001. Dependence of *Sitona lepidus* (Coleoptera: Curculionidae) larvae on abundance of white clover *Rhizobium* nodules. Bull. Entomol. Res. 91: 149–52.

Godfrey, L. D., K. V. Yeargan, and R. B. Muntifering. **1987**. Digestibility, protein content, and nutrient yields of alfalfa stressed by selected early season insect pests and diseases. J. Econ. Entomol. 80: 257–262.

Godfrey, L. D., and K. V Yeargan. **1987**. Effects and interactions of early season pests on alfalfa yield in Kentucky. J. Econ. Entomol. 80: 248–256.

Goldson, S. L., G. W. Bourdot, and J. R. Proffitt. **1987**. A study of the effects of *Sitona discoideus* (Coleoptera: Curculionidae) larval feeding on the growth and development of lucerne (*Medicago sativa*). J. Appl. Ecol. 24: 153–161.

Goldson, S. L., P. D. Jamieson, and G. W. Bourdôt. **1988**. The response of field-grown lucerne to a manipulated range of insect-induced nitrogen stresses. Ann. Appl. Biol. 113: 189–196.

Gotlieb, A., N. Pellett, and B. Parker. **1987**. Sitona/*Fusarium*/cold hardiness interaction in alfalfa. Phytopathology. 77: 1615.

Graham, J. H., and R. C. Newton. **1960**. Relationship between injury by the clover root curculio and incidence of *Fusarium* root rot in Ladino white clover. Plant Dis. Report. 44: 534–535.

Griffin, G. D. 1980. Interrelationship of *Meloidogyne hapla* and *Ditylenchus dipsaci* on resistant and susceptible alfalfa. J. Nematol. 12: 287–293.

Griffin, G. D., and J. H. Elgin. **1977**. Penetration and development of *Meloidogyne hapla* in resistant and susceptible alfalfa under differing temperatures. J. Nematol. 9: 51–66.

Hackell, D. L., and P. J. Gerard. **2004**. Nodule preference by first instar clover root weevil. New Zeal. Plant Prot. 57: 319=322.

Havlíčková, H. 1980. Causes of different feeding rates of pea leaf weevil *Sitona lineatus* on three pea cultivars. Entomol. Exp. Appl. 27: 287–292.

Hill, R. R., R. C. Newton, K. E. Zeiders, and J. H. Elgin. **1969**. Relationships of the clover root curculio, *Fusarium* wilt, and bacterial wilt in alfalfa. Crop Sci. 9: 327–329.

Hunter, M. D. 2001. Out of sight , out of mind: the impacts of root-feeding insects in natural and managed systems. Agric. For. Entomol. 3: 3–9.

Johnson, S. N., A. N. E. Birch, P. J. Gregory, and P. J. Murray. **2006**. The “mother knows best” principle: Should soil insects be included in the preference-performance debate? Ecol. Entomol. 31: 395–401.

Kafle, D., A. Hänel, T. Lortzing, A. Steppuhn, and S. Wurst. **2017**. Sequential above- and belowground herbivory modifies plant responses depending on herbivore identity. BMC Ecol. 17: 1–10.

Kaloshian, I., W. H. Lange, and V. M. Williamson. **1995**. An aphid-resistance locus is tightly linked to the nematode-resistance gene, *Mi*, in tomato. Proc. Natl. Acad. Sci. U. S. A. 92: 622–625.

Kaplan, I., R. Halitschke, A. Kessler, S. Sardanelli, and R. F. Denno. **2008**. Constitutive and induced defences to herbivory in above- and below plant tissues. Ecology. 89: 392–406.

Kreitner, G. L., and E. L. Sorensen. **1979**. Glandular trichomes on *Medicago* species. Crop Sci. 19: 380–384.

Leath, K. T., and A. A. Hower. **1993**. Interaction of *Fusarium oxysporum* f.sp. *medicaginis* with feeding activity of clover root curculio larvae in alfalfa. Plant Dis. 77: 345–348.

Lucas, P. W., I. M. Turner, N. J. Dominy, and N. Yamashita. **2000**. Mechanical defences to herbivory. Ann. Bot. 86: 913–920.

Maureira, I. J., and T. C. Osborn. **2005**. Molecular markers in genetics and breeding: Improvement of alfalfa (*Medicago sativa* L.), pp. 139–154. *In* Lorz, H., Wenzel, G. (eds.), Mol. Marker Syst. Plant Breed. Crop Improv. Heidelberg.

Morrill, W. L., G. D. Kushnak, P. L. Bruckner, and J. W. Gabor. **1994**. Wheat stem sawfly (Hymenoptera: Cephidae) damage, rates of parasitism, and overwinter survival in resistant wheat lines. J. Econ. Entomol. 87: 1373–1376.

Mowat, D. J., and S. Clawson. **1996**. Oviposition and hatching of the clover weevil *Sitona lepidus* Gyll. (Coleoptera: Curculionidae). Grass Forage Sci. 51: 418–423.

NAFA. **2017**. Alfalfa variety ratings: Winter survival, fall dormancy and pest resistance ratings for alfalfa varieties. National Alfalfa & Forage Alliance.

Neally, J., R. Deubler, J. L. Hansen, E. Thomas, J. Yaeger, and D. R. Viands. **2006**. Heritability of clover root curculio resistance in alfalfa. *In* 2006 North Am. Alfalfa Improv. Conf. Bloomington, MN.

Nombela, G., V. M. Williamson, and M. Muñiz. **2003**. The root-knot nematode resistance gene *Mi-1.2* of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. Mol. Plant-Microbe Interact. 16: 645–649.

Pedersen, M. W., D. K. Barnes, E. L. Sorensen, G. D. Griffin, M. W. Nielson, R. R. Hill, F. I. Frosheiser, R. M. Sonoda, C. H. Hanson, O. J. Hunt, R. N. Peaden, J. H. Elgin, T. E. Devine, M. J. Anderson, B. P. Goplen, L. J. Elling, and R. E. Howarth. **1976**. Effects of Low and high saponin selection in alfalfa on agronomic and pest resistance traits and the interrelationship of these traits. Crop Sci. 16: 193–199.

Pedersen, M. W., E. L. Sorensen, and M. J. Anderson. **1975**. A comparison of pea aphid-resistant and susceptible alfalfas, for field performance, saponin concentration, digestibility, and insect resistance. Crop Sci. 15: 254–256.

Platt, A. W., C. W. Farstad, and J. A. Callenbach. **1948**. The reaction of rescue wheat to sawfly damage. Sci. Agric. 28: 154–161.

Postnikova, O. A., M. Hult, J. Shao, A. Skantar, and L. G. Nemchinov. **2015**. Transcriptome analysis of resistant and susceptible alfalfa cultivars infected with root-knot nematode *Meloidogyne incognita*. PLoS One. 10: e0123157.

Potenza, C., S. H. Thomas, and C. Sengupta-Gopalan. **2001**. Genes induced during early response to *Meloidogyne incognita* in roots of resistant and susceptible alfalfa cultivars. Plant Sci. 161: 289–299.

Price, S. J. 2017. Understanding the biology of clover root curculio and improving their management using biofumigation in alfalfa. M.S. Thesis. Utah State University, Logan, UT.

Quinn, M. A., and M. H. Hall. **1992**. Compensatory response of a legume root-nodule system to nodule herbivory by *Sitona hispidulus*. Entomol. Exp. Appl. 64: 167–176.

Quinn, M. A., and A. A. Hower. **1986a**. Effects of root nodules and taproots on survival and abundance of *Sitona hispidulus* (Coleoptera: Curculionidae) on *Medicago sativa*. Ecol. Entomol. 11: 391–400.

Quinn, M. A., and A. A. Hower. **1986b**. Alfalfa rhizosphere: The unknown world of clover root curculio, pp. 115–129. *In* 16th Natl. Alfalfa Symp. Fort Wayne, Indiana.

Ramanujan, K. 2014. New alfalfa variety resists ravenous local pest. Phys.org. (https://phys.org/news/2014-04-alfalfa-variety-resists-ravenous-local.html).

Ramirez, R. A., and L. R. Spears. **2014**. Stem nematode counteracts plant resistance of aphids in alfalfa, *Medicago Sativa*. J. Chem. Ecol. 40: 1099–1109.

Ranger, C. M., and A. a Hower. **2001**. Glandular morphology from a perennial alfalfa clone resistant to the potato leafhopper. Crop Sci. 41: 1427–1434.

Ranger, C. M., R. E. K. Winter, E. A. Backus, G. E. Rottinghaus, M. R. Ellersieck, and D. W. Johnson. **2005**. Discrimination by the potato leafhopper (Hemiptera: Cicadellidae) of host volatiles from resistant and susceptible alfalfa, *Medicago* *sativa* L.. Environ. Entomol. 34: 271–280.

Rim, K., S. J. Price, E. J. Wenninger, R. Long, and R. A. Ramirez. **2019**. Biology and management of clover root curculio (Coleoptera: Curculionidae). J. Integr. Pest Manag. 10: 1–14.

Rodriguez-Saona, C. R., R. O. Musser, H. Vogel, S. M. Hum-Musser, and J. S. Thaler. **2010**. Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. J. Chem. Ecol. 36: 1043–1057.

Rossi, M., F. L. Goggin, S. B. Milligan, I. Kaloshian, D. E. Ullman, and V. M. Williamson. **1998**. The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid, pp. 9750–9754. *In* Proc. Natl. Acad. Sci. U. S. A.

Sen, S., H. P. S. Makkar, and K. Becker. **1998**. Alfalfa saponins and their implication in animal nutrition. J. Agric. Food Chem. 46: 131–140.

Shade, R. E., T. E. Thompson, and W. R. Campbell. **1975**. An alfalfa weevil larval resistance mechanism detected in *Medicago*. J. Econ. Entomol. 68: 399–404.

Snapp, S. S., and C. P. Vance. **1986**. Asparagine biosynthesis in alfalfa (*Medicago sativa* L.) root nodules. Plant Physiol. 82: 390–395.

Soler, R., T. M. Bezemer, W. H. Van Der Putten, L. E. M. Vet, and J. A. Harvey. **2005**. Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. J. Anim. Ecol. 74: 1121–1130.

Stout, M. J., K. V Workman, R. M. Bostock, and S. S. Duffey. **1998**. Specificity of induced resistance in the tomato, *Lycopersicon esculentum*. Oecologia. 113: 74–81.

Sulc, R. M., E. Van Santen, K. D. Johnson, C. C. Sheaffer, D. J. Undersander, L. W. Bledsoe, D. B. Hogg, and H. R. Willson. **2001**. Glandular-haired cultivars reduce potato leafhopper damage in alfalfa. Agron. J. 93: 1287–1296.

Teuber, L., J. Jernstedt, and K. Foord. **1988**. Alfalfa growth and development, pp. 39–44. *In* Proc. 18th Calif. Alfalfa Symp. Modesto, CA.

Tietz, N. 2012. Mysterious alfalfa menace: Clover root curculio sneak-attacks Western fields. Hay Forage Grow. 14–15.

Vankosky, M. A., H. A. Cárcamo, R. H. McKenzie, and L. M. Dosdall. **2011**. Integrated management of *Sitona lineatus* with nitrogen fertilizer, *Rhizobium*, and thiamethoxam insecticide. Agron. J. 103: 565–572.

Viands, D. R., J. L. Hansen, E. M. Thomas, and J. L. Neally. **2004a**. Registration of ’ Oneida Ultra ’ alfalfa. Crop Sci. 44: 1484–1485.

Viands, D. R., J. L. Hansen, E. M. Thomas, and J. L. Neally. **2004b**. Registration of ’ Seedway 9558 ’ alfalfa. Crop Sci. 44: 1485.

Vos, P., G. Simons, T. Jesse, J. Wijbrandi, L. Heinen, R. Hogers, A. Frijters, J. Groenendijk, P. Diergaarde, M. Reijans, J. Fierens-Onstenk, M. De Both, J. Peleman, T. Liharska, J. Hontelez, and M. Zabeau. **1998**. The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. Nat. Biotechnol. 16: 1365–1369.

Wenninger, E. J., and G. E. Shewmaker. **2014**. Clover root curculio in alfalfa: Identification, biology, and management. Pacific Northwest Ext. PNW 663: 1–5.

Williamson, V. M., and A. Kumar. **2006**. Nematode resistance in plants: the battle underground. Trends Genet. 22: 396–403.

Wolfson, J. L. 1987. Impact of *Rhizobium* nodules on *Sitona hispidulus,* the clover root curculio. Entomol. Exp. Appl. 43: 237–243.

Wondafrash, M., N. M. Van Dam, and T. O. G. Tytgat. **2013**. Plant systemic induced responses mediate interactions between root parasitic nematodes and aboveground herbivorous insects. Front. Plant Sci. 4: 87.

Wurst, S., and W. H. van der Putten. **2007**. Root herbivore identity matters in plant-mediated interactions between root and shoot herbivores. Basic Appl. Ecol. 8: 491–499.

York, A. C., G. G. Gyrisco, and C. M. Edmonds. **1971**. The status of the alfalfa snout beetle in New York state. J. Econ. Entomol. 64: 1332–1333.

Tables and Figures

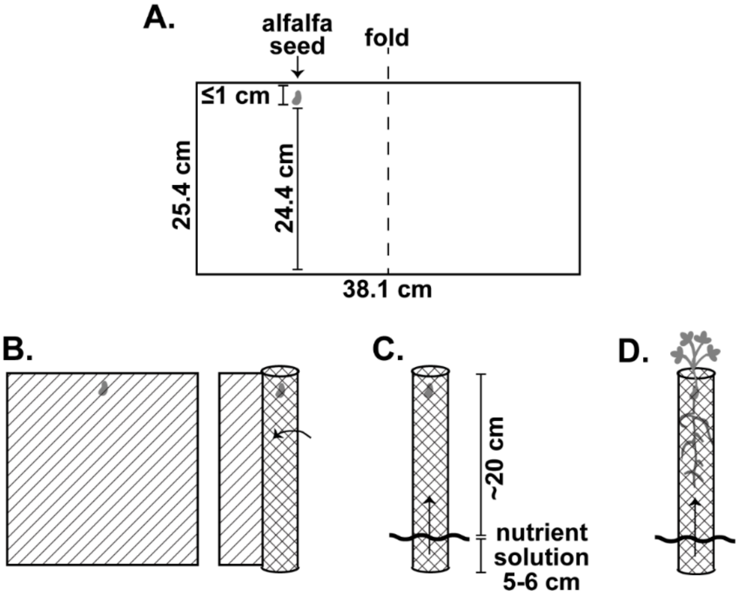


Fig. 4-1. *Medicago sativa* plants were grown hydroponically to be tested against *Sitona hispidulus* larvae. (A) One rhizobia-inoculated (Nitragin® Gold, Monsanto, St. Louis, MO) alfalfa seed was placed no more than 1 cm from the top edge of a moistened germination paper (38.1 × 25.4 cm) (Anchor Paper Inc, St. Paul, MN), which was then folded in half. (B) Papers with seeds were rolled into cylinders, and (C) placed into containers filled with a hydroponic growth solution (0 N-10 P-10 K, Alaska Morbloom, Central Garden and Pet Company, Walnut Creek, CA; Immunox Multi-purpose Fungicide, Spectracide, Spectrum Brands, Madison, WI) covering the bottom 5-6 cm of papers. Capillary action through the papers appropriately moistened seeds for germination and growth. (D) Plants were grown for approximately 2-4 months prior to use in larval trials.

Table 4-1. Larval *Sitona hispidulus* feeding and development on alfalfa check variety and root pathogen-resistant commercial alfalfa varieties. Mean (± SEM) number of nodules, number of nodules consumed by larval *S. hispidulus*, proportion of nodules consumed, and larval *S. hispidulus* head capsule width for each variety. *CRC = clover root curculio, RKN = root knot nematode, R = Resistant, HR = Highly resistant.

Variety	**Resistance/ Susceptibility***	**No. Nodules**	**No. Nodules Consumed**	**Proportion of nodules consumed**	**Larval head capsule width (mm)**
Ranger	CRC and RKN susceptible	5.78 ± 0.46	2.56 ± 0.53	0.48 ± 0.10	0.45 ± 0.04
PGI437	RKN-HR, *Phytopthora*-R	5.18 ± 0.30	3.18 ± 0.30	0.64 ± 0.07	0.55 ± 0.03
PGI459	RKN-HR, *Phytopthora*-HR	5.21 ± 0.30	2.14 ± 0.30	0.41 ± 0.05	0.48 ± 0.03

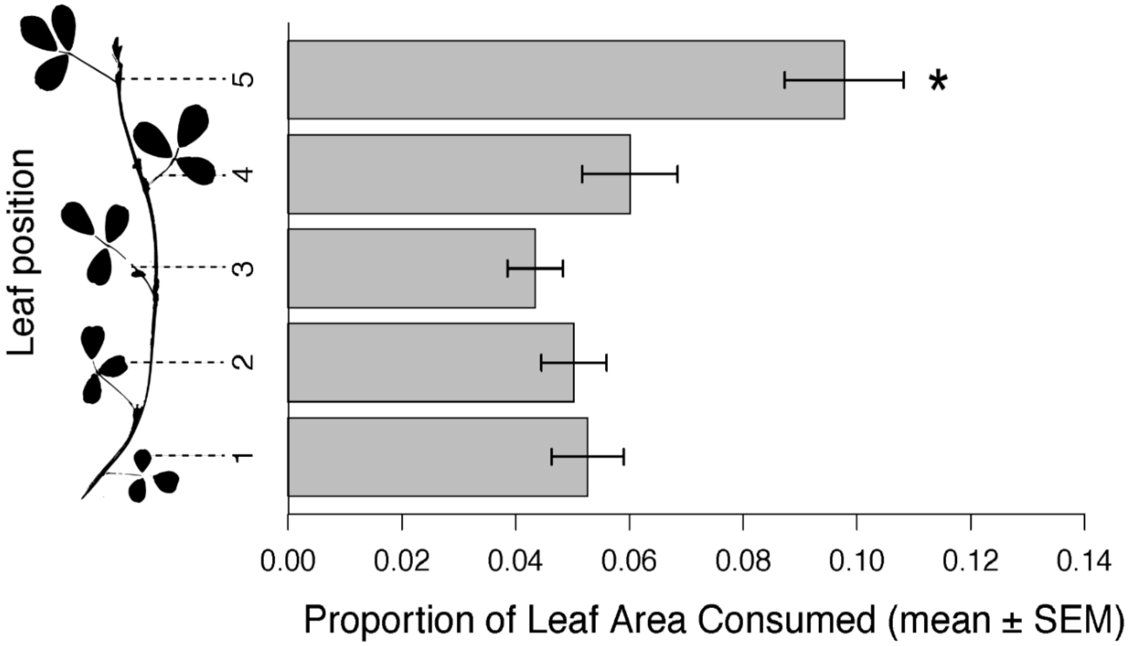


Fig. 4-2. Comparison of leaf area consumed by adult *Sitona hispidulus* (Ranger, PGI437, and PGI459 combined) by leaf position (1 being the basal most trifoliate leaf and 5 the apical trifoliate leaf). Values shown are the mean (± SEM) proportion of leaf area consumed by caged mated pairs of adults. Asterisk indicates significant difference via Tukey test ($P < 0.05$).

Table 4-2. Mean (± SEM) alfalfa leaf area, area consumed, and proportion of area consumed by adult *Sitona hispidulus* on root knot nematode-resistant commercial alfalfa varieties and Ranger. Proportion of leaf area consumed was calculated by dividing leaf area consumed by total leaf area.

Variety	**Leaf Area (mm^2)**	**Leaf Area Consumed (mm^2)**	**Proportion of Leaf Area Consumed**
Ranger	1,761.44 ± 175.19	109.98 ± 14.71	0.06 ± 0.01
PGI437	2,335.96 ± 445.59	126.83 ± 25.02	0.05 ± 0.01
PGI459	1,816.43 ± 201.27	112.64 ± 14.70	0.07 ± 0.01

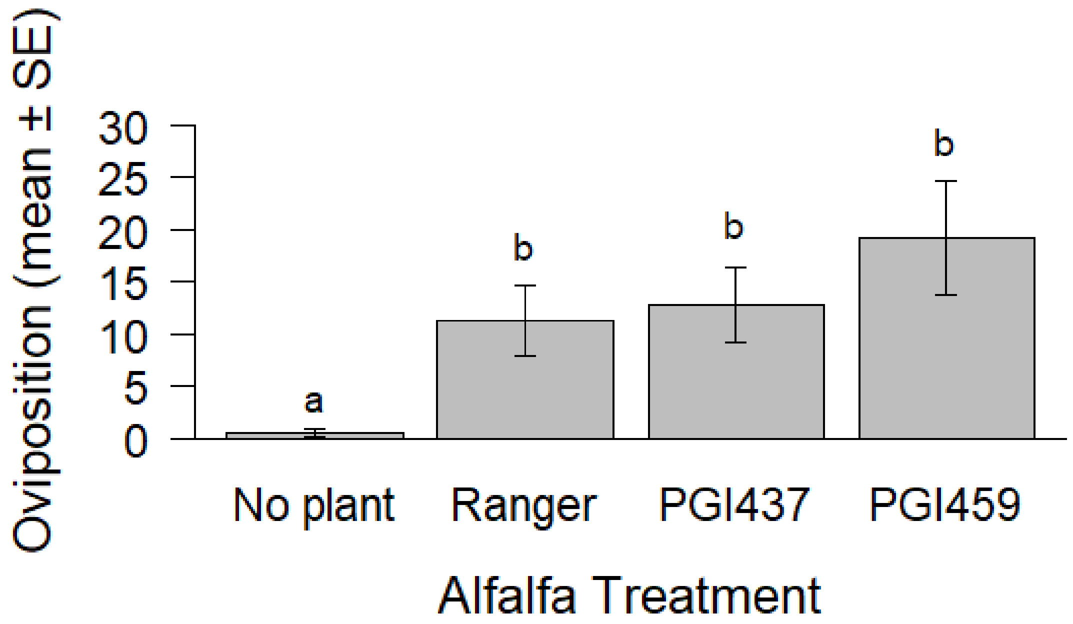


Fig. 4-3. Mean (± SE) number of eggs laid by adult female *Sitona hispidulus* oviposition on the no-plant treatment, Ranger, PGI437, and PGI459. Different letters above bars indicate significant differences via a Tukey test with a 95% confidence index (MultComp package).

Table 4-3. Larval *Sitona hispidulus* feeding and development on Cornell University developmental lines. Mean (± SEM) total number of nodules, number of nodules consumed by larval *S. hispidulus*, proportion of nodules consumed, and larval *S. hispidulus* head capsule width for each experimental alfalfa line. CRC = clover root curculio. *Different letters within column indicate significantly different means via Kruskall Wallis rank sum test ($p \leq 0.05$).

Line	**Resistance/ Susceptibility**	**No. Nodules**	**No. Nodules Consumed**	**Proportion of nodules consumed ***	**Larval head capsule width (mm)**
NY1718	CRC-susceptible (1 cycle)	4.33 ± 0.33	2.33 ± 0.88	0.57 ± 0.23ab	0.59 ± 0.05
NY1717	CRC-susceptible (1 cycle)	4.67 ± 0.43	2.17 ± 0.54	0.44 ± 0.09ab	0.50 ± 0.04
NY1713	CRC-resistant (4 cycle)	9.20 ± 1.32	1.53 ± 0.17	0.22 ± 0.04a	0.43 ± 0.03
NY1720	CRC-resistant (4 cycle)	5.11 ± 0.39	3.00 ± 0.41	0.64 ± 0.11b	0.50 ± 0.05
NY1719	CRC-resistant (3 cycle)	5.40 ± 0.58	2.30 ± 0.42	0.48 ± 0.10ab	0.43 ± 0.04

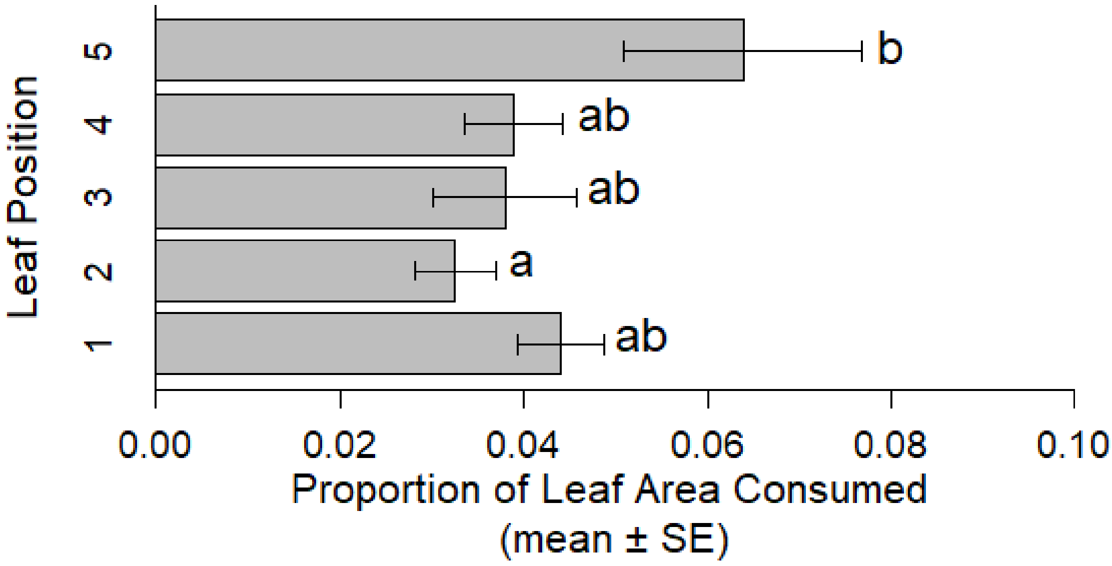


Fig. 4-4. Comparison of leaf area consumed by adult *Sitona hispidulus* (NY1718 and NY1713 combined) by leaf position (1 being the basal most trifoliate leaf and 5 the apical trifoliate leaf). Values shown are the mean (± SEM) proportion of leaf area consumed by caged mated pairs of adults. Different letters above bars indicate significant differences via a Tukey test with a 95% confidence index.

Table 4-4. Mean (± SEM) total leaf area, area consumed, proportion of area consumed, and oviposition by adult *Sitona hispidulus* on CRC-susceptible (NY1718) and CRC-resistant (NY1713) developmental lines. Proportion of leaf area consumed was calculated by dividing leaf area consumed by total leaf area.

Line	**Total Leaf Area (mm^2)**	**Leaf Area Consumed (mm^2)**	**Proportion of Leaf Area Consumed**	**Oviposition**
NY1718	1,774.58 ± 140.57	67.67 ± 7.78	0.04 ± 0.01	22.53 ± 5.11
NY1713	2,198. 80 ± 175.11	78.65 ± 9.78	0.04 ± 0.01	23.76 ± 4.67

CHAPTER V

SUMMARY AND CONCLUSION

Summary

The current knowledge and available pest control tactics for clover root curculio (CRC) primarily comes from historic research on eastern U.S. populations. Novel studies have provided valuable information about CRC biology in the Intermountain West and now that updated phenological information can inform monitoring and collection protocols, research can now focus on the development of updated control strategies. However, management of belowground pests can be challenging and synthetic insecticides have not been registered to target CRCs susceptible larval stage belowground. Integrated pest management of similar belowground weevil pests (*e.g.*, alfalfa snout beetle and black vine weevil) has found success through the isolation and application of biological insecticides. Furthermore, host plant resistance in alfalfa, especially towards pathogens and hemipteran pests, is well-studied but we lack an understanding of alfalfa resistance towards chewing insects such as CRC.

In the first study, we surveyed northern Utah alfalfa fields with known resident populations of CRC for entomopathogenic nematodes (EPNs) and fungi. Similar to previous reports, we found *Beauveria* spp. fungi most commonly infecting CRC immatures. We did not isolate EPNs from alfalfa field soil, and we posit this may be a result of soil textures and low soil moistures resulting from alfalfa management that are unsupportive of EPN survival. We urge future research to evaluate locally-adapted strains of *Beauveria* spp. for potential augmentative applications in CRC immature management.

In the second field study, we evaluated the direct and indirect suppressive effects of biological insecticides (*Steinernema riobrave*, *Beauveria bassiana*, and *Bacillus thuringiensis* spp. *galleriae*) and a synthetic systemic (flupyradifurone) insecticide on field populations of CRC immatures across two phenological times (before larval peak and during larval peak) and two field seasons (2018-2019). Surprisingly, we found that neither biological nor systemic insecticides reduced resident CRC populations or affected larval development compared to untreated controls, and overall, application timing did not improve insecticide efficacy. Root damage was also similar across control and insecticide treatments. Differing mechanisms may exist for the lack of success of these products. For instance, flupyradifurone translocation to belowground systems is not well understood. Further, many biotic and abiotic variables affect biological insecticide establishment and persistence. For instance, penetration of *Bacillus thuringiensis* in soil strata is reduced in soils with high clay. To offset biological agent mortality due to the low soil moisture and high UV, heat, and temperature levels in Intermountain West climates, EPN application rates may need to increase, but this could be cost prohibitive. Further, CRC immatures' cryptic belowground nature, aggregated population distributions, and low sample numbers make evaluating field suppression challenging.

In the final laboratory study, three commercial alfalfa varieties with varying resistance toward root pathogens were evaluated for potential cross-resistance, and five developmental alfalfa lines were tested for their purported resistance or susceptibility to CRC adults and larvae. Adults and larvae were able to feed, oviposit, and develop on all test varieties and lines. Neither differences in adult leaf consumption and oviposition nor differences in larval development and nodule consumption were observed in cross-resistance trials, suggesting root-pathogen resistance may not impact CRC. Likewise, adult leaf consumption and oviposition were similar between CRC-resistant and CRC-susceptible lines. Although larval development did not differ between lines, the proportion of nodules consumed was significantly lower on one CRC-resistant line due to its fast nodule growth overall. Increased nodulation may lead to increased root vigor and improved plant health making this line more tolerant of CRC larval damage.

Conclusion

Coincident with the federal ban on the soil-active insecticide, carbofuran, CRC has become a major pest throughout the U.S.; the need to develop modern management programs has increased. The research presented herein represents the foundations of contemporary integrated pest management and host-plant resistance for CRC in Intermountain West alfalfa production. In agreement with previous research, we have identified *Beauveria* spp. fungi as the most prominent entomopathogenic fungi naturally occurring in Intermountain West CRC-infested alfalfa. Knowing this, various virulent strains can be collected, tested, and developed into a CRC-specific and locally adapted biological control agent, ideally better at targeting damaging CRC larvae and reducing synthetic insecticide inputs. However, this research outlines some of the challenges in targeting CRC larvae with insecticides and some of the specific hurdles for biological insecticide applications in similarly hot and dry climates. In anticipation of these challenges, continued product testing, especially of biological insecticides, should use innovative techniques to aid in insecticide soil penetration and biological survival. From this work, we can also begin to understand and speculate about the mechanisms of alfalfa resistance or tolerance to CRC and better inform the continued development of CRC-resistant alfalfa cultivars. Overall, this research offers some insight into the intricacies and challenges of CRC belowground management; laying the foundation for future investigations into integrated management of CRC.

APPENDICES

APPENDIX A

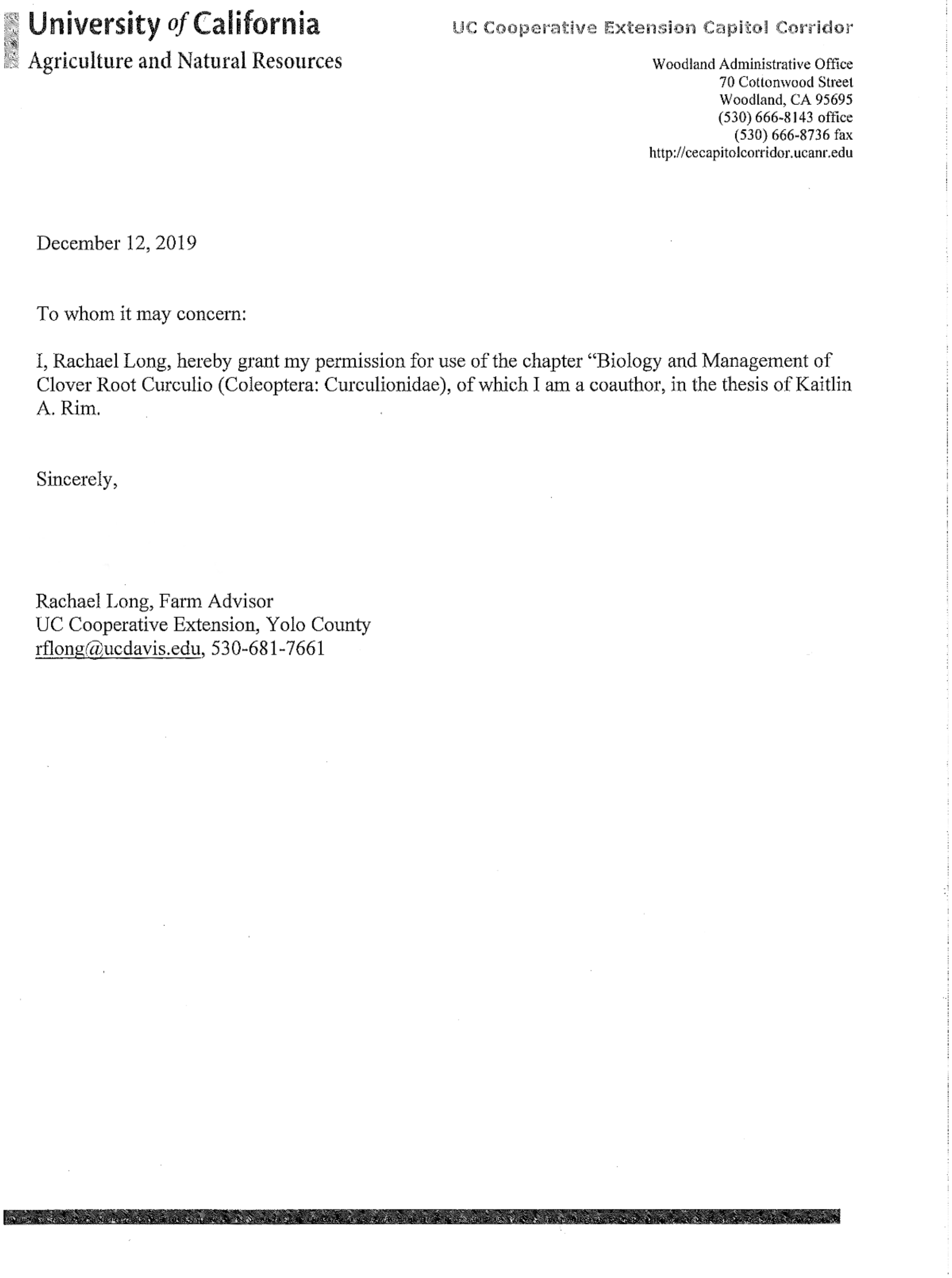
AUTHORSHIP AND CITATION OF PUBLISHED CHAPTERS

Chapter I:

This is a pre-copyedited, author-produced version of an article accepted for publication in the *Journal of Integrated Pest Management* following peer review. The version of record **Rim, K., S. J. Price, E. J. Wenninger, R. Long, and R. A. Ramirez**. 2019. Biology and management of clover root curculio (Coleoptera: Curculionidae). Journal of Integrated Pest Management 10: 1-14 is available online at: https://doi.org/10.1093/jipm/pmz020.

APPENDIX B

LETTERS OF PERMISION



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December 12, 2019

To whom it may concern:

I, Rachael Long, hereby grant my permission for use of the chapter “Biology and Management of Clover Root Curculio (Coleoptera: Curculionidae), of which I am a coauthor, in the thesis of Kaitlin A. Rim.

Sincerely,

Rachael Long, Farm Advisor
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December 16, 2019

To whom it may concern,

I, Steven Price, hereby grant my permission for use of the chapter "Biology and Management of Clover Root Curculio (Coleoptera: Curculionidae)", of which I am a coauthor, in the thesis of Kaitlin A. Rim.

Sincerely,

Steven J. Price
Extension Assistant Professor of Agriculture, Natural Resources & 4-H

APPENDIX C

ROOT-PATHOGEN RESISTANT COMMERCIAL VARIETY AND CLOVER ROOT CURCULIO-RESISTANT DEVELOPMENTAL LINE SAPONIN CONTENT

Materials and Methods

Saponin levels were measured following the methods described in Kendall (1964) for all commercial varieties in cross-resistance trials (Ranger, PGI437, PGI459) and two clover root curculio (CRC)-resistant lines, NY1713 and NY1719. Briefly, 3 g of fresh plant material and 150 mL of distilled water was placed in a laboratory blender and plant tissue was ground on high for 2 min. Some foam was created during this process. The solvent was transferred to a beaker (1,000 mL) and another 150 mL of distilled water was added. This solution was mixed with an electric food mixer on high for 2 min to create foam. After mixing, the solution was immediately transferred to a 1,000 mL graduated cylinder, allowed to stand for 2 min, and then shaken to eliminate air spaces left by large unstable bubbles. The total volume (mL) of foam was then taken as a rough measure of the saponin level for that plant material. Saponin foam levels were measured for both the roots and shoots of each plant and repeated for 15 plants of each variety.

Commercial varieties and developmental CRC-resistant lines were analyzed separately using R software (RStudio). Saponin foam volume data were $\sqrt{x}$ transformed to obtain data normality and homoscedasticity. To compare the volume of saponin among the roots and shoots of alfalfa varieties and lines, a two-way Analysis of Variance (ANOVA) was performed where plant part (root or shoot) and variety or line were the two main factors. Following significant ANOVAs, Tukey HSD with a 95% confidence index was performed to separate significantly different group means.

Results

Commercial Varieties

Saponin foam volume was significantly affected by plant part (root or shoot) and variety (plant part × variety interaction: F = 3.42, df = 2, 96, $P = 0.037$) (Fig. B1-A). The roots of Ranger and the moderate root-pathogen resistant variety, PGI437, had the highest saponin foam levels at 285.3 ± 29.8 and 300.4 ± 48.4 mL of saponin foam, respectively (Fig. B1-A). Contrastingly, Ranger shoots as well as the roots and shoots of PGI459, the variety with high resistance to root pathogens, had the lowest saponin foam volumes (Fig. B1-A).

CRC-Resistant Lines

Similarly, there was a significant interaction between plant part and CRC-resistant line (plant part × line interaction: F = 5.65, df = 1, 48, $P = 0.022$) (Fig. B1-B). Saponin levels in the roots of NY1713 were approximately half that of its shoots ($P < 0.001$) or either tissue in NY1719 (roots: $P = 0.02$, shoots: $P < 0.001$) (Fig. B1-B).

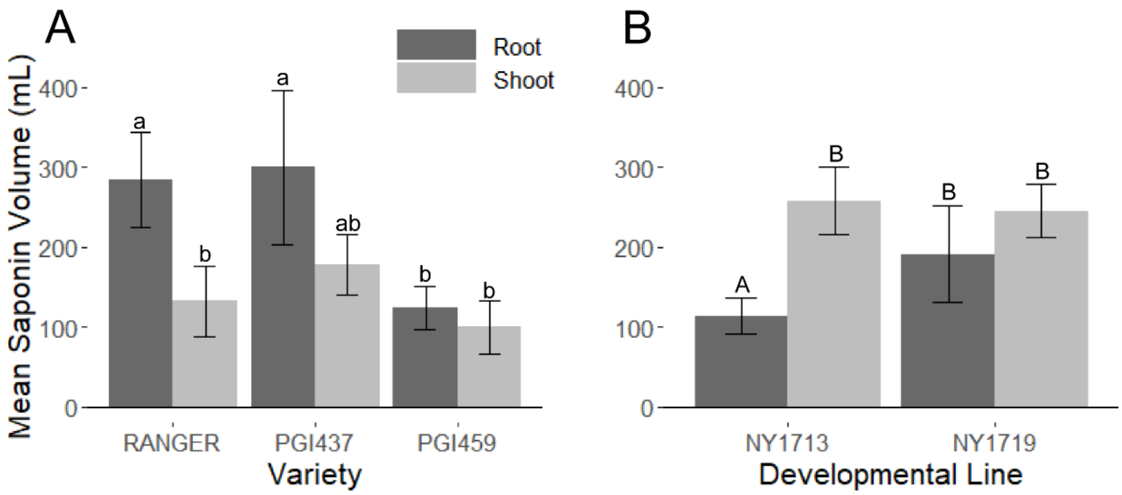

Fig. B1: Mean (±SE) saponin foam volume (mL) for the roots and shoots of (A) commercial alfalfa varieties and (B) CRC-resistant developmental lines. Different letters above bars indicate significant differences via Tukey test with a 95% confidence index.

Conclusions

The insecticidal activity of alfalfa saponins is well-known (Nozzolillo et al., 1997; Oleszek et al. 1990; Tava and Odoardi, 1996), and previous research by Pedersen et al. (1976) demonstrated that alfalfa plants selected to express overall higher levels of saponin were positively correlated with CRC resistance. Further research revealed that the potentially CRC-resistant Lahontan variety (40-48% resistance) exhibited higher root saponins than 7 out of the 11 varieties evaluated despite its being among those varieties with the lowest foliage saponin levels (Pedersen et al. 1975). Based on these results, we estimated saponin levels in the roots and shoots of all commercial varieties (Ranger, PGI437, PGI459) and two CRC-resistant developmental lines (NY1713 and NY1719) tested against CRC adults and larvae in Chapter IV.

Adult feeding was similar among the commercial varieties (Chapter IV, Table 4-2) and similarly, shoot saponin levels across these varieties were statistically indistinguishable. However, while root saponin volume was approximately two times higher in Ranger and PGI437, when compared to PGI459 with high resistance to root pathogens, the proportion of nodules consumed by CRC larvae and larval head capsule widths (larval development) (Chapter IV, Table 4-1) were not affected by saponin differences. Similarly, nodule consumption and larval head capsule widths did not differ among NY1713 and NY1719 (Chapter IV, Table 4-3), although saponin foam volume in NY1713 roots was half that of NY1719 roots.

These results suggest that the correlation between saponins and CRC resistance may not be as strong as previously assumed, or that more precise saponin measurement techniques are needed to fully understand this relationship. Historic research formed hypotheses on CRC-resistance ratings calculated from an arbitrary 0-5 scale (0 = “no symptoms of damage”, 5 = “severe damage”), did not directly sample CRC immature populations, and often did not statistically analyze CRC data to determine differences (Pedersen et al. 1975, 1976). Furthermore, while the methods described in Kendall (1964) are simple and allow for assaying large sample sizes quickly, it only provides a crude look at total saponins. Saponins are a broad class of chemicals and further work would be necessary to isolate specific saponins important for suppression. Lastly, many advancements in our understanding of host-plant resistance and defense have occurred since these publications in the 1970s. Thus, although CRC-resistance in Lahontan was seemingly correlated with increased root saponins, other physical or chemical defenses may have also contributed to resistance.

References

Hanson, C. H., Kohler, G. O., Dudley, J. W., Sorensen, E. L., Van Atta, G. R., Taylor, K. W., Pedersen, M. W., Carnahan, H. L., Wilsie, C. P., Kehr, W. R., Lowe, C. C., Standford, E. H., & Yungen, J. A. (1963) *Saponin content of alfalfa as related to location, cutting, variety, and other variables.* [Bulletin].Washington, DC: USDA- ARS.

Kendall, W.A. (1964) Foam formation from forages in vitro. *Crop Science, 4*:391-393.

Nozzolillo, C., Arnason, J.T., Campos, F., Donskov, N., Jurzysta, M. (1997) Alfalfa leaf saponins and insect reistance. *Journal of Chemical Ecology, 23*(4): 995–1002. doi: 1023/B:JOEC.0000006384.60488.94

Oleszek, W., Price, K.R., Colquhoun, I.J., Jurzysta, M., Ploszynski, M., Fenwick, G.R. (1990) *Journal of Agricultural and Food Chemistry, 38*: 1810–1817. doi: abs/10.1021/jf9901237

Pedersen, M. W., Sorensen, E. L., & Anderson, M. J. (1975) A comparison of pea aphid-resistant and susceptible alfalfas for field performance, saponin concentration, digestibility, and insect resistance. *Crop Science, 15*: 254–256.

Pedersen, M. W., Barnes, D. K., Sorensen, E. L., Griffin, G. D., Nielson, M. W., Hill, R. R., Frosheiser, F. I., Sonoda, R. M., Hanson, C. H., Hunt, O. J.,. Peaden, R. N, Elgin, J. H., Devine, T. E., Anderson, M. J., Goplen, B. P., Elling, L. J., & Howarth R. E. (1976) Effects of low and high saponin selection in alfalfa on agronomic and pest resistance traits and the inter- relationship of these traits. *Crop Science, 16*: 193–199.

Tava, A. & Odoardi, M. (1996) Saponins from *Medicago* spp.: Chemical characterization and biological activity against insects. In G. Walter and K. Yamasaki (Eds.), *Saponins Used in Food and Agriculture* (pp. 97-109). New York, NY: Plenum Press.

APPENDIX D

ADULT CLOVER ROOT CURCULIO MANAGEMENT AND EFFECTS ON SUBSEQUENT EGG POPULATIONS

Materials and Methods

A field experiment was conducted at the Universtity of California Research and Extension Center in Tulelake, California targeting the adult CRC in early fall after aestivation as they migrate back into alfalfa fields with the aim of reducing egg subsequent egg populations. This was a parallel component of Chapter III evaluating insecticide applications targeting larvae. The experiment was performed in a fourth-year alfalfa stand (variety, WL372HQ Roundup Ready, fall dormancy 5) established in 2014. Each of five replicated blocks (randomized complete block design) consisted of three 8.2 × 6.1 m plots (experimental unit). Plots were randomly assigned one of three treatments: untreated control, lambda-cyhalothrin, and lambda-cyhalothrin combined with a spinosad. Treatments were applied at 33.6 g ai/ha lambda-cyhalothrin (Warrior II®, Syngenta, Basel, Switzerland) and 33.6 g ai/ha lambda-cyhalothrin (Warrior II) + 224.2 g ai/ha Spinosad (Entrust®, Corteva, Wilmington, DE, USA) on September 10, 2018 using a backpack sprayer and a 2.7 m boom. Spray equipment was operated at 207 kPa (187 L/ha) for all treatments.

Adult CRCs were monitored weekly for one month post-application using a standard sweep net and counting the number of weevils from ten, 180^{o} sweeps per plot. Egg populations were estimated approximately 3 weeks post-application (October 3, 2018) and again the following Spring (April 17, 2019) by removing two soil core samples (~20 cm deep × ~20 cm diameter) from each plot with a shovel. All samples were shipped on ice overnight to UC Davis for soil core processing according to the methods described by Rim et al. (2019) to determine egg numbers. Four samples were taken from the field pre-application to determine CRC populations and stages present. Across all pre-application samples, 2 eggs were collected, and no adults were detected from sweeps. Lastly, alfalfa yields (kg/ha) were estimated from the final (4th) harvest of the season on September 26, 2019 to reflect an entire season of CRC immature damage post-application.

Adult CRC data were not analyzed because >60% of sweep samples collected at each time point were devoid of adults, thus, adult data was >70% zeroes and could not be accurately analyzed. Egg collections in fall and spring were analyzed by separate generalized linear mixed models (GLMMs) with the $\log_{10}$-link and Poisson distribution error where treatment was the main factor and block was considered a random effect. Yield data were analyzed by a linear mixed-effects model with treatment as the main factor and block as a random effect. Following significant analyses, Tukey HSD with a 95% confidence index was performed using the MultComp package for multiple comparisons. All data were analyzed using R software (RStudio).

Results

On average, less than 1 adult CRC were collected from lambda-cyhalothrin (Warrior II) and lambda-cyhalothrin + spinosad (Warrior II + Entrust) treatments, while samples from untreated control plots typically collected >1 adult CRC from sweeps (Fig. C1). Further, mean adults steadily decreased in control plots over time (Fig. C1). Significantly fewer eggs were collected from insecticide treated plots compared to the untreated control at 3-weeks post application in fall 2018 (df = 2, 12, $P < 0.001$) (Fig. C2). Similar trends were observed in eggs collected the following April (spring 2019) where significantly fewer eggs (15.2 ± 5.2 eggs) were collected from lambda-cyhalothrin + spinosad (Warrior II + Entrust) treated plots compared to the untreated control (36.6 ± 5.4 eggs), and an intermediate number of eggs (21.8 ± 5.9 eggs) were collected from lambda-cyhalothrin (Warrior II) treated plots (df = 2, 12 , $P = 0.044$) (Fig. C2). Significant differences in yield (kg/ha) were not observed among treatments from the September 2019 harvest (F = 3.42, df = 2,8, $P = 0.084$) (Fig. C3).

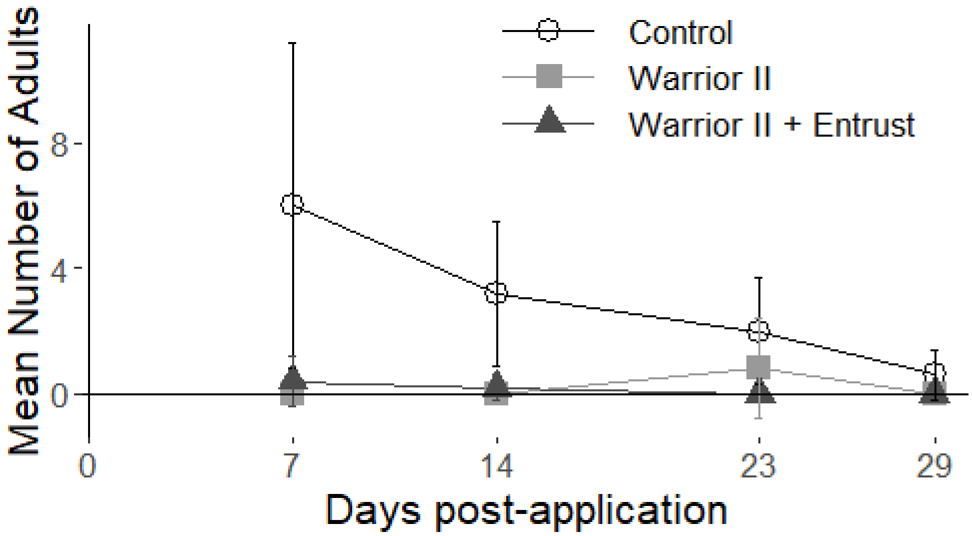

Fig. C1: Mean (±SE) number of adult *Sitona hispidulus* collected for each treatment at various time points after insecticide application on September 10, 2018 to alfalfa at UC Research and Extension Center in Tulelake, California.

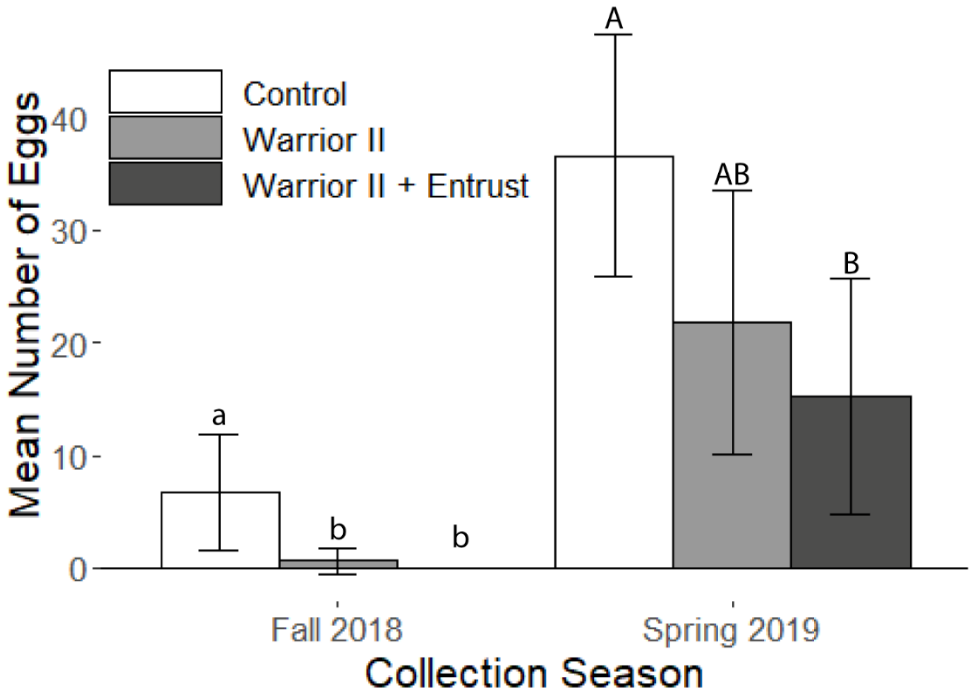

Fig. C2: Mean (±SE) number of *Sitona hispidulus* eggs collected in fall (Oct 3, 2018) and spring (Apr. 17, 2019) for treatments applied to alfalfa at UC Research and Extension Center in Tulelake, California on September 10, 2018. Fall and Spring data were analyzed by separate GLMMs; different letters above bars indicate significant differences via a Tukey test with a 95% confidence index (MultComp package) for each sampling time.

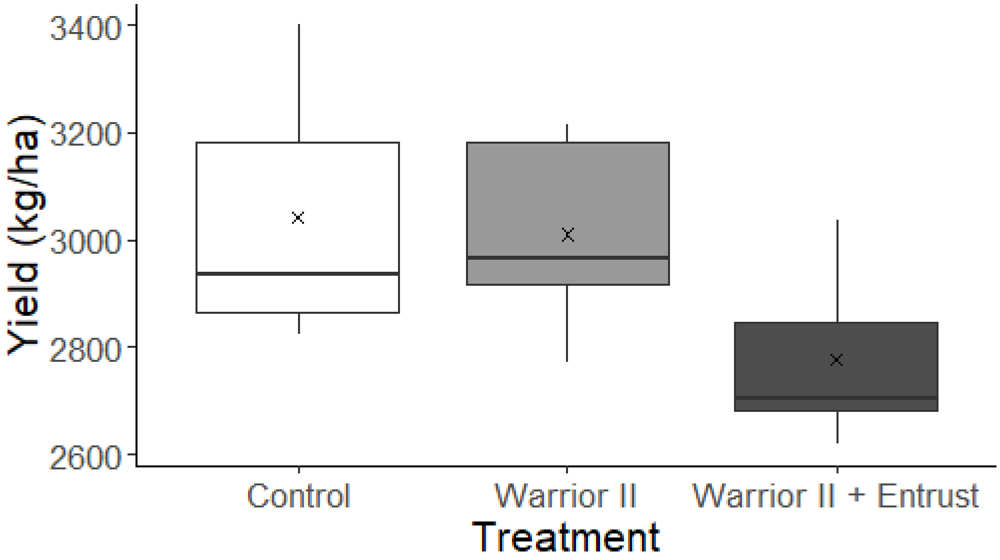

Fig. C3: Alfalfa yield (kg/ha) harvested on September 26, 2019 for treatments applied to alfalfa at UC Research and Extension Center in Tulelake, California the previous fall on September 10, 2018. Significant differences among treatments were not detected at the α = 0.05 by linear mixed-effects model.

Conclusions

As discussed in Chapter III, CRC immature management is difficult, mainly due to their cryptic soil-association. Contrastingly, adult CRC may be easier to access as they are present aboveground and can be easily accessed for monitoring and management. Indeed, several insecticides are registered to target adult CRC, but spring applications are not recommended due to the non-target effects on biocontrol agents and natural enemies of other major alfalfa pests (e.g., alfalfa weevil, aphids) (Rim et al., 2019; Wenninger and Shewmaker, 2014). Further, historic research on the efficacy of prophylactic fall adult reductions has been inconsistent (Godfrey and Yeargan, 1987; Waters, 1964). Therefore, this study aimed to determine if reducing post-aestivation adult populations leads to subsequent immature reductions and potential yield increases.

Clover root curculio adult populations in control plots steadily declined over time, likely a result of natural CRC adult phenology (Rim et al., 2019). As expected, insecticide treatments suppressed CRC adult populations in the fall, even to zero at some collection times. Equally promising reductions of egg populations (<1 egg detected per plot) also occurred in insecticide treated plots in fall 2018, reflecting the decreased number of adults. Overall, egg populations increased in spring, likely due to surviving overwintered adults ovipositing in early spring (Rim et al. 2019), but insecticide treated plots still trended towards reduced egg populations. However, it was still unclear if these reduced egg densities (15-20 eggs/sample) were effective for significantly reducing subsequent larval outbreaks and damage. In fact, yield was unaffected by insecticide treatments, suggesting that observed egg reductions may not suppress CRC larval populations and root damage associated with yeild decreases. But, difficulties in determining yield differences may also be a result of the already existing four years of accumulative CRC damage (Dickason et al., 1968; Hower et al., 1995).

Initial results from this study indicate fall adult management has the potential to reduce subsequent immature populations, although the impact on yield was inconsistent. However, future studies should continue to investigate this trend by testing other registered products for adult CRC (Reitz, 2018) in a similar fashion, and determining the cumulative effects of adult management throughout the life of the alfalfa stand. For example, Godfrey and Yeargan (1987) found yearly fall granular carbofuran treatments for adults combined with spring granular diazinon applications for larvae significantly reduced root damage and larval densities by 75-80% every spring for the 3-year study. Lastly, because adult monitoring and management programs are accessible and likely to be adopted by producers, monitoring techniques (e.g., degree day models) and action thresholds should be developed to better inform insecticide applications.

References

Dickason, E.A., Leach, C.M., Gross, A.E. (1968) Clover root curculio injury and vascular decay of alfalfa roots. *Journal of Economic Entomology, 61*(5): 1163–1168. doi: 10.1093/jee/61.5.1163

Godfrey, L.D., Yeargan, K.V. (1987) Effects and interaction of early season pests on alfalfa yield in Kentucky. *Journal of Economic Entomology, 80*(1): 248–259. doi: 10.1093/jee/80.1.248

Hower, A.A., Quinn, M.A., Alexander, S.D., Leath, K.T. (1995) Productivity and persistence of alfalfa in response to clover root curculio (Coleoptera:

Curculionidae) injury in Pennsylvania. *Journal of Economic Entomology, 88*(5): 1433–1440. doi: 10.1093/jee/88.5.1433

Reitz, S. (2018) Alfalfa hay – Clover root curculio. In *Pacific Northwest Insect Management Handbook* (p. E7). Corvallis, Oregon. Retrieved from https://pnwhandbooks.org

Rim, K., Price, S. J., Wenninger, E. J., Long, R, & Ramirez, R. A. (2019) Biology and management of clover root curculio (Coleoptera: Curculionidae). *Journal of Integrated Pest Management*, *10*: 1-14. doi: 10.1093/jipm/pmz020

Waters, N.D. (1964) Effects of *Hypera nigrirostris*, *Hylastinus obscurus,* and *Sitona hispidula* populations on red clover in Southwestern Idaho. *Journal of Economic Entomology, 57*(6): 907–910. doi: 10.1093/jee/57.6.907

Wenninger, E.J., and Shewmaker, G.E. (2014) Clover root curculio in alfalfa: Identification, biology, and management. *Pacific Northwest Extension*, *PNW663*: 1-5.