PLANT-INSECT INTERACTIONS BETWEEN YELLOW TOADFLAX, *LINARIA VULGARIS*, AND A POTENTIAL BIOCONTROL AGENT, THE GALL-FORMING WEEVIL, *RHINUSA PILOSA*.

EMILY C. BARNEWALL
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ABSTRACT

Yellow toadflax, *Linaria vulgaris* (L.) Mill. (Plantaginaceae), is a non-native invasive plant. *Rhinusa pilosa* Germar (Coleoptera: Curculionidae) is a proposed biocontrol agent. Gall development by *R. pilosa* was described using histological methods and compared between plant populations from native and introduced ranges. Key stages of oviposition were isolated histologically to determine their importance in gall induction. *Rhinusa pilosa* galled and developed on four geographically distinct Canadian populations in a pre-release quarantine study. Low agent densities only negatively affected one population. High densities of *R. pilosa* reduced potential reproductive output and plant biomass. Conducting detailed investigations into the biology, impact, and development of *R. pilosa* on populations from invasive and native ranges may help predict the efficacy of *R. pilosa* in the field if approved for release and.goes beyond current pre-release testing requirements.

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CHAPTER 1: GENERAL INTRODUCTION AND THESIS OBJECTIVES

Classical weed biological control and agent selection

Classical weed biological control involves the introduction of foreign natural enemies to control an exotic invader (Harris, 1991; McFadyen, 2005). The goal of classical weed biological control is not to completely eradicate the weed, but to reduce the levels below economic and environmental thresholds (Wilson & McCaffrey, 1999). The objective is that the arthropod(s), once released, will become a sustained population over time, resulting in an equilibrium between host and arthropod; an equilibrium that is more like that found in the native range (McFadyen, 1998). One hypothesis for why some introduced plants become invasive is their lack of natural enemies in the invaded ecosystem; i.e., the enemy release hypothesis (Keane & Crawley, 2002). By introducing specialist herbivores of the noxious weed, it is hoped that these organisms (i.e., typically arthropods) will weaken the plant so as to be less competitive with more desirable vegetation (Müller-Schärer & Schaffner, 2008; Wilson & McCaffrey, 1999).

Biological control agent selection is an evolving process with multiple goals, including improved prediction of agent safety and efficacy. One method of selecting agents is the "lottery approach" where many agents are released and one controls the plant, effectively "winning the lottery" (Denoth, Frid, & Myers, 2002; Sheppard, 2003). Alternatively, the additive effects (cumulative stress model) of multiple agents attacking different parts of the plant can control the weed (Harris, 1991). However, releasing multiple agents that are not effective in controlling the target weed is expensive and can

lead to an increased probability of non-target and indirect ecological effects (Louda, Pemberton, Johnson, & Follett, 2003; Morin et al., 2009).

The majority of current pre-release testing is focused on determining the potential risk to non-target plant species (McFadyen, 1998). Proposed candidate biocontrol agent feeding and development on non-target species, especially on economic or threatened and endangered plant species, has become the most critical agent screening tool in recent years and is mandatory in most countries (Sheppard, 2003). Historically, there has been a shift in the non-target plant species tested, from major agricultural and horticultural test species to indigenous plant species that are closely related to the target weed, especially plants that may be listed as threatened or endangered (Briese, 2003). Non-target feeding by biocontrol agents has resulted in major criticisms of classical weed biocontrol (Louda, et al., 2003). For example, the flower head-galling weevil *Rhinocyllus conicus* (Froelich), released to control thistles in the genera *Carduus* and *Cirsium*, was found to feed and develop on thistles native to North America during host range testing (McFadyen, 1998; Zwölfer & Harris, 1984). The threat of the invasive thistle was greater than the desire to protect native thistles at the time and the agent was approved for release in North America. After release, *R. conicus* was found feeding and developing on native thistles (Turner, Pemberton, & Rosenthal, 1987). Better prediction of host range through improved testing of non-target species is a critical issue for investigators of classical weed biocontrol.

Criticisms of classical weed biological control also have occurred due to negative direct or indirect effects caused by biocontrol agents (Louda, et al., 2003). An example of

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indirect effects occurring is with the seed head-galling flies, *Urophora* spp. (Diptera: Tephritidae) used to control knapweed, *Centaurea* spp. (Asteraceae). The flies have been successful at building up high densities, but due to the longevity of the plant population and seed bank, the agents require greater than 20 years of continual attack to control the weed (Story, Smith, Corn, & White, 2008). Deer mice (*Peromyscus maniculatus* Wagner) overwintering survival rates have increased due to subsidized feeding on *Urophora* within seed heads (Pearson & Callaway, 2005). Deer mice can be vectors for hantavirus, a human pathogen, thus if the virus is present in the mice population, outbreaks of hanta virus could be linked to mice feeding on *Urophora*-containing seed-heads (Pearson & Callaway, 2005). The availability of this subsidized food source will be a temporary problem as *Urophora*-containing seed heads are reduced due to knapweed control by *Urophora* spp. and other seed head-feeding insects (Story, et al., 2008). The current paradigm in selecting candidate biocontrol agents is shifting to reduce the release of ineffective agents (McFadyen, 1998; Morin, et al., 2009). This is accomplished by choosing agents that are very host specific and that negatively affect the target weed in pre-release studies.

Pre-release efficacy assessments

While reducing the risk of non-target effects is a very important aspect in biocontrol agent screening, McClay and Balciunas (2005) also makes the recommendation of evaluating the potential biological control agent's impact before release. Post-release assessments of biocontrol agents show that close to 45 % of insects that establish fail to control their target weed (McFadyen, 2005). Even in high densities, post-release studies have indicated that some agents have actually increased the host

plant's survival rather than decreased it (Callaway, DeLuca, & Belliveau, 1999).

Therefore, it is important to have some preliminary data on the interaction of the

candidate agent with its host plant before release. Pre-release efficacy assessments

(PREA) can be conducted in the field, greenhouse, or laboratory (i.e., within quarantine)

settings. However, PREAs are more easily and commonly conducted in the native range

of the biocontrol agent and the target weed (Djamankulova, Khamraev, & Schaffner,

2008; Morin, et al., 2009). Densities of the candidate agent can be manipulated through

the use of exclusion cages or insecticide treatments (Briese, Pettit, & Walker, 2004;

Goolsby, Zonneveld, & Bourne, 2004). In some cases, biocontrol workers are unable to

conduct field trials in the native range and must rely on quarantine or greenhouse studies

(Balciunas & Smith, 2006; Goolsby, Spencer, & Whitehand, 2009; Klöppel, Smith, &

Syrett, 2003). Quarantine facilities also can serve as valuable tools, allowing a controlled

and safe environment for pre-release assessments.

The ability of the candidate agent to attack and develop on multiple populations

from the introduced range also must be considered to improve agent efficacy (Müller-

Schärer & Schaffner, 2008). Some biocontrol agents are highly specific in that they can

only develop on certain genotypes of their target weed (Clement, 1994; Lym & Carlson,

2002). For example, post-release studies found variable establishment rates of leafy

spurge flea beetles, *Aphthona* spp. (Coleoptera: Chrysomelidae) on leafy spurge,

Euphorbia esula L., in North America since host genotype affected the development of

the different species of flea beetles (Lym & Carlson, 2002). Identifying the source

populations from the invasive range (i.e., Europe), and testing agents from those source

locations in the native range may improve efficacy by matching agent with host plant

genotypes (Goolsby et al., 2006). In cases where there have been multiple introductions from source populations over long periods of time from the native range, there could be higher genetic diversity than if there were only a single or a few introductions making pre-release testing on multiple plant populations essential (Dlugosch & Parker, 2008). Alternatively, where there have been limited introductions, multiple population testing would not be as important. This will also help determine the intra-specific host-specificity of the proposed agent. The Japanese knotweed, *Fallopia japonica* (Houtt.) Ronse Decr. (Polygonaceae), infestation in England is composed of one clone (Hollingsworth & Bailey, 2000), thus pre-release impact testing of the psyllid, *Aphalara itadori* Shinji (Hemiptera: Psylloidea), in Britain would require only assessing this clone. Thus, if the target species is composed of multiple genetic populations in the invasive range, pre-release assessments should include multiple populations from the invasive range to determine that agent's intra-specific host range (i.e., can the agent develop on a broad range or only a few biotypes of the target weed from the invasive range?).

Galls

Galls are defined as atypical plant growths induced by host-specific organisms such as bacteria, mites, and insects (Abrahamson & Weis, 1987). In this interaction, the insect provides the stimulus and the plant initiates the growth response (Weis & Abrahamson, 1986). In some instances, a plant will defend itself against the intruder by initiating a hypersensitive response, which is a type of host plant rejection (Abrahamson, McCrea, Whitwell, & Vernieri, 1991), and in others, normal gall development will occur. This can lead to a range of responses from the host plant, from being highly susceptible to highly resistant to the galling organism. The gall phenotype is believed to be an

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extension of the gall-inducer genotype in that no two species can produce a gall with the same morphological structure. For instance, five different cynipid wasps in the genus *Diplolepis* produce distinct galls on the same host species (Shorthouse, Leggo, Sliva, & Lalonde, 2005). Galls can be induced by a wide variety of organisms, from bacteria to insects, and galls can range in complexity from slight swellings to the more complex and ornate galls induced by insects in the Families Cecidomyiidae (Diperta) or Cynipidae (Hymenoptera) (Dreger-Jauffret & Shorthouse, 1992). Leaves are the most common plant organ galled but galls can develop on all plant organs (Dreger-Jauffret & Shorthouse, 1992).

There are an estimated 13,000 gall-inducing arthropods in the world (Dreger-Jauffret & Shorthouse, 1992). The most common gall-inducing arthropods belong to the insect Families Cecidomyiidae (Diptera) and Cynipidae (Hymenoptera) (Dreger-Jauffret & Shorthouse, 1992). The Order Coleoptera, or beetles, has the largest diversity within the insects, Class Insecta, with an estimated 360,000 number of species worldwide (Romoser & Stoffolano, 1998). However, the beetles are not a common gall-inducing insect order with only about 109 species of gall-inducing Coleoptera known worldwide (Ramamurthy, 2007). The most common Coleopteran gall-inducers belong to the Families Apionidae, Brentidae, Buprestidae, Chrysomelidae, Curculionidae, and Scolytidae (Korotyaev, Konstantinov, Lingafelter, Mandelshtam, & Volkovitsh, 2005). Galling beetles tend to produce simple galls in comparison to the more complex galls induced by the Cynipidae or Cecidomyiidae (Rohfritsch, 1992). Few detailed Coleopteran gall development studies have been conducted, and of those, the beetles are generally either biocontrol agents (Florentine, Raman, & Dhileepan, 2002; Raman, Cruz,

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Muniappan, & Reddy, 2007; Shorthouse & Lalonde, 1984) or are economic pests (Le Pape & Bronner, 1987). Further studies are required to elucidate gall development in this unconventional gall-inducing group.

There are four general stages in arthropod gall development: 1) initiation, 2) growth and development, 3) maturation, and 4) dehiscence (Rohfritsch, 1992). Initiation is the most critical stage in gall development due to the physiological modifications of host cells and tissues (Rohfritsch, 1992). The gall-inducing substance causes a reaction from the affected host tissue during the initiation stage. Typically, larval feeding is crucial for normal insect gall initiation and development (Rohfritsch, 1992). Gall initiation and creation of a "procecidium" (i.e., a preformed gall) via ovipositional fluid has only been previously reported for the Cynipidae and Tenthredinidae (Hymenoptera), but in both cases, continued larval feeding is required to complete gall development (Dreger-Jauffret & Shorthouse, 1992). Gall growth also can be initiated by mechanical damage due to female oviposition or egg deposition (i.e., sawflies, Smith, 1970), or adult feeding (i.e., aphids, Lewis & Walton, 1947). During the gall growth and development phase, the majority of the increased gall volume is due to cell expansion (hypertrophy) and cell division (hyperplasy). This stage is mainly due to larval feeding via salivary secretions to liquefy cell contents (i.e., aphids, Rohfritsch & Anthony, 1992) or to modify the cell walls (i.e., cynipids, Bronner, 1992), which in turn stimulates hypertrophy and hyperplasy. Nutritive tissue, the specialized cells surrounding the larval chamber, develops during this stage in more complex galls. Nutritive cells contain a reduced vacuole and dense cytoplasm, hypertrophied nucleus, and an increased number of organelles (Bronner, 1992). During gall maturation in some complex galls, a

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sclerenchyma sheath develops between the nutritive and vascular tissue creating inner and outer gall regions (Rohfritsch, 1992). The inner region contains the actively feeding larva(e). During dehiscence or gall-opening, the insect leaves the gall. This can occur as simply as the insect chewing a small exit hole, or chemical cues from the maturing insect that induces major physical modifications such as the spontaneous separation of the inner and outer regions of the gall, allowing the inner region to separate from the plant (Rohfritsch, 1992).

Gall-inducing arthropods are believed to have evolved from arthropods utilizing new niches on their host plant with which they have had a long evolutionary association (Schaefer, Raman, & Withers, 2005). Three hypotheses have been developed to explain gall development and morphology: 1) Nutrition Hypothesis, 2) Microenvironment Hypothesis, and 3) Enemy Hypothesis (Price, Fernandes, & Waring, 1987; Stone & Schönrogge, 2003). The Nutrition Hypothesis states that galls provide increased surface area for feeding or contain more nutritious food. This hypothesis can help explain gall existence in general. Gall morphology for gall-inducing thrips also can be explained using this hypothesis in that the increased surface area of the gall allows more feeding sites for the insects (Crespi, Carmean, & Chapman, 1997). Galls also can contain nutritive tissue that can be more nutritious and contain less defensive compounds than other plant organs (Bronner, 1992). The Microenvironment Hypothesis states that galls provide suitable microenvironments for the developing larvae, mostly due to high humidity, which prevents desiccation of the larvae. The California gall wasp, *Andricus quercuscalifornicus* (Bassett) (Hymenoptera: Cynipidae), larva increased the humidity within its gall to about 40 % higher than the external environment to increase larval

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survivorship (Miller III, Ivey, & Shedd, 2009). The Enemy Hypothesis states that the gall protects the gall inducer from attack by natural enemies and this hypothesis has been favoured to explain the large diversity in gall morphology (Stone & Schönrogge, 2003). Selection for medium-sized galls induced by *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae) on goldenrod, *Solidago altissima* L., (Asteraceae) was due to increased parasitism rates of smaller galls by hymenopteran parasitiods, while larger galls were more susceptible to attack by avian predators (Weis & Abrahamson, 1986). Stone and Schönrogge (2003) suggested four gall structural traits help prevent galler mortality from parasitoids and/or predators: 1) increased gall hardness, 2) increased gall thickness, 3) hairs surrounding the exterior of the gall, and 4) gall guarding (i.e., the gall is protected by secreting substances that attract ant guards, Seibert, 1993). Other gall development hypotheses, such as the Plant Protection Hypothesis (the plant isolates the inducer in space and time to reduce negative impacts to the plant) and the Mutual Benefit Hypothesis (where both the gall inducer and the host plant benefit from the interaction) have been rejected (Price, et al., 1987).

Gall-inducing insects in biological control

Harris and Shorthouse (1996) suggested that gall-forming arthropods could be preferable candidates for biological control since gall makers typically have a narrow host range and can negatively affect host plants. Galls can reduce the health of the target species by creating resource sinks within the host plant (Harris & Shorthouse, 1996). Resource sinks within galls can be formed due to plant cell differentiation into vascular tissue within the gall (Harris & Shorthouse, 1996). Resources from other portions of the plant are redirected to the gall, thereby stunting the plant's normal growth and

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reproduction (McCrea, Abrahamson, & Weis, 1985). For example, the gall wasp *Trichilogaster acaciaelongifoliae* (Froggatt) (Hymenoptera: Pteromalidae) has been successful in controlling its target weed, *Acacia longifolia* (Andr.) Willd. (Fabaceae), in South Africa (Dennill, 1988). This gall wasp reduces the reproductive potential of its host by 89 % and vegetative growth by 53 % (Dennill, 1988). The weevil, *R. conicus*, which produces simple callus galls on the flower heads of *C. nutans* (Shorthouse & Lalonde, 1984), has been recognized as the fourth most successful biocontrol agent in the world based on the ability of the agent to control the weed, and the area of control in relation to the pre-release severity of the weed (Crawley, 1989).

Documenting gall development patterns of proposed biocontrol agents in their preferred host is important because: 1) it can potentially determine if the gall may act as a resource sink (i.e., increased vasculature within the gall); and 2) the gall development pattern on the preferred host can be used as a standard to compare against gall development patterns in non-target plant species, and thus predict host range. The gall development patterns could give insights into how the plant tissues respond to galling, which can indicate how the gall may act as a resource sink. For example, gall development rates are positively related to the time the gall acts as a resource sink (Abrahamson & Weis, 1987). Increased vasculature directed towards the gall also is an indication that the gall may be acting as a resource sink (Harris & Shorthouse, 1996). Host range can be determined by conducting a histological assessment of gall development. For example, if the galler develops within an enlarged pith region of a stem gall in the preferred host, but in a less suitable host, the pith tissue may not be the plant tissue that responds to gall induction, thus reducing the suitability of this plant as a host.

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Assessing detailed gall development studies on plants from the introduced range also is important in that some agents are not as well adapted to plants from the invasive range (Cullen & Moore, 1983). The gall-inducing ability of the mite, *Aceria chondrillae* Canestrini (Acarina: Eriophyidae), on *Chondrilla juncea* L. (Compositae) was dependent on host genotype and growth conditions of the plant (Cullen & Moore, 1983).

Biology of the study organisms

Linaria vulgaris

Yellow toadflax, *Linaria vulgaris* (L.) Mill, is a non-native, invasive plant in North America, originating from the Mediterranean region of Europe (Saner, Clements, Hall, Doohan, & Crompton, 1995). The phylogeny of *Linaria vulgaris* has recently been changed; *L. vulgaris* has been transferred from the Family Scrophulariaceae to the Plantaginaceae as a result of recent molecular analyses (see Albach, Meudt, & Oxelman, 2005; Olmstead et al., 2001). Flowers are similar to those of snapdragon and are yellow with a bright orange "lip" and the flowering period is from May until late fall in North America (Wilson et al., 2005). *Linaria vulgaris* is self-incompatible and readily hybridizes with closely related species such as the invasive, introduced species, *Linaria dalmatica* (Ward, Fleischmann, Turner, & Sing, 2009). The weed is pollinated by long-tongued insects such as bumble bees (Hymenoptera: Apidae) (Wilson, et al., 2005). The weed reproduces by seed, producing up to 30,000 seeds per plant annually, and vegetatively by rhizomes (Saner, et al., 1995). The large majority of seeds fall within half a meter of the parent plant (Nadeau & King, 1991). *Linaria vulgaris* has low seed germination rates (10 %), but seeds can remain dormant for 10 years (Lageunesse, 1999). *Linaria vulgaris* stands can be maintained for long periods of time due to a combination

of plant characteristics; i.e., *L. vulgaris* are short lived perennials (approximately 4 years) but can maintain patches due to clonal growth (Lageunesse, 1999).

Linaria vulgaris was introduced into North America in the mid-1700's (Wilson, et al., 2005) for use as yellow dye (Mitich, 1993), medicinal purposes (ILieva, Handjieva, & Popov, 1992), and as a garden ornamental (Parchoma, 2002). It has been hypothesized that multiple introductions have occurred and may help explain the large genetic diversity of *L. vulgaris* in North America (Ward, Reid, Harrington, Sutton, & Beck, 2008). *Linaria vulgaris* is currently listed as a noxious weed in four Canadian provinces and eight US states and is distributed across Canada and the continental United States (USDA, 2010). It is a common weed in dark soils of perennial and annual crops (Saner, et al., 1995), in other disturbed habitats such as railway rights-of-way (Lageunesse, 1999), and in minimally disturbed environments, such as native grasslands (Ward, et al., 2008). This plant competes with native grasses, negatively affecting biodiversity (Parchoma, 2002) and causes reduced forage for cattle and big game species such as elk and deer (Lageunesse, 1999). New infestations are commonly a result of seed germination, but also can be initiated with small root pieces (i.e., tillage can break up the root system and move root pieces to uninfested areas). Transport of seeds in contaminated feed, on or in animals (muddy hooves or stomachs), on muddy machines, or in gravel also are important sources of new infestations (Lageunesse, 1999).

Linaria vulgaris is difficult to control by chemical and mechanical means. The weed has developed resistance to common herbicides (Saner, et al., 1995). It is hypothesized that the high genetic diversity found in this species contributes to the speed at which it becomes resistant to herbicides (Sebastian & Beck, 1998; Ward, et al., 2008).

Using cattle and other grazing animals to control *L. vulgaris* has not been successful due to the mild toxicity of a glucoside in *L. vulgaris* (Morishita, 1991). Mowing has shown little success in controlling the weed due to regeneration of patches through the horizontal root system, and mowing cannot reduce the seed bank (Parchoma, 2002). Hand pulling patches for 5 or 6 years can control small toadflax infestations, but yearly site visits may be required for up to 15 years to remove seedlings arising from dormant seeds (Lageunesse, 1999).

   Linaria vulgaris has been a target for biological control since the 1960's (McClay & De Clerck-Floate, 2002). To date, eight host-specific biocontrol agents have been either intentionally released to control *L. vulgaris* in North America or were accidentally introduced from Europe and occur adventively. The full list includes: a defoliating noctuid moth, *Calophasia lunula* (Hufnagel) (Lepidoptera: Noctuidae), two root-feeding moths, *Eteobalea serratella* Treitschke and *E. intermediella* Riedl (Lepidoptera: Cosmopterigidae), a shoot-boring weevil, *Mecinus janthinus* Germar (Coleoptera: Curculionidae), three fruit-feeding beetles (*Brachypterolus pulicarious* L. (Coleoptera: Kateretidae)*, Rhinusa antirrhini* (Paykull), and *R. neta* (Germar) (Coleoptera: Curculionidae), and a root-feeding weevil, *R. linariae* (Panzer) (Sing, Peterson, Weaver, Hansen, & Markin, 2005; Wilson, et al., 2005). These insects have had little impact on populations of *L. vulgaris* in Canada (McClay & De Clerck-Floate, 2002). A potential reason for failure of some of these agents is that it was initially believed that the species were capable of surviving on both *L. vulgaris* and *L. dalmatica* in overseas testing. However, post-release assessments found that some insects preferred one host over another (i.e., *M. janthinus* being successful in controlling *L. dalmatica* and not *L. vulgaris*

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(Ward, et al., 2008)), either suggesting the presence of insect biotypes or cryptic speciation.

Rhinusa pilosa

Rhinusa pilosa Gyllenhal (Coleoptera: Curculionidae), a stem-galling weevil originating from Europe, is one of two agents presently being considered as a potential biocontrol agent for *L. vulgaris*. Research began in the mid-1990's with *Rhinusa thapsicola* Germar (then *Gymnetron thapsicola* Germar) and *R. pilosa* (then *G. hispidum* Brulle) (Toševski, Gassmann, & Desančić, 2004). Due to high numbers of *R. thapsicola* collected in field galls and low numbers of *R. pilosa*, it was initially hypothesized that *R. thapsicola* was the gall former and that *R. pilosa* was an inquiline, utilizing the galls that *R. thapsicola* produced. After repeated failures in coaxing *R. thapsicola* to induce galls during laboratory trials in Europe, it was then discovered that the reverse was true; *R. pilosa* was the gall inducer and that *R. thapsicola* was utilizing the *R. pilosa* galls (Toševski, et al., 2004).

The biology, taxonomy, and distribution of *R. pilosa* has been described mainly through the efforts of Ivo Toševski with the Toadflax Biocontrol Program in Serbia (Toševski, et al., 2004). *Rhinusa pilosa* is distributed in northern Europe from France to Russia (Caldara, Desančić, Gassmann, & Toševski, 2008). Adults of the species emerge from diapause in early March in Serbia, during which time they feed on young toadflax shoots, mate, and commence oviposition (Toševski, et al., 2004). Eggs are deposited inside the stem pith tissue in the apical (i.e., meristematic) region of young, growing stems. Externally, gall development is visible within 48 hours after oviposition. Gall

growth is complete 8 to 10 days later when reared at 22°C in artificial environmental

conditions. The gall is generally light green in color, but can be reddish-green, and

smooth in texture. For slower growing stems, the gall can be positioned at the apex of the

shoot, halting further vertical growth of the plant. For faster growing stems, the gall can

be in the middle of the stem, as the apical meristem continues to grow beyond the gall.

Galls at the apex tend to be oval, symmetrical, and tapering towards the apical meristem,

whereas galls located in the middle of the stem are either symmetrical or asymmetrical

(i.e., with one side of the gall larger than the other). Galls are generally multilocular,

averaging four individuals per gall (Toševski, et al., 2004). Preliminary studies found the

average gall width was 9.1 ± 0.5 mm and length was 18.7 ± 1.7 mm (mean ± standard

deviation) (Chapter 2). Studies by I. Toševski provide the most complete record of

biology within the *R. pilosa – L. vulgaris* system (Toševski, et al., 2004). The

development from egg to adult of *R. pilosa* is complete within the gall. When reared at a

constant 22°C, the eggs hatch 8 days after oviposition, followed by two larval molts,

which occur every 7 - 8 days. The third and final instar pupates inside the gall and the

adults eclose 10 days later. The adults feed on the remaining gall tissue for 2 weeks

before creating a "window" or emergence hole, from where they exit the gall. This period

of feeding is believed to be most critical for the winter survival of adults (I. Toševski,

personal communication). Once the adults emerge from the gall, they periodically feed on

the leaves and stem of the host plant prior to winter diapause (Toševski, et al., 2004).

Thesis objectives

This thesis has four objectives: 1) describe the patterns of gall formation within

the *R. pilosa – L. vulgaris* system in detail, 2) document the early stages of gall induction,

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3) explore the biocontrol efficacy of *R. pilosa* on multiple populations of *L. vulgaris* from the invasive range, and 4) determine the biocontrol efficacy of high densities of *R. pilosa* on a previously galled population of *L. vulgaris*, thus simulating a post-release impact assessment.

The first section of the thesis (Chapter 2) describes normal gall development by *R. pilosa* on *L. vulgaris* from its native range (Serbia). Gall development in the *R. pilosa - L. vulgaris* system has yet to be described in the published scientific literature. Gall induction by *R. pilosa* is quite unusual in that: 1) the causative agent is a weevil (Coleoptera: Curculionidae); and 2) gall induction appears to be initiated by female ovipositional fluid or the egg since the gall is already formed by the time the larvae have hatched from their eggs. For this study, I collected, preserved, and sectioned galled plant material at set intervals over time (control, less than 24 hours, 48 hours, 4, 8, 12, 16, 20, 28, and 35 days post oviposition). I examined tissue and cellular level changes during gall induction, growth, and maturation phases on *L. vulgaris*. In addition to studying gall development patterns on *L. vulgaris* plants from the native range, galled samples also were collected from *L. vulgaris* plants from the invasive range (British Columbia, Canada). A brief comparison of gall development on plants from the native and introduced range was included to determine if there were differences in plant responses to *R. pilosa*. These results could provide a standard for comparison with other gall development/plant response patterns on: 1) different *L. vulgaris* genotypes (i.e., *L. vulgaris* populations from the invasive range), or 2) other closely-related plant species (i.e., within the same genus or tribe). This is one method to explain, and potentially predict, host range.

The second section (Chapter 3) examined, using histological methods, the key steps involved in gall induction in the *R. pilosa* – *L. vulgaris* system. It has been observed that gall development on *L. vulgaris* induced by *R. pilosa* is near completion by the time the egg of *R. pilosa* has hatched. The ovipositional sequence of interest here involved the female *R. pilosa* chewing a small ovipositional chamber into the apical region of the stem where she deposited her ovipositional fluid and an egg. In an attempt to experimentally isolate the host plant's response to different stages of gall induction, mated, ovipositing females were removed at different stages during the ovipositional sequence. Replicate samples were collected for fixation and sectioning 3 and 5 days after treatment (one treatment had an additional collection period, 10 days after treatment) to allow time for the plant to respond. This study could help to elucidate the critical stage of gall induction by *R. pilosa*. Results from this study will contribute valuable information on gall induction by a non-conventional gall-former. The results of this study could help predict the ecological host range of the insect. For example, if specific characteristics of *L. vulgaris* are necessary for gall induction in this system, the host range could be quite narrow. Most host-specificity tests do not examine the plant's initial reaction to the insect in this detail.

The third section (Chapter 4) asked two questions: 1) Can *R. pilosa* successfully induce galls and develop on multiple *L. vulgaris* populations from the invasive range?; and 2) What is the impact of low densities of *R. pilosa* on these populations in terms of toadflax growth and potential reproductive output? These questions were tested by exposing a mated female to randomly collected *L. vulgaris* plants from four

geographically distinct populations in western Canada and one population from the native range (Serbia). To determine the ability of *R. pilosa* to gall and develop on the different *L. vulgaris* populations, gall response variables (proportion of galled stems, gall volume, dry gall mass, proportion of live adults, average number of adults per gall, and fresh adult mass) were compared among the different populations. Impact was assessed using multiple plant response variables (final stem length, proportion of flowering stems, proportion of stems with lateral shoots, proportion of dead stems, proportion of new stems, above-ground biomass, and below-ground biomass) comparing population and treatment level effects. *Linaria vulgaris* plants are composed of multiple stems from the same plant and not all stems were galled. Thus, intra-plant effects also were assessed to determine how the plant responded to galling (i.e., galled vs. ungalled stems within the treatment plant). Ward et al. (2008) recently found more genetic variation within and between populations of *L. vulgaris* than expected due to sexual reproduction via seeds, contrary to the common belief that *L. vulgaris* reproduces mainly clonally with rhizomes at the patch level. Multiple introductions of *L. vulgaris* are suspected, and *R.* pilosa, as a biocontrol agent, will be exposed to a large variety of genotypes. If *R. pilosa* is to be considered an effective biocontrol agent for *L. vulgaris*, it will be important to document multiple population responses to galling by *R. pilosa* based on the genetic variability of *L. vulgaris*. Differences in *R. pilosa* performance on the four geographically distinct populations may predict variable establishment in the field due to variation in *L. vulgaris* populations. The second question of this experiment represents the first year of a release (i.e., low densities of the candidate agent on previously unexposed plants). It is of interest to investigate the host response to low densities of the candidate agent. Testing the

impact of *R. pilosa* on its host from the native range will provide a standard that can be

compared with populations from the invasive range. Overall, this experiment will provide

valuable pre-release information on the multi-population-galling ability of this agent and

the impact of low densities of this agent on its host plant.

The final section (Chapter 5) examined the impact of high densities of mated

female *R. pilosa* on one previously galled, susceptible population of *L. vulgaris* from the

invasive range. Impact was assessed by comparing a variety of plant response variables

(final stem length, final stem width, proportion of flowering stems, proportion of stems

with lateral shoots, proportion of dead stems, proportion of new stems, above-ground

biomass, and below-ground biomass) on the effect of treatment. This experiment was

designed to simulate potential post-release field conditions more than one year after a

release. If high densities of this agent are not successful in controlling this weed, then *R.*

pilosa may not be an effective agent in the field. High densities of an ineffective agent

pose greater potential for non-target and/or indirect risks. It is likely that a perennial plant

will experience multiple years of galling before the agent densities or the cumulative

impact with repeated, year-to-year attack are great enough to effectively control the

weed. The results of this study may predict the efficacy of this agent to control *L.*

vulgaris in the field, if approved for release.

This thesis provides valuable baseline data that can be applied to an assessment of

biocontrol efficacy within the *R. pilosa* – *L. vulgaris* system. In addition to empirically

examining the impact and multi-population-galling ability of *R. pilosa*, I examined the

details of the biology of the system, elucidating the potential mechanisms of gall

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induction within this plant-insect interaction. This approach is innovative and is of immediate and lasting impact for investigators of this biocontrol system.

REFERENCES

Abrahamson, W. G., McCrea, K. D., Whitwell, A. J., & Vernieri, L. A. (1991). The role of phenolics in goldenrod ball gall resistance and formation. *Biochemical Systematics and Ecology, 19*(8), 615-622.

Abrahamson, W. G., & Weis, A. E. (1987). Nutritional Ecology of Arthropod Gallmakers. In F. J. Slansky & J. G. Rodriquez (Eds.), *Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates* (pp. 235-258). New York: John Wiley & Sons, Inc.

Albach, D. C., Meudt, H. M., & Oxelman, B. (2005). Piecing together the "new" Plantaginaceae. *American Journal of Botany, 92*(2), 297-315.

Balciunas, J., & Smith, L. (2006). Prerelease efficacy assessment, in quarantine, of a tephritid gall fly being considered as a biological control agent for Cape-ivy (*Delairea odorata*). *Biological Control, 39*, 516-524.

Briese, D. T. (2003). The Centrifugal Phylogenetic Method Used to Select Plants for Host-Specificity Testing of Weed Biological Control Agents: Can and Should it Be Modernised? In H. Spafford Jacob & D. T. Briese (Eds.), *Improving the Selection, Testing, and Evaluation of Weed Biological Control Agents* (pp. 23-33). Adelaide: Cooperative Research Centre for Australian Weed Management.

Briese, D. T., Pettit, W. J., & Walker, A. (2004). Evaluation of the biological control agent, *Lixus cardui*, on *Onopordum* thistles: experimental studies on agent demography and impact. *Biological Control, 31*, 165-171.

Bronner, R. (1992). The Role of Nutritive Cells in the Nutrition of Cynipids and Cecidomyiids. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 118-140). New York: Oxford University Press.

Caldara, R., Desančić, M., Gassmann, A., & Toševski, I. (2008). On the identity of *Rhinusa hispida* (Brullé) and its current synonyms (Coleoptera: Curculionidae). *Zootaxa, 1805*, 61-68.

Callaway, R. M., DeLuca, T. H., & Belliveau, W. M. (1999). Biological control herbivores may increase competitive ability of the noxious weed *Centaurea maculosa. Ecology, 80*(4), 1196-1201.

Clement, S. L. (1994). Resistance among populations of yellow starthistle to thistle-head insects: results from garden plots in Italy. *Biological Control, 4*, 149-156.

Crawley, M. J. (1989). The successes and failures of weed biocontrol using insects. *Biocontrol News and Information, 10*, 213-223.

Crespi, B. J., Carmean, D. A., & Chapman, T. W. (1997). Ecology and evolution of galling thrips and their allies. *Annual Reviews in Entomology, 42*, 51-71.

Cullen, J. M., & Moore, A. D. (1983). Influence of three populations of *Aceria chondrillae* on three forms of *Chondrilla juncea*. *Journal of Applied Ecology, 20*(1), 235-243.

Dennill, G. B. (1988). Why a gall former can be a good biocontrol agent: The gall wasp *Trichilogaster acaciaelongifoliae* and the weed *Acacia longifolia*. *Ecological Entomology, 13*, 1-9.

Denoth, M., Frid, L., & Myers, J. H. (2002). Multiple agents in biological control: improving the odds? *Biological Control, 24*(1), 20-30.

Djamankulova, G., Khamraev, A., & Schaffner, U. (2008). Impact of two shoot-galling biological control candidates on Russian knapweed, *Acroptilon repens*. *Biological Control, 46*, 101-106.

Dlugosch, K. M., & Parker, I. M. (2008). Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Molecular Ecology, 17*, 431-449.

Dreger-Jauffret, F., & Shorthouse, J. D. (1992). Diversity of Gall-Inducing Insects and their Galls. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 8-33). New York: Oxford University Press.

Florentine, S. K., Raman, A., & Dhileepan, K. (2002). Responses of the weed *Parthenium hysterophorus* (Asteraceae) to the stem gall-inducing weevil *Conotrachelus albocinereus* (Coleoptera: Curculionidae). *Entomologia Generalis 26*(3), 195-206.

Goolsby, J. A., De Barro, P. J., Makinson, J. R., Pemberton, R. W., Hartley, D. M., & Frohlich, D. R. (2006). Matching the origin of an invasive weed for selection of a herbivore haplotype for a biological control programme. *Molecular Ecology, 15*, 287-297.

Goolsby, J. A., Spencer, D., & Whitehand, L. (2009). Pre-release assessment of impact on *Arundo donax* by the candidate biological control agents *Tetramesa romana* (Hymenoptera: Eurytomidae) and *Rhizaspidiotus donacis* (Hemiptera: Diaspididae) under quarantine conditions. *Southwestern Entomologist, 34*(4), 359-376.

Goolsby, J. A., Zonneveld, R., & Bourne, A. (2004). Prerelease assessment of impact on biomass production of an invasive weed, *Lygodium microphyllum* (Lygodiaceae: Pteridophyta), by a potential biological control agent, *Floracarus perrepae* (Acariformes: Eriophyidae). *Environmental Entomology, 33*(4), 997-1002.

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Harris, P. (1991). Classical biocontrol of weeds: Its definitions, selection of effective agents, and administrative-political problems. *The Canadian Entomologist, 123*, 827-849.

Harris, P., & Shorthouse, J. D. (1996). Effectiveness of gall inducers in weed biological control. *The Canadian Entomologist, 128*, 1021-1055.

Hollingsworth, M. L., & Bailey, J. P. (2000). Evidence for massive clonal growth in the invasive weed *Fallopia japonica* (Japanese Knotweed). *Botanical Journal of the Linnean Society, 133*, 463-472.

Ilieva, E. I., Handjieva, N. V., & Popov, S. S. (1992). Iridoid glucosides from *Linaria vulgaris*. *Phytochemistry, 31*, 1040-1041.

Keane, R. M., & Crawley, M. J. (2002). Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology and Evolution, 17*(4), 164-170.

Klöppel, M., Smith, L., & Syrett, P. (2003). Predicting the impact of the biocontrol agent *Aulacidea subterminalis* (Cynipidae) on growth of *Hieracium pilosella* (Asteraceae) under differing environmental conditions in New Zealand. *Biocontrol Science and Technology, 13*, 207-218.

Korotyaev, B. A., Konstantinov, A. S., Lingafelter, S. W., Mandelshtam, M. Y., & Volkovitsh, M. G. (2005). Gall-inducing Coleoptera. In A. Raman, C. W. Schaefer & T. M. Withers (Eds.), *Biology, Ecology, and Evolution of Gall-inducing Arthropods* (Vol. 1, pp. 239-272). Plymoth, UK: Science Publishers, Inc.

Lageunesse, S. (1999). Dalmatian and Yellow Toadflax. In L. Sheley & J. K. Petroff (Eds.), *Biology and Management of Noxious Rangeland Weeds* (pp. 219-260). Corvallis: Oregon State University Press.

Le Pape, H., & Bronner, R. (1987). The Effects of *Ceuthorrhynchus napi* (Curculionidae, Coleoptera) on Stem Tissues of *Brassica napus* var *oleifera*. In V. Labeyrie, G. Fabres & D. Lachaise (Eds.), *Insects-Plants: Proceedings of the 6th International Symposium on Insect-Plant Relationships (PAU 1986)* (pp. 207-212). Dordrecht: Dr W. Junk Publishers.

Lewis, I. F., & Walton, L. (1947). Initiation of the cone gall of witch hazel. *Science, 106*(2757), 419-420.

Louda, S. M., Pemberton, R. W., Johnson, M. T., & Follett, P. A. (2003). Nontarget effects-the achilles' heel of biological control? Retrospective analyses to reduce risk associated with biocontrol introductions. *Annual Review in Entomology, 48*, 365-396.

Lym, R. G., & Carlson, R. B. (2002). Effect of leafy spurge (*Euphorbia esula*) genotype on feeding damage and reproduction of *Aphthona* spp.: Implications for biological weed control. *Biological Control, 23*, 127-133.

McClay, A. S., & Balciunas, J. K. (2005). The role of pre-release efficacy assessment in selecting classical biological control agents for weeds - applying the Anna Karenina principle. *Biological Control, 35*, 197-207.

McClay, A. S., & De Clerck-Floate, R. A. (2002). *Linaria vulgaris* Miller, Yellow Toadflax (Scrophulariaceae). In P. G. Mason & J. T. Huber (Eds.), *Biological Control Programmes in Canada, 1981-2000* (pp. 375-382). New York: CABI Publishing.

McCrea, K. D., Abrahamson, W. G., & Weis, A. E. (1985). Goldenrod ball gall effects on *Solidago altissima*: 14C translocation and growth. *Ecology, 66*(6), 1902-1907.

McFadyen, R. E. (1998). Biological control of weeds. *Annual Review in Entomology, 43*, 369-393.

McFadyen, R. E. (2005). Does Ecology Help in the Selection of Biocontrol Agents? In H. Spafford Jacob & D. T. Briese (Eds.), *Improving the selection, testing and evaluation of weed biological control agents* (pp. 5-9). Glen Osmond, Australia: CRC for Australian Weed Management.

Miller III, D. G., Ivey, C. T., & Shedd, J. (2009). Support for the microenvironment hypothesis for adaptive value of gall induction in the California gall wasp, *Andricus quercuscalifornicus*. *Entomologia Experimentalis et Applicata, 132*, 126-133.

Mitich, L. W. (1993). Yellow toadflax. *Weed Technology, 7*(3), 791-793.

Morin, L., Reid, A. M., Sims-Chilton, N. M., Buckley, Y. M., Dhileepan, K., Hastwell, G. T., et al. (2009). Review of approaches to evaluate the effectiveness of weed biological control agents. *Biological Control, 51*, 1-15.

Morishita, D. W. (1991). Dalmatian Toadflax, Yellow Toadflax, Black Henbane, and Tansy Mustard: Importance, Distribution, and Control. In L. F. James, J. O. Evans, M. H. Ralphs & R. D. Child (Eds.), *Noxious Range Weeds*. Boulder: Westview Press.

Müller-Schärer, H., & Schaffner, U. (2008). Classical biological control: exploiting enemy escape to manage plant invasions. *Biological Invasions, 10*, 859-874.

Nadeau, L. B., & King, J. R. (1991). Seed dispersal and seedling establishment of *Linaria vulgaris* Mill. *Canadian Journal of Plant Science, 71*, 771-782.

Olmstead, R. G., DePamphilis, C. W., Wolfe, A. D., Young, N. D., Elisons, W. J., & Reeves, P. A. (2001). Disintegration of the Scrophulariaceae. *American Journal of Botany, 88*(2), 348-361.

Parchoma, G. (2002). Yellow Toadflax *Guide to Weeds in British Columbia* (pp. 192-195). Burnaby: Province of British Columbia.

Pearson, D. E., & Callaway, R. M. (2005). Indirect nontarget effects of host-specific biological control agents: Implications for biological control. *Biological Control, 35*, 288-298.

Price, P. W., Fernandes, G. W., & Waring, G. L. (1987). Adaptive nature of insect galls. *Environmental Entomology, 16*, 15-24.

Ramamurthy, V. V. (2007). Faunistic, ecological, biogeographical and phylogenetic aspects of coleoptera as gall-inducers and associates in plant galls in the orient and eastern palearctic. *Oriental Insects, 41*, 93-119.

Raman, A., Cruz, Z. T., Muniappan, R., & Reddy, G. V. P. (2007). Biology and host specificity of gall-inducing *Acythopeus burkhartorum* (Coleoptera: Curculionidae), a biological-control agent for the invasive weed *Coccinia grandis* (Cucurbitaceae) in Guam and Saipan. *Tijschrift voor Entomologie, 150*, 181-191.

Rohfritsch, O. (1992). Patterns in Gall Development. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 60-86). New York: Oxford University Press.

Rohfritsch, O., & Anthony, M. (1992). Strategies in Gall Induction by Two Groups of Homopterans. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 102-117). New York: Oxford University Press.

Romoser, W. S., & Stoffolano, J. G. (1998). *The Science of Entomology* (4th ed.). Boston: WCB/McGraw-Hill

Saner, M. A., Clements, D. R., Hall, M. R., Doohan, D. J., & Crompton, C. W. (1995). The biology of Canadian weeds. 105. *Linaria vulgaris* Mill. *Canadian Journal of Plant Science, 75*, 525-537.

Schaefer, C. W., Raman, A., & Withers, T. M. (2005). Galls and Gall-inducing Arthropods: Ecological Issues and Evolutionary Problems. In A. Raman, C. W. Schaefer & T. M. Withers (Eds.), *Biology, Ecology, and Evolution of Gall-inducing Arthropods* (Vol. 2, pp. 761-767). Plymoth, UK: Science Publishers, Inc.

Sebastian, J. R., & Beck, K. G. (1998). Yellow toadflax control with metsulfuron, metsulfuron tank mixes, picloram, quinclorac, 2,4-D, or dicamba. *Western Society of Weed Science*, 24.

Seibert, T. F. (1993). A nectar-secreting gall wasp and ant mutualism: selection and counter-selection shaping gall wasp phenology, fecundity and persistence. *Ecological Entomology, 18*, 247-253.

Sheppard, A. W. (2003). Prioritising Agents Based on Predicted Efficacy: Beyond the Lottery Approach. In H. Spafford Jacob & D. T. Briese (Eds.), *Improving the Selection, Testing and Evaluation of Weed Biological Control Agents* (pp. 11-22). Adelaide: Cooperative Research Centre for Australian Weed Management.

Shorthouse, J. D., & Lalonde, R. G. (1984). Structural damage by *Rhinocyllus conicus* (Coleoptera: Curculionidae) within the flowerheads of nodding thistle. *The Canadian Entomologist, 116*, 1335-1343.

Shorthouse, J. D., Leggo, J. J., Sliva, M. D., & Lalonde, R. G. (2005). Has egg location influenced the radiation of *Diplolepis* (Hymenoptera: Cynipidae) gall wasps on wild roses? *Basic and Applied Ecology, 6*(5), 423-434.

Sing, S. E., Peterson, R. K. D., Weaver, D. K., Hansen, R. W., & Markin, G. P. (2005). A retrospective analysis of known and potential risks associated with exotic toadflax-feeding insects. *Biological Control, 35*, 276-287.

Smith, E. L. (1970). Biosystematics and morphology of symphyta. II. Biology of gall-making nematine sawflies in the California region. *Annals of the Entomological Society of America, 61*(1), 36-51.

Stone, G. N., & Schönrogge, K. (2003). The adaptive significance of insect gall morphology. *Trends in Ecology and Evolution, 18*(10), 512-522.

Story, J. M., Smith, L., Corn, J. G., & White, L. J. (2008). Influence of seed head-attacking biological control agents on spotted knapweed reproductive potential in western Montana over a 30-year period. *Environmental Entomology, 37*(2), 510-519.

Toševski, I., Gassmann, A., & Desančić, M. (2004). *Annual Report 2003: Biological Control of Dalmatian and Yellow Toadflaxes, Linaria dalmatica and L. vulgaris*. Delemont: CABI Publishing.

Turner, C. E., Pemberton, R. W., & Rosenthal, S. S. (1987). Host utilization of native *Cirsium* thistles (Asteraceae) by the introduced weevil *Rhinocyllus conicus* (Coleoptera: Curculionidae) in California. *Environmental Entomology, 16*, 111-115.

USDA, N. R. C. S. (2010). The PLANTS Database. Retrieved 5 October, 2010, from http://plants.usda.gov/java/profile?symbol=LIVU2

Ward, S. M., Fleischmann, C. E., Turner, M. F., & Sing, S. E. (2009). Hybridization between invasive populations of Dalmatian toadflax (*Linaria dalmatica*) and yellow toadflax (*Linaria vulgaris*). *Invasive Plant Science and Management, 2*, 369-378.

Ward, S. M., Reid, S. D., Harrington, J., Sutton, J., & Beck, K. G. (2008). Genetic variation in invasive populations of yellow toadflax (*Linaria vulgaris*) in the western United States. *Weed Science, 56*, 394-399.

Weis, A. E., & Abrahamson, W. G. (1986). Evolution of host-plant manipulation by gall makers: ecological and genetic factors in the *Solidago-Eurosta* system. *American Naturalist, 127*, 681-695.

Wilson, L. M., & McCaffrey, J. P. (1999). Biological Control of Noxious Rangeland Weeds. In R. L. Sheley & J. K. Petroff (Eds.), *Biology and Management of Noxious Rangeland Weeds* (pp. 97-115). Corvallis: Oregon State University Press.

Wilson, L. M., Sing, S. E., Piper, G. L., Hansen, R. W., De Clerck-Floate, R. A., MacKinnon, D. K., et al. (2005). *Biology and Biological Control of Dalmatian and Yellow Toadflax*: USDA Forest Service, FHTET-05-13.

Zwölfer, H., & Harris, P. (1984). Biology and host specificity of *Rhinocyllus conicus* (Froel.) (Col., Curculionidae), a successful agent for biocontrol of the thistle, *Carduus nutans* L. *Zeitschrift für Angewandte Entomologie, 97*, 36-62.

CHAPTER 2: HISTOLOGICAL INVESTIGATIONS INTO THE GALL DEVELOPMENT OF *RHINUSA PILOSA* (COLEOPTERA: CURCULIONIDAE) ON *LINARIA VULGARIS* (PLANTAGINACEAE), FROM THE NATIVE AND INVASIVE RANGE.

INTRODUCTION

Galls are defined as atypical plant growths induced by host-specific organisms such as bacteria, mites, and insects (Abrahamson & Weis, 1987). In this interaction, the insect provides the stimulus and the plant initiates the growth response (Weis & Abrahamson, 1986). Galls can be induced by a wide variety of organisms, from bacteria to insects, and can range in complexity from slight swellings to the more complex and ornate galls induced by insects in the Family Cecidomyiidae (Diperta) or Cynipidae (Hymenoptera) (Dreger-Jauffret & Shorthouse, 1992). Leaves are the most common plant organ galled but galls can develop on all plant organs (Dreger-Jauffret & Shorthouse, 1992). There are four general stages in arthropod gall development: 1) initiation, 2) growth and development, 3) maturation, and 4) dehiscence. Initiation is the most critical stage in gall development due to the physiological modifications of host cells (Rohfritsch, 1992). In most gall-inducing arthropods, larval feeding is generally required for gall induction and the gall-inducing substance causes a reaction from the host tissue. The chemical nature or the exact mode of action of the substance has not been elucidated for the majority of gall inducers (Rohfritsch & Shorthouse, 1982). During the gall growth and development phase, the majority of the increased gall volume is due to cell expansion

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(hypertrophy) and cell division (hyperplasy). Nutritive tissue develops during this stage and is comprised of specialized cells surrounding the larval chamber in more complex galls, which contain a reduced vacuole, dense cytoplasm, hypertrophy of nucleus, and an increased number of organelles (Bronner, 1992). During gall maturation in more complex gall inducers, a sclerenchyma sheath develops between the nutritive and vascular tissue creating inner and outer gall regions, with the inner region containing the feeding larva (Rohfritsch, 1992). During dehiscence, or gall-opening, the insect leaves the gall. This can occur as simply as the insect chewing a small exit hole, or major physical modifications such as the separation of the inner and outer layers due to a chemical cue from the maturing insect causing the gall to open (Rohfritsch, 1992).

Harris and Shorthouse (1996) suggested that gall-forming insects are suitable candidates for biological control since gall makers can have a negative impact on the host plant and typically have a narrow host range. For instance the flower bud-galling wasp, *Trichilogaster acaciaelongifoliae* (Froggatt) (Hymenoptera: Pteromalidae), has been successful in controlling its target weed, *Acacia longifolia* (Andr.) Willd. (Fabaceae), in South Africa (Dennill, 1988). This gall wasp reduces the reproductive potential of its host by 89 % and vegetative growth by 53 % (Dennill, 1988). The weevil, *Rhinocyllus conicus* (Froelich) (Coleoptera: Curculionidae), induces simple, callus-growth galls in the flower heads of *Carduus nutans* L. (Shorthouse & Lalonde, 1984), and has been stated as the fourth most successful biocontrol agent in the world based on the degree and area of control in relation to the pre-release severity of the weed (Crawley, 1989).

Studying gall development patterns of a proposed biocontrol agent using histological methods can help determine the host range of the gall inducer and may help elucidate if the gall acts as a resource sink. The intimate plant-insect interaction of the gall maker and its host can be isolated, at the cellular level, using histology. The gall development pattern can be used to compare against gall development in non-target plant species, and thus predict host range. Assessing detailed gall development studies on plants from the introduced range also is important in that some agents are not as well adapted to plants from the invasive range (Cullen & Moore, 1983). The gall-inducing ability of the mite, *Aceria chondrillae* Canestrini (Acarina: Eriophyidae), on *Chondrilla juncea* L. (Compositae) was dependent on host genotype and growth conditions of the plant (Cullen & Moore, 1983). Galls can have a negative impact on the host plant through the formation of resource sinks that can be formed due to plant cell differentiation into vascular tissue within and to the gall (Harris & Shorthouse, 1996). Resources from other portions of the plant can be redirected to the gall, thereby stunting the plant's normal growth and reproduction (McCrea, Abrahamson, & Weis, 1985). However, not all galls create resource sinks sufficient enough to negatively affect the host plant (Harris & Shorthouse, 1996). The capitula gall induced by the biocontrol agent *Tephritis dilacerate* Loew (Diptera: Tephritidae) on *Sonchus arvensis* L. (Asteraceae) does not develop new vascular tissue within the gall. The reproductive output of the ungalled capitula on the host plant is not reduced, only the seed output from the galled capitula (Harris & Shorthouse, 1996). Thus, it is important to document gall development patterns of proposed biocontrol agents in its preferred host through histology, which can then potentially be used to assess if the gall will act as a resource sink. By assessing the gall

development of a proposed biocontrol agent on its host from the native range, this will become a standard that can be used to compare to that of galls induced on plants from the invasive range. This will contribute knowledge towards predicting agent efficacy in the field.

Yellow toadflax, *Linaria vulgaris* (L.) Mill (Plantaginaceae), is a non-native invasive plant that originated in Europe and was introduced into North America in the mid-1700s (Wilson et al., 2005). It is currently listed as a noxious weed in four Canadian provinces and eight US states and is distributed across Canada and the continental United States (USDA, 2010). *Linaria vulgaris* reproduces vegetatively by rhizomes and also by seed (Saner, Clements, Hall, Doohan, & Crompton, 1995). Flowers are similar to those of a snapdragon, yellow with a bright orange "lip", and the flowering period is from May until late fall (Wilson, et al., 2005). *Linaria vulgaris* is a common weed in dark soils of perennial and annual crops (Saner, et al., 1995), in other disturbed soils, such as railway rights-of-ways (Lageunesse, 1999), and in minimally disturbed environments, such as native grasslands (Ward, Reid, Harrington, Sutton, & Beck, 2008). *Linaria vulgaris* is difficult to control by chemical and mechanical means (Parchoma, 2002; Saner, et al., 1995). The weed has been an attractive candidate for biological control studies since the 1960's (McClay & De Clerck-Floate, 2002). To date, eight host-specific biocontrol agents have been either intentionally released to control *L. vulgaris* in North America, or were accidentally introduced from Europe and occur adventively on *L. vulgaris* (McClay & De Clerck-Floate, 2002; Wilson, et al., 2005). None of these agents have been successful at controlling *L. vulgaris* in Canada (McClay & De Clerck-Floate, 2002).

Currently, *Rhinusa pilosa* Gyllenhal (Coleoptera: Curculionidae) is one of two biological control agents being assessed as potential biological control agents for *L. vulgaris*. *Rhinusa pilosa* induces stem galls on various species in the genus *Linaria,* but its preferred host is *L. vulgaris* (Caldara, Desančić, Gassmann, & Toševski, 2008). The biology, taxonomy, and distribution of *R. pilosa* has been described mainly through the efforts of the toadflax biocontrol program (Toševski, Gassmann, & Desančić, 2004). Adults of the species emerge from diapause in early March in Serbia, during which time they feed on young toadflax shoots, mate, and commence oviposition. Eggs are deposited inside the stem pith tissue in the apical (i.e., meristematic) region of young, growing stems. The development from egg to adult of *R. pilosa* is complete within the multilocular gall. When reared at a constant 22˚C, the egg hatches after 8 days followed by two larval molts occurring every 7 - 8 days. The third and final instar larva pupates inside the gall and the adult ecloses 10 days later. The adult feeds on the remaining gall tissue before creating an emergence hole, or window, from which it will leave the gall. The adult continues to periodically feed on the leaves and stem of the host plant during the summer prior to winter diapause (Toševski, et al., 2004).

The main purpose of this study was two-fold. The first was to conduct a detailed, histological assessment of gall development in the *L. vulgaris - R. pilosa* system. The second was to compare gall development on plants from the native range (i.e., Serbia) to that of plants from the invasive range (i.e., Canada). *Rhinusa pilosa* is currently being investigated as a potential biocontrol agent for *L. vulgaris* in Canada, thus a detailed gall development study involving populations from the invasive range is essential to determining the suitability of this agent. Gall development should be studied because this

system is unusual in that: 1) the causative agent is a weevil (Coleoptera: Curculionidae) (most common insect gall formers belonged to the Families Cecidomyiidae (Diptera) and Cynipidae (Hymenoptera) (Dreger-Jauffret & Shorthouse, 1992), and detailed Coleopteran gall development studies have been lacking in the published literature (Ramamurthy, 2007); and 2) the gall induction process appears to be initiated by female ovipositional fluid or secretions from the egg since the gall is already formed by the time the larvae hatch from their eggs. Thus, results from this study will elucidate the gall development patterns of a potential biocontrol agent, *R. pilosa*, on its host, *L. vulgaris*, from the invasive and introduced range. This study also will contribute to the knowledge of cecidogenesis for an unconventional gall-inducing insect.

MATERIALS AND METHODS

Test plants of *L. vulgaris* from the native range were collected from three sites in the Belgrade region of Serbia in the spring of 2008. Seeds of *L. vulgaris* from the invasive range in Canada were collected from two locations in southern British Columbia (Kamloops and Chase) in September 2007 and germinated on wet sand in the early spring of 2008. Each plant was planted into 10 cm tall by 8 cm diameter plastic pots with a soil mixture of 4:1 of potting soil to sand. Prior to the test, all plants were initially grown under ambient greenhouse conditions with ambient day length in Zemun, Serbia. Plants for use in the study were mixed by location (i.e., the three Serbian populations were pooled and the two Canadian populations were pooled). In addition to the young control tissue collected in Serbia, mature control stem tissue was required. A second collection of ungalled control stems was conducted in Lethbridge, Alberta, Canada in the spring of

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2009 using Serbian plants grown from seed and field collected roots from Mountain View, Alberta, Canada plants grown in a greenhouse. Replicate stems from Serbian and Canadian material were approximately 30 cm tall and a small piece of stem tissue (5 mm long) was sampled 10 mm below the apical meristem and 10 mm above the base of the stem to represent young and mature control tissue samples, respectively.

Rhinusa pilosa adults were collected from outdoor overwintering cages in the spring of 2008 in Zemun, Serbia. One to four mated females were placed onto the apical portion of a plant for 24 hours. White sand was sprinkled onto the soil for ease of finding the female(s) when they dropped off the plant. Pots were covered with a plastic cylinder with a mesh lid (10 cm tall by 8 cm in diameter) that fit snugly around the pot to prevent the female(s) from escaping. Females were periodically monitored for oviposition. After 24 hours, the plants were checked for oviposition marks and the female(s) were then transferred to a new plant. Oviposition marks appeared as small oval or dark spots on the stem surface. The number of ovipositional marks and stems oviposited into were recorded. A total of 17 females induced 88 collected galls on 48 Serbian plants while 13 females induced 49 collected galls on 14 Canadian plants. The study was conducted in a growth chamber set at a constant 23°C with 16 /8 hours day/night regime.

Histology

Galls were collected at set time intervals after oviposition to determine gall development over time: control, less than 24 hours, 48 hours, 4 days, 8 days, 12 days, 16 days, 20 days, 28 days, and mature galls at 35 days after oviposition. Five to 10 samples per stage were collected. Samples were cut into sections (less than 5 mm^3) and fixed in

formalin acetic acid alcohol (FAA) (Ruzin, 1999; Schichnes, Nemson, & Ruzin, 2001). Galls were fixed for 3 - 6 months with the solution changed 2 to 4 times within the first month depending on the sample size until a clear solution remained to ensure proper fixation. Samples were embedded in wax using the protocol outlined by Schichnes, et al. (2001). In summary, galls were washed three times in de-ionized water for 10 minutes followed by an ethanol graded dehydration series for 30 – 90 minutes per dilution. Eosin dye was added during the final stage of dehydration for better visualization of the samples. Protocol SafeClear II (Fisher Scientific) was substituted for xylene for safety reasons as the intermediate solvent. The samples were then slowly infiltrated with paraffin using a heat plate until SafeClear II was saturated with wax. Samples were transferred to a small oven (incubator, Fisher Scientific 3510FS) set at 37°C with the wax being changed every 4 hours to once a day until no SafeClear II was detected. Following wax infiltration, samples were embedded into plastic molds and sectioned (9 to 35 μm depending on hardness of the tissues) with a glass blade on a microtome. Three or four galls per stage were randomly selected for sectioning. Microscope slides were then left over night on a hot plate to ensure that the sections fused to the slide.

Slides were stained using a modified Safranin-Fast Green protocol (Schneider, 1981). Slides were soaked two times in SafeClear II for 15 minutes each and rehydrated using a graded ethyl alcohol series and stained in Safranin for 90 minutes and counterstained with fast green for 2 minutes. Slides were soaked in two 15-minute washes of SafeClear II to destain.

Coverslips were attached with Poly-Mount® (Polysciences, Inc). Attempts were made to stain fixed material with potassium iodide (IKI) and Sudan IV for starches and fats, respectively, but staining was unsuccessful and no fresh material was available.

Data collection and statistical analysis

At the time of gall collection, gall width and length, as well as stem width 1 cm below the gall were recorded. Insect development stages were noted whenever possible during gall dissections. Gall volume was calculated using the volume of a prolate spheroid

$$4/3\pi(w/2)^2 l \hspace{3cm} (1)$$

modified from Woods (1996) and De Souza et al. (2001). Where $\pi = 3.14$, w is the widest part of the gall, and l is gall length.

After histological processing, photographs were taken of each sample. The tissue widths were measured from photographs of cross-sections at either 100 x (controls and samples collected 48 hours after oviposition) or 50 x (samples collected 4 days after oviposition and older) magnification using a micrometer.

Statistical analyses were conducted using the statistics program R version 2.10.1 (R Development Core Team, 2009). ANOVA was used to compare differences in means followed by Tukey's HSD for pairwise comparisons while linear regression was used to determine the relationship between variables. For the linear regressions, data from the two plant sources were pooled and only included measurements from mature galls (12 to greater than 35 days after oviposition) that were sectioned. The chi-squared test was used to determine if there was an equal proportion of categorical response variables between

the two source regions. Data were averaged per plant if more than one sample was sectioned from the same plant at the same collection time. All variables were checked for normality using histograms and q-q plots. Data that were positively skewed were $\log_{10}$ transformed where needed (cortex width only) to meet assumptions of normality.

RESULTS

Oviposition sequence

The oviposition sequence involved the female chewing a small hole near the apex of the stem with her head pointed towards the base of the plant. After chewing the oviposition canal, she turned around and placed her ovipositor into the oviposition canal. She remained still during oviposition, but when she was finished, she retracted her ovipositor and tapped her antennae on the stem. The female repeated this sequence multiple times spiraling down the stem to create multilocular galls (Chapter 3).

Insect development

The egg stage was observed in all galls up until 4 days after oviposition. At day eight (the next gall collection time), eggs and first instar larvae were observed. By day 12 after oviposition, second instar larvae were found. Head capsules were not consumed by the larvae facilitating counting of molts to determine larval instar when galls were dissected. Sixteen days after oviposition, there was a mixture of second and third instar larvae within the galls. By day 20 after oviposition, all galls collected contained third instar larvae and all samples contained pupae by day 28 after oviposition. Adults were noticed at final gall collection (greater than 35 days after oviposition). Adults fed on the

gall parenchyma within the gall before they chewed a small exit hole from the gall. Usually, the exit hole was covered by the epidermis prior to adult emergence.

Ungalled stem description

Within young stems of *L. vulgaris* at approximately 5 mm below the shoot apex (which corresponds to the location of female oviposition), the vascular tissue was arranged in a ring with small bundles and interfascicular parenchyma (Fig. 2.1). The primary xylem cells were clearly visible due to the cell wall thickenings and red stain. Epidermal cells were larger than the cortex cells and occasionally the epidermal cells had a multicellular trichome (not shown). Cells of the cortex and pith tissue were composed of parenchyma cells (Fig. 2.1). The majority of parenchyma cells had a large central vacuole and green-stained plastids, most likely to be chloroplasts due to their large size (5 µm) (Evert, 2006). The pith cells were generally larger and had irregular edges in comparison to the smooth edges of the cortex cells when viewed in cross-section (Fig. 2.1). When viewed longitudinally (Fig. 2.2), the cortex cells were equal in length to width, but the pith parenchyma cells were elongated.

In mature control stems (1 cm above the base of an ungalled stem), the vascular tissue was a continuous ring with xylem, phloem, and parenchyma cells (Fig. 2.3). The epidermal cells had some slight wall thickenings. The cells in the cortex resembled the same structure as in the young stem tissue with chloroplasts and large central vacuole, but mature cortex cells were larger. The phloem fibers were present as denoted by the thickened cell walls. Within the xylem, the secondary xylem cells were larger than the neighboring ray parenchyma cells, both of which appeared to be lignified due to the red-

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staining of the safraninin to the cell walls (Schneider, 1981). The pith parenchyma cells were large and round with a large central vacuole and the cell walls stained green due to the fast green staining the cellulose within the cell wall (Schneider, 1981). The histology of ungalled Canadian stems was similar to the ungalled Serbian stems (not shown).

Initiation phase (less than 24 hours to 48 hours after oviposition)

Cellular changes were observed as early as 24 hours after oviposition (Figs. 2.4 – 2.6). The initial plant response was a wound response with increased cell division of the cortical and cambial cells above the entrance to the oviposition canal. Similar responses were noted in the Canadian galls (not shown).

However, 48 hours after oviposition, more dramatic changes occurred. The number of cell layers within the cambium increased due to hyperplasy (i.e., increased cellular divisions), and this caused the xylem to be pushed towards the pith (Fig. 2.7). Hypertrophy of parenchyma cells within the cortex and some cell wall separation created small intercellular spaces. The vascular tissue from the more responsive plants had more hyperplasy of the cambium and minor hypertrophy of the xylem than the less responsive plants at this time (Fig. 2.8). The callus tissue had completely filled the entrance to the oviposition canal in most of the Serbian and Canadian galls sectioned.

Growth phase (4 to 8 days after oviposition)

By day 4 after oviposition, external stem swelling was observed in both the Serbian and Canadian galls. Internally, the parenchyma cells within the cortex had continued to hypertrophy, as well as the intercellular spaces within the cortex continued to expand (Fig. 2.9). The pith cells also broke apart at the cell wall to produce large,

multi-branched cavities (Fig. 2.9). These cavities were observed macroscopically when galls were dissected (not shown). By this time, some of the less responsive galls exhibited hyperplasied fascicular cambium (Fig. 2.10). In some samples, a second layer of cambium or "gall meristem" (Lalonde & Shorthouse, 1984) was located proximal to the pith (Fig. 2.9). Pith parenchyma cells that surrounded the egg chamber were smaller and more densely packed than in control stem pith. The pith parenchyma cells exhibited greater densities of plastids but retained the large central vacuole, thus it does not appear to be nutritive tissue. The callus tissue that had filled the oviposition canal had not been replaced with vascular tissue (Fig. 2.10). Within the less reactive plants, the vascular tissue, located in closer proximity to the egg, appeared to react more than the vascular tissue further away. For example, in Fig. 2.10 the vascular tissue located proximal to the egg underwent hyperplasy while the vascular bundles on the opposite side of the stem resembled that of control stems. Additionally, the pith cells further away from the egg retained similar characteristics as control pith while the parenchyma cells closer to the egg were smaller and contained plastids.

At this point in time, the more responsive galls showed xylem differentiation within the gall pith parenchyma (Fig. 2.11). New lignified xylem cells were differentiated within the pith. The larvae hatched from their eggs about 8 days after oviposition and the larvae fed on the pith or gall parenchyma cells (Fig. 2.12). The gall parenchyma cells were densely filled with plastids and a large central vacuole and was observed in both the Canadian and Serbian galls. As the galls grew, the pith cells became more densely packed with plastids, over many cell layers, surrounding the egg/larva (Fig. 2.12). The

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parenchyma cells within the vascular tissue also started to expand in some galls (Figs. 2.13 and 2.14). Gall meristem also was observed in the Canadian galls (Fig. 2.13).

Maturation and dehiscence phase (12 days to greater than 35 days after oviposition)

Externally, the mature galls were light green in colour and smooth in texture (Fig. 2.15) while the internal pith and parenchyma cells were greenish-yellow when dissected (Fig. 2.16). Galls were either positioned at the apex of the shoot, halting further vertical growth of the plant, or galls were in the middle of the stem depending on the growth rate of the plant. Galls at the apex tended to be oval, symmetrical and tapered acropetally. The width of mature galls were not significantly different between the two plant sources, while the Canadian galls were significantly longer (by 24 %) than the Serbian galls (ANOVA, $F_{1,24} = 4.41$, $P = 0.046$) (Table 2.1). Gall volume was not significantly different between the two source locations (Table 2.1). The Serbian and Canadian galls did not have a significantly different number of oviposition marks per stem (Table 2.1).

There were two types of vascular tissue organization (named by the author as "band" and "large bundle") observed in the maturation stage. Band vascular tissue had vascular tissue that was less than 500 μm in width in cross-sections in a continuous band, while large bundle vascular tissue was greater than 500 μm in width and was generally composed of large, more distinct bundles. Of the mature galls sectioned, 46 % (6/13) contained band vascular tissue from Serbia while 58 % (9/14) of the galls from Canada contained band vascular tissue. Proportions of band vascular tissue were not significantly different between the two source regions ($\chi^2 = 0.6$, P-value = 0.44). The exterior of a gall

with band vascular tissue organization was depicted in Fig. 2.15. Within this gall, a second instar larva was present within the pith region of the gall (Fig. 2.16). The vascular tissue was quite narrow and there was a large cortex region (Fig. 2.16). Microscopically, the vascular tissue was composed of a double layer of cambium (gall meristem) giving the appearance of a continuous band of vascular tissue and many large intercellular spaces were observed in the cortex (Fig. 2.17). In longitudinal-section of a different gall with band vascular tissue, the vascular tissue was a straight strip down the sides of the gall (Fig. 2.18). When observed at a higher magnification, a second layer of meristematic cells (i.e., gall meristem) was detected that appeared to produce xylem cells towards the outer region of the gall (Figs. 2.18 and 2.19). Frass has been observed in the larval chamber (Fig. 2.18).

The second example of vascular tissue organization was the development of large bundle vascular tissue organization. Large bundle vascular tissue consisted of 54 % (7/13) of the Serbian galls and 42 % (5/14) of the Canadian galls sectioned. Proportions of large bundle vascular tissue were not significantly different between the two source regions ($\chi^2 = 0.33$, P-value $= 0.56$). The exterior of a gall with large bundle vascular tissue was depicted in Fig. 2.20. When dissected, the gall contained large white sections of vascular tissue, surrounded by a thin layer of cortical cells (Fig. 2.21). The large width of the vascular cambium was observed to be the result of cell hyperplasy (Fig. 2.22), hypertrophy (Fig. 2.23) and/or de novo origin of meristematic regions within the vascular tissue (Figs. 2.24 and 2.25).

In some of the galls sectioned from both vascular tissue organizations, gall meristem was observed inside the gall pith region (Figs. 2.18, 2.19, and 2.25). The gall meristem was generally parallel to the vascular cambium but produced cells in the opposite orientation to the vascular cambium. Periclinal cell divisions produced cells that differentiated into xylem cells towards the outer region of the gall rather than towards the pith (Figs. 2.18, 2.19, and 2.25). In other galls, small islands of meristematic regions occurred within the pith (Fig. 2.24).

During the maturation phase, the pith and cortex cells retained the same characteristics as during the late gall growth phase. The pith cells retained the high density of plastids and large central vacuole (Fig. 2.19). The gall parenchyma cells that were distally located from the larval chamber were larger and contained fewer plastids than the gall parenchyma cells proximal to the larval chamber (not shown). The cortex cells completed cell expansion early in the gall maturation developmental phase (Figs. 2.17 and 2.22).

As the gall aged, it became very hard and there were greater amounts of lignin within the cells of the vascular tissue (Figs. 2.26 and 2.27) in comparison to the younger galls (Fig. 2.9). In the early maturation stage (12 to 16 days after oviposition), lignification was observed in a minor portion of the cells within the vascular tissue of the galls (Fig. 2.19). In these galls, lignin deposition occurred in a similar pattern to normal ungalled stems (Figs. 2.22 and 2.23). The vascular cells positioned towards the pith lignified before the vascular cells located towards the outer region of the gall. During the mid-maturation phase, almost all of the galls sectioned had some lignified cells within the

vascular tissue where the younger vascular cells matured before the older vascular tissue (Fig. 2.26). At the last gall development stage collected (greater than 35 days after oviposition), the vascular tissue from nearly all galls sectioned contained lignin (Figs. 2.27 and 2.28). Although a few galls, generally those with band vascular tissue organization, did not show extensive lignification within the vascular tissue (Fig. 2.29).

For the dehiscence stage or adult emergence, no samples were collected post-adult emergence. Samples were collected during the post-eclosion adult-feeding stage (greater than 35 days after oviposition) and have been described during the late maturation stage above. It was observed that the adults leave the gall by chewing an exit hole out of the gall.

Gall volume increased over time and differed in its relation to the plant response variables measured (width of stem below gall, number of oviposition marks, width of cortex, width of vascular tissue, and width of pith of mature galls from both populations). Gall volume steadily increased during the gall initiation, growth, and maturation phase (Fig. 2.30). Gall volume was greatest during the gall maturation phase (Fig. 2.30). Gall volume was significantly different among the gall development stages (1-way ANOVA, $F_{3,40} = 13.10$, $P < 0.000$) (Fig. 2.30). Gall volume was not associated with stem width 1 cm below the gall (linear regression, $F_{1,22} = 0.06$, $P = 0.80$, $r^2 = 0.00$) (Fig. 2.31). Gall volume was positively related to number of oviposition marks (linear regression, $F_{1,24} = 6.90$, $P = 0.01$, $r^2 = 0.22$) (Fig. 2.32). Within the gall, gall volume had a positive relationship with the width of the cortex ($\log_{10}$) (linear regression, $F_{1,23} = 4.57$, $P = 0.04$, $r^2 = 0.17$) (Fig. 2.33) and width of the pith (linear regression, $F_{1,13} = 6.6$, $P = 0.02$, r^2

=0.34) (Fig. 2.34). Vascular tissue width was not relate to gall volume (linear regression, $F_{1,23} = 0.00$, $P = 0.99$, $r^2 = 0.00$) (Fig. 2.35). Since there was a significant positive relationship between gall volume and gall width (linear regression, $F_{1,24} = 48.5$, $P < 0.000$, $r^2 = 0.67$) and gall length (linear regression, $F_{1,24} = 38.1$, $P < 0.000$, $r^2 = 0.61$) (not shown), the other plant response variables tested above (i.e., number of oviposition marks and gall tissue widths, etc.) were only assessed against gall volume and not gall width nor gall length.

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As vascular tissue width overall was not related to gall volume, the vascular tissue organizational type was investigated in relation to gall volume. As mentioned previously, galls exhibited two types of vascular tissue organization that were easily classified during the maturation phase (Figs. 2.17 and 2.22). Within each plant source (Serbia vs. Canada), the plant tissue type (cortex, vascular tissue, and pith) was subdivided into vascular tissue organization (band or large bundle) (Fig. 2.36). Cortex width was the same regardless of population or vascular tissue organization (Fig. 2.36). The average band and large bundle vascular tissue width was significantly different in both plant sources (ANOVA, Serbia: $F_{1,10} = 5.61$, $P = 0.04$; Canada: $F_{1,12} = 31.36$, $P < 0.000$) but not between plant sources (i.e., band vascular tissue width was not significantly different between Serbian and Canadian galls (Fig. 2.36). The width of the pith was equivalent between vascular tissue organization and plant sources (Fig. 2.36).

DISCUSSION

Gall development in the *L. vulgaris – R. pilosa* system from Serbian and Canadian populations was novel in that: 1) gall development had begun prior to larval

feeding, 2) multiple vascular tissue organizations (small band of vascular tissue or large vascular bundles composed of hypertrophied cells within the vascular tissue), 3) cellular differentiation of gall parenchyma cells that contained increased numbers of plastids and a large central vacuole but no true nutritive tissues (cells with dense cytoplasm, large nuclei, and an increased density of organelles), and 4) post-eclosion adult feeding within the gall.

Within the Coleoptera, gall induction prior to larval feeding is rare since larval feeding is generally required to induce galls (Florentine, Raman, & Dhileepan, 2002; Raman, Cruz, Muniappan, & Reddy, 2007). At least one other weevil studied has exhibited gall initiation prior to larval hatch, *Ceuthorrhynchus napi* Gyll. (Coleoptera: Curculionidae) on *Brassica napus* (Brassicaceae) (Le Pape & Bronner, 1987). Le Pape and Bronner (1987) found that the initial host response (after wound callus growth in response to the oviposition wound) was due to the egg's exochorion. Previous researchers diagnosed the exochorion as the ovipositional fluid secreted by the female (Deubert, 1955). Ovipositional fluid has been suspected as the gall-inducing substance in the *R. pilosa – L. vulgaris* interaction due to the fluid's ability to induce galls in other systems, such as the cynipid wasps (Rohfritsch, 1992). Ovipositional fluid also was thought to be responsible for gall induction due to the observation of normal-looking galls that upon dissection, lacked insects (Chapter 3). However, since no dark staining of a substance was observed beside the egg in our system, it is possible that the egg and not the ovipositional fluid provided the stimulus to activate hyperplasy in the vascular tissue. It is also possible that the ovipositional fluid was present, just not stained by the staining procedure or was washed away during histological processing (Chapter 3). Based on

these observations, gall induction in this system was studied in more detail in Chapter 3 of this thesis.

Two types of vascular tissue organizations were observed in the galls induced by *R. pilosa* and are quite unique for a gall-former. The big bundle vascular tissue organization was significantly wider than the band vascular tissue organization. The big bundle vascular tissue can be composed of hypertrophied cells, and/or cell hypertropy and hyperplasy. The hypertrophied vascular cells proximal to the cortex could be a means for the plant to re-direct nutrients around the gall (Fig. 2.24). In many other gall-inducing systems, vascular tissue modifications occur with increased vasculature directed towards the gall chamber (Dorchin, Freidberg, & Aloni, 2002; Lalonde & Shorthouse, 1984; Rohfritsch, 1992). Vascular differentiation around the gall chamber was induced by *Izeniola obesula* Dorchin (Diptera: Cecidomyiidae) larvae on *Suaeda monoica* Gmelin (Chenopodiaceae) (Dorchin, et al., 2002). The increased vasculature observed in the *R. pilosa* induced galls could bring more resources to the gall or be an adaptive measure by the plant to circumvent damage from the feeding larvae (Rohfritsch, 1992).

In the *R. pilosa* gall, no true nutritive tissue appears to be present as in more advanced gall formers. Nutritive tissue is defined as specialized cells that surround the larval chamber (Bronner, 1992). These cells contain large nuclei and nucleolus, dense cytoplasm with a reduced central vacuole, and increased numbers of organelles (Bronner, 1992). Nutritive tissue has been found in a portion of the weevil galls that have been studied in detail (Florentine, et al., 2002; Le Pape & Bronner, 1987; Raman, et al., 2007). Gall parenchyma cells near feeding *Conotrachelus albocinereus* Fiedler (Coleoptera:

Curculionidae) larvae on *Parthenium hysterophorus* L. (Asteraceae) become

cytoplasmically dense and contained prominent nuclei (Florentine, et al., 2002). In galls

induced by *R. pilosa* on *L. vulgaris*, a large region of green cells, spanning multiple cell

layers, surrounded the insect through all stages of development. These cells contained an

increased density of plastids and a large central vacuole. The organelles are most likely to

be chloroplasts due to their large size (approximately 5 μm) (Evert, 2006). This area of

plastid-containing cells could be a "green island", a region of green tissue surrounding

the insect (Walters, McRoberts, & Fitt, 2008). Green islands have been found to contain

higher concentrations of nutrients than the surrounding tissue (Walters, et al., 2008).

Intracellular studies to determine the presence of starches and lipids within the gall

parenchyma cells and cell ultrastructural examinations would be an ideal next step to

determine the nutritional value for the developing insects. Additionally, in order to

determine if the gall is acting as a resource sink, conducting translocation studies using

radio-labeled carbon would be beneficial. Tracking changes in carbon-14 levels both

above and below the gall in galled and ungalled plants can give us an indication of the

carbon allocation patterns occurring in this system. Carbon-14 levels have been measured

during gall development studies in the *Eurosta - Solidago* system to help determine

carbon allocation patterns (McCrea, et al., 1985).

After eclosion, the adults continued to feed on the gall parenchyma cells, an

unusual behaviour for gall-formers. In other more complicated, insect gall-inducing

systems, the parenchyma cells surrounding the adult chamber lignified to produce a

sclerenchyma sheath making post-eclosion feeding unlikely (Brooks & Shorthouse, 1998;

Lalonde & Shorthouse, 1984). In Canada thistle stem galls induced by the fly, *Urophora*

cardui L. (Diptera: Tephritidae), the pith parenchyma cells surrounding the larval chamber become lignified during the gall maturation phase (Lalonde & Shorthouse, 1984). Mature galls produced by the weevil, *Acythopeus burkhartorum* O' Brien (Coleoptera: Curculionidae) on *Coccinia grandis* (L.) Voigt (Cucurbitaceae) have cortical sclerenchyma (Raman, et al., 2007). The hard lignified cells are potentially more difficult to consume and digest than the more nutritious nutritive cells. In the *R. pilosa* system, if the adult or late instar larva has not depleted the gall parenchyma cells, the gall parenchyma cells do not lignify (Fig. 2.29). The gall parenchyma cells provided the adult with additional nourishment.

Rhinusa pilosa is currently being considered as a potential biological control agent for *L. vulgaris*. It is important to document the histological changes occurring during gall development in the native and introduced ranges of the host plant. This is to confirm that *R. pilosa* will be able to induce galls on *L. vulgaris* plants from the introduced range. Canadian galls tended to be longer than Serbian galls even though they contained the same number of oviposition marks (Table 2.1). This could be an indication that galls on Canadian plants could be larger nutrient sinks and thus, be a good target for biocontrol by *R. pilosa*.

Galls induced by *R. pilosa* are assumed to be energetically expensive since resources may be directed towards the gall due to increased vascular tissue and creation of de novo meristematic regions, both due to gall meristem and hyerplasy of gall parenchyma cells. De novo zones of meristematic cells are maintained or stimulated from egg to adult indicate that the insect was actively providing a stimulus to maintain

meristematic activity. Also, meristematic areas are energetically expensive due to the continual energy required for cell division (Evert, 2006).

The gall induced by *R. pilosa* on *L. vulgaris* was a "mark gall" as defined by Rohfritsch (1992) since the egg was enclosed in the stem at the beginning of gall development. Using the shoot-axis gall classification developed by Pjayaraman (1989), based on the location of the gall-inducer and type of tissues that were modified during gall development, galls induced by *R. pilosa* do not fall into one distinct category. The eggs of *R. pilosa* were positioned within the vascular tissue and pith region of the stem. As we have seen, gall development of *R. pilosa* induced galls involved modifications to these two tissues as well to the cortex. According to the classification by Pjayaraman (1989), gall development patterns were a combination of "lignogenous" and "medullogenous" cecidia. Lignogenous cecidia involved proliferation of the xylem parenchyma cells, while medullogenous cecidia involved proliferation of the pith parenchyma cells.

The first *L. vulgaris* response to *R. pilosa* was the production of callus tissue, undifferentiated cells that formed in response to wounding (Evert, 2006), that filled the oviposition wound. A similar wound response has been observed in Canada thistle stems in response to tunneling by *U. cardui* larvae (Lalonde & Shorthouse, 1984). The callus tissue remained undifferentiated over time in both the *L. vulgaris* and the Canada thistle plants. The undifferentiated callus tissue was utilized by both gall-inducing insects as a means to exit the gall. The callus tissue in the Canada thistle stem degraded allowing the gall former to escape, while *Rhninusa pilosa* may chew an exit hole through the callus

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tissue. Since the tissue surrounding the *R. pilosa* and *U. cardui* insects become hardened, consuming the undifferentiated callus tissue, rather than the hardened tissue, is a more efficient way to exit the gall. Pre-gall-inducing tunneling behaviour of some weevils also caused the host plant to produce callus tissue, such as the stem galls induced by *C. albocinereus* on *P. hysterophorus* (Florentine, et al., 2002). Callus tissue production after insect feeding, both by gall-inducing and non-gall-inducing endophagous insects (i.e., stem borers or leaf miners), appears to be a general plant wound response (Hewett, 1977; Shorthouse & Lalonde, 1984).

Gall growth in this system was due to a combination of hyperplasy and large intercellular space development in the cortex tissue as well as hypertrophy of pith parenchyma cells. Galls produced by *A. burkhartorum* (Raman, et al., 2007) and *C. albocinereus* (Florentine, et al., 2002) showed pith cell hyperplasy similar to what was observed in galls produced by *R. pilosa*. However, the cortex tissue response of the host plant differs with these two weevils. The cortex cells in *C. albocinereus* also contributed to the bulk of the gall, but did so through hyperplasy of the cortex cells rather than by hypertrophy and creation of intercellular spaces. No changes of cortex tissue were mentioned in galls of *A. burkhartorum*, but to allow gall expansion, some cellular modifications would be expected.

Frass that lined the larval chamber had been observed to contain fragments of xylem, indicating that the larva consumed the vascular tissue in addition to gall parenchyma cells. Feeding on the vascular tissues disrupts the flow of nutrients in the stem. Death of galled stems has been observed due to wilting, which indicates that

damage by *R. pilosa* to the vascular tissue can cause stem mortality. Larval feeding of *Coelocephalapion camarae* Kissinger (Coleoptera: Brentidae) on the vascular tissue within its petioles galls has resulted in the desiccation of *Lantana camara* L. (Verbenaceae) leaves, making this insect a more effective biocontrol agent (Baars, Hill, Heystek, Neser, & Urban, 2007).

CONCLUSION

The first objective of this study was to document gall development patterns in the *R. pilosa – L. vulgaris* system using plants from the native range. Galls induced by *R. pilosa* were novel in that: 1) gall development had begun prior to larval feeding when larval feeding is generally required for gall induction; 2) multiple vascular tissue organizations (small band of vascular tissue or large vascular bundles composed of hypertrophied cells within the vascular tissue) developed; 3) no nutritive tissue developed but the gall parenchyma cells surrounding the developing insect contained increased numbers of plastids; and 4) adults that emerged within the gall were able to feed on the pith parenchyma cells that had not lignified as is typically observed in other gall formers. The second objective of this study was to compare gall development patterns between the invasive and introduced range. No significant differences in gall development patterns were observed between the two plant regions although the Canadian galls were longer in comparison to the Serbian galls. Results from this study will also contribute to Coleopteran gall-induction and development studies, as weevils are an unconventional gall-inducing group.

REFERENCES

Abrahamson, W. G., & Weis, A. E. (1987). Nutritional Ecology of Arthropod Gallmakers. In F. J. Slansky & J. G. Rodriquez (Eds.), *Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates* (pp. 235-258). New York: John Wiley & Sons, Inc.

Baars, J. R., Hill, M. P., Heystek, F., Neser, S., & Urban, A. J. (2007). Biology, oviposition preference and impact in quarantine of the petiole-galling weevil, *Coelocephalapion camarae* Kissinger, a promising candidate agent for biological control of *Lantana camara*. *Biological Control, 40,* 187-195.

Bronner, R. (1992). The Role of Nutritive Cells in the Nutrition of Cynipids and Cecidomyiids. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 118-140). New York: Oxford University Press.

Brooks, S. E., & Shorthouse, J. D. (1998). Developmental morphology of stem galls of *Diplolepis nodulosa* (Hymenoptera: Cynipidae) and those modified by the inquiline *Periclistus pirata* (Hymenoptera: Cynipidae) on *Rosa blanda* (Rosaceae). *Canadian Journal of Botany, 76*, 365-381.

Caldara, R., Desančić, M., Gassmann, A., & Toševski, I. (2008). On the identity of *Rhinusa hispida* (Brullé) and its current synonyms (Coleoptera: Curculionidae). *Zootaxa, 1805*, 61-68.

Crawley, M. J. (1989). The successes and failures of weed biocontrol using insects. *Biocontrol News and Information, 10*, 213-223.

Cullen, J. M., & Moore, A. D. (1983). Influence of three populations of *Aceria chondrillae* on three forms of *Chondrilla juncea*. *Journal of Applied Ecology, 20*(1), 235-243.

De Souza, A. L. T., Tanaka, M. O., Fernandes, G. W., & Figueira, J. E. C. (2001). Host plant response and phenotypic plasticity of a galling weevil (*Collabismus clitellae*: Curculionidae). *Austral Ecology, 26*, 173-178.

Dennill, G. B. (1988). Why a gall former can be a good biocontrol agent: The gall wasp *Trichilogaster acaciaelongifoliae* and the weed *Acacia longifolia*. *Ecological Entomology, 13*, 1-9.

Deubert, K. H. (1955). Beiträge zu den beziehungen zwischen *Ceuthorrhynchus napi* Gyll. (Col. Curc.) und winterraps hinsichtlich der gallenbildung mit ovarienuntersuchungen an verschiedenen *Ceuthorrhynchus*-Arten. *Wissenschaftliche Zeitschrift Der Martin-Luther-Universität Halle-Wittenberg, 4*, 909-932.

Dorchin, N., Freidberg, A., & Aloni, R. (2002). Morphogenesis of stem gall tissues induced by larvae of two cecidomyiid species (Diptera: Cecidomyiidae) on *Suaeda monoica* (Chenopodiaceae). *Canadian Journal of Botany, 80*, 1141-1150.

Dreger-Jauffret, F., & Shorthouse, J. D. (1992). Diversity of Gall-Inducing Insects and their Galls. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 8-33). New York: Oxford University Press.

Evert, R. F. (2006). *Esau's Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function, and Development* (3rd ed.): Wiley-Interscience.

Florentine, S. K., Raman, A., & Dhileepan, K. (2002). Responses of the weed *Parthenium hysterophorus* (Asteraceae) to the stem gall-inducing weevil *Conotrachelus albocinereus* (Coleoptera: Curculionidae). *Entomologia Generalis 26*(3), 195-206.

Harris, P., & Shorthouse, J. D. (1996). Effectiveness of gall inducers in weed biological control. *The Canadian Entomologist, 128*, 1021-1055.

Hewett, E. W. (1977). Some effects of infestation on plants: A physiological viewpoint. *The New Zealand Entomologist, 6*(3), 235-243.

Lageunesse, S. (1999). Dalmatian and Yellow Toadflax. In L. Sheley & J. K. Petroff (Eds.), *Biology and Management of Noxious Rangeland Weeds* (pp. 219-260). Corvallis: Oregon State University Press.

Lalonde, R. G., & Shorthouse, J. D. (1984). Developmental morphology of the gall of *Urophora cardui* (Diptera, Tephritidae) in the stems of Canada thistle (*Cirsium arvense*). *Canadian Journal of Botany, 62*(1372-1384).

Le Pape, H., & Bronner, R. (1987). The Effects of *Ceuthorrhynchus napi* (Curculionidae, Coleoptera) on Stem Tissues of *Brassica napus* var *oleifera*. In V. Labeyrie, G. Fabres & D. Lachaise (Eds.), *Insects-Plants: Proceedings of the 6th International Symposium on Insect-Plant Relationships (PAU 1986)* (pp. 207-212). Dordrecht: Dr W. Junk Publishers.

McClay, A. S., & De Clerck-Floate, R. A. (2002). *Linaria vulgaris* Miller, Yellow Toadflax (Scrophulariaceae). In P. G. Mason & J. T. Huber (Eds.), *Biological Control Programmes in Canada, 1981-2000* (pp. 375-382). New York: CABI Publishing.

McCrea, K. D., Abrahamson, W. G., & Weis, A. E. (1985). Goldenrod ball gall effects on *Solidago altissima*: 14C translocation and growth. *Ecology, 66*(6), 1902-1907.

Parchoma, G. (2002). Yellow Toadflax *Guide to Weeds in British Columbia* (pp. 192-195). Burnaby: Province of British Columbia.

Pjayaraman, P. (1989). Histogenetic patterns and classification of the shoot-axis galls. *Proceedings of the Indian Academy of Science (Plant Science), 99*(4), 391-403.

R Development Core Team (2009). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Ramamurthy, V. V. (2007). Faunistic, ecological, biogeographical and phylogenetic aspects of coleoptera as gall-inducers and associates in plant galls in the orient and eastern palearctic. *Oriental Insects, 41*, 93-119.

Raman, A., Cruz, Z. T., Muniappan, R., & Reddy, G. V. P. (2007). Biology and host specificity of gall-inducing *Acythopeus burkhartorum* (Coleoptera: Curculionidae), a biological-control agent for the invasive weed *Coccinia grandis* (Cucurbitaceae) in Guam and Saipan. *Tijschrift voor Entomologie, 150*, 181-191.

Rohfritsch, O. (1992). Patterns in Gall Development. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 60-86). New York: Oxford University Press.

Rohfritsch, O., & Shorthouse, J. D. (1982). Insect Galls *Molecular Biology of Plant Tumors*: Academic Press Inc.

Ruzin, S. E. (1999). *Plant Microtechnique and Microscopy*. New York: Oxford University Press.

Saner, M. A., Clements, D. R., Hall, M. R., Doohan, D. J., & Crompton, C. W. (1995). The biology of Canadian weeds. 105. *Linaria vulgaris* Mill. *Canadian Journal of Plant Science, 75*, 525-537.

Schichnes, D., Nemson, J. A., & Ruzin, S. E. (2001). Microwave Paraffin Techniques for Botanical Tissues. In R. T. Giberson & R. S. J. Demaree (Eds.), *Microwave Techniques and Protocols* (pp. 181-189). Totowa: Humana Press.

Schneider, H. (1981). Plant Anatomy and General Botany. In G. Clark (Ed.), *Staining Procedures* (pp. 315-333). Baltimore: Williams & Wilkins.

Shorthouse, J. D., & Lalonde, R. G. (1984). Structural damage by *Rhinocyllus conicus* (Coleoptera: Curculionidae) within the flowerheads of nodding thistle. *The Canadian Entomologist, 116*, 1335-1343.

Toševski, I., Gassmann, A., & Desančić, M. (2004). *Annual Report 2003: Biological Control of Dalmatian and Yellow Toadflaxes, Linaria dalmatica and L. vulgaris*. Delemont: CABI Publishing.

USDA, N. R. C. S. (2010). The PLANTS Database. Retrieved 5 October, 2010, from http://plants.usda.gov/java/profile?symbol=LIVU2

Walters, D. R., McRoberts, N., & Fitt, B. D. L. (2008). Are green islands red herrings? Significance of green islands in plant interactions with pathogens and pests. *Biological Review, 83*, 79-102.

Ward, S. M., Reid, S. D., Harrington, J., Sutton, J., & Beck, K. G. (2008). Genetic variation in invasive populations of yellow toadflax (*Linaria vulgaris*) in the western United States. *Weed Science, 56*, 394-399.

Weis, A. E., & Abrahamson, W. G. (1986). Evolution of host-plant manipulation by gall makers: ecological and genetic factors in the *Solidago-Eurosta* system. *American Naturalist, 127*, 681-695.

Wilson, L. M., Sing, S. E., Piper, G. L., Hansen, R. W., De Clerck-Floate, R. A., MacKinnon, D. K., et al. (2005). *Biology and Biological Control of Dalmatian and Yellow Toadflax*: USDA Forest Service, FHTET-05-13.

Woods, J. O., Carr, T. G., Price, P. W., Stevens, L. E., & Cobb, N. S. (1996). Growth of coyote willow and the attack and survival of a mid-rib galling sawfly, *Euura* sp. *Oecologia, 108*, 714-722.

Figures 2.1 – 2.3: Ungalled *Linaria vulgaris* Serbian stem. **2.1**. Cross-section of a young

Serbian stem 5 mm below the apical meristem, the location that corresponds to *R. pilosa*

oviposition. **2.2**. Longitudinal-section of a young Serbian *L. vulgaris* stem. Notice on the

left the section goes through a vascular bundle while on the right, the section shows the

interfascicular space. **2.3**. Cross-section through a mature Serbian *L. vulgaris* stem. Here

the vascular tissue has connected into a continuous ring. The secondary xylem is

interdispersed with ray parenchyma, both of which exhibit scleratization. The pith cells

are larger and seem to have a reduced number of plastids in comparison to the younger

tissue. Cortex (C); epidermis (E); interfascicular space (IF); phloem (Ph); pith (P);

vascular bundle (VB).
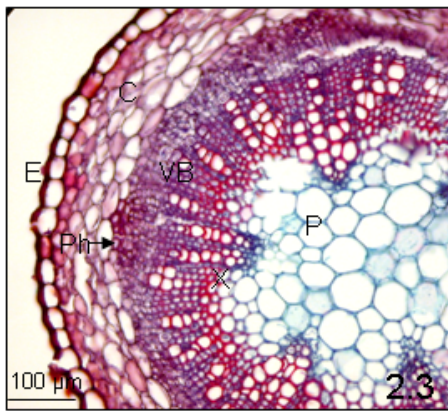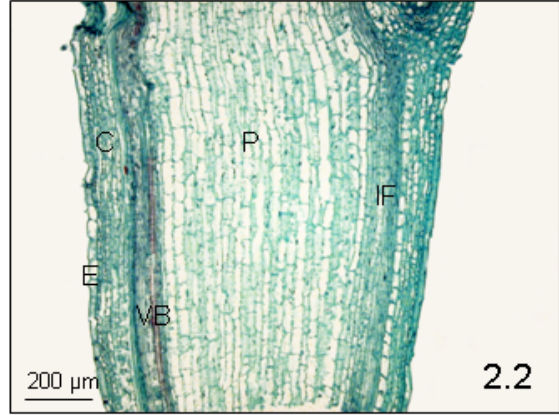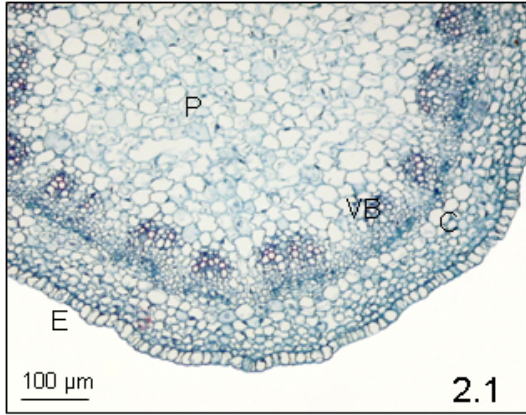
Figures 2.4 – 2.8: Gall initiation in the *Rhinusa pilosa* galls (less than 24 to 48 hours after oviposition). **2.4**. Cross-section of egg within a young Serbian stem less than 24 hours after oviposition, 16 μm above the entrance to the oviposition canal (See Fig. 3.6 for a schematic diagram of the oviposition canal). **2.5**. Increased magnification of wound response to oviposition from Figure 2.4 showing hyperplasy. **2.6**. Longitudinal-section of egg less than 24 hours after oviposition in a Serbian stem. **2.7**. Cross-section of an egg in a young Serbian stem, 48 hours after oviposition. The xylem beside the egg is being pushed towards the pith by the dividing fascicular cambium. Cells in the cortex and pith are separating to create air pockets. **2.8**. Cross-section of a *Linaria vulgaris* Canadian stem with an egg 48 hours after oviposition. The entrance to the oviposition canal has now been filled with callus tissue. The increased numbers of cells due to cell division within the vascular tissue can be observed pushing the xylem towards the pith. Note the frass beside the oviposition canal. Callus tissue (CT); cortex (C); egg (Eg); egg chamber (EC); epidermis (E); frass (F); hyperplasy (Hp); intercellular space (IS); pith (P); vascular tissue (VT); xylem (X).

Figure 2.4

Figure 2.5

Figure 2.6
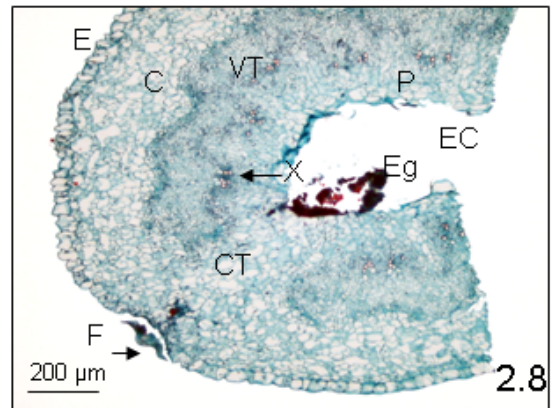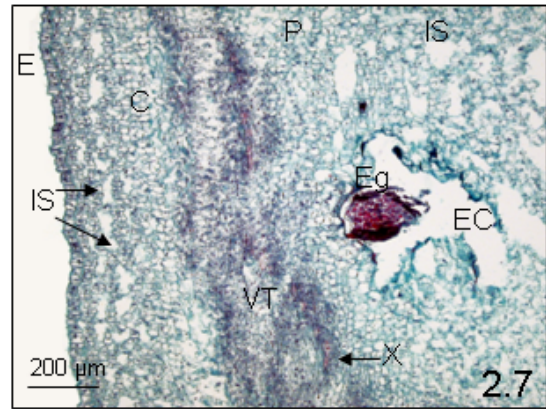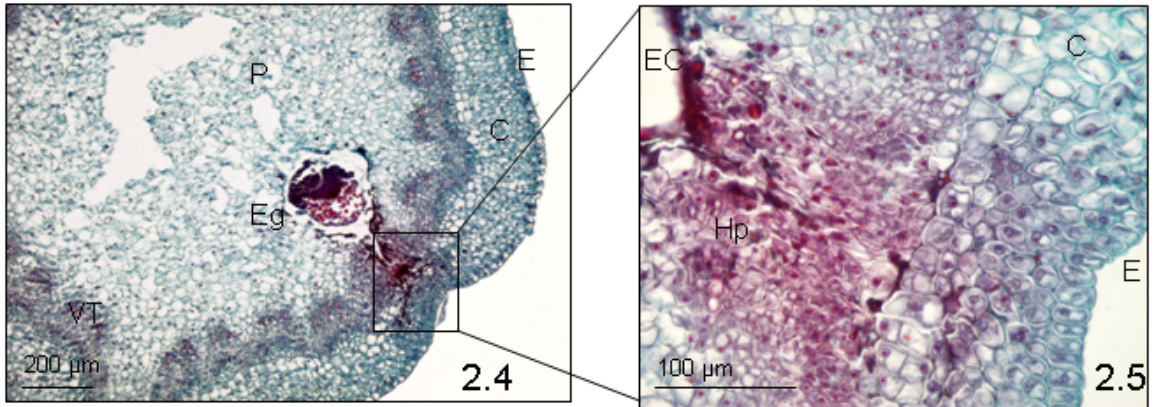
Figure 2.7

Figure 2.8

Figures 2.9 – 2.14: Early gall growth (four to eight days after oviposition) **2.9**. Longitudinal-section of a Serbian gall 4 days after oviposition showing egg and further cortical cell separation. Increased egg cavity in the pith and the vascular tissue shows gall meristem. **2.10**. Cross-section of a Serbian gall 4 days after oviposition showing unequal responsiveness of plant tissues. Note the hyperplasy of cells within the vascular tissue proximal to the egg and the normal vascular tissue away from the egg. **2.11**. Cross-section of a Canadian gall 4 days after oviposition showing xylem differentiation within the pith. **2.12**. Cross-section of a first instar larva feeding on gall parenchyma cells lining the larval chamber 8 days after oviposition within a Serbian gall. Gall parenchyma tissue is compact with a large central vacuole and a large density of plastids. **2.13**. Cross-section of a Canadian gall 4 days after oviposition. Note double layer of cambium and minor hypertrophy of undifferentiated vascular tissue cells. **2.14**. Cross-section of a Canadian gall 4 days after oviposition with large hypertrophied vascular cells and presence of gall parenchyma. Callus tissue (CT); cavity (Ca); cortex (C); egg (Eg); egg chamber (EC); epidermis (E); gall meristem (GM); gall parenchyma (GP); hyperplasy cells (HP); hypertrophied cells (HT); intercellular space (IS); larva (L); larval chamber (LC); normal vascular tissue (NV); pith (P); vascular cambium (VC); vascular tissue (VT); xylem (X).
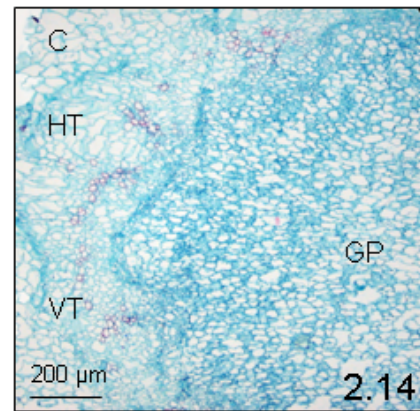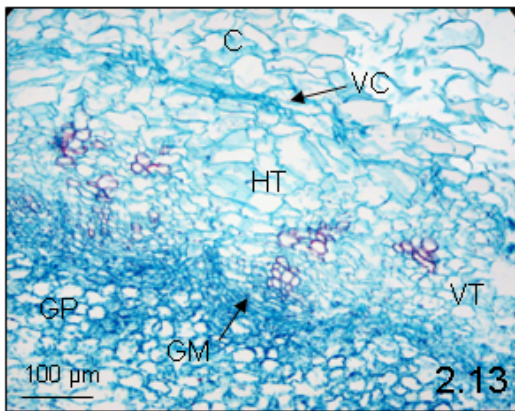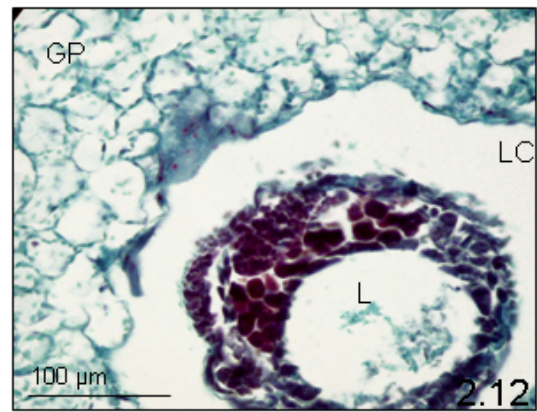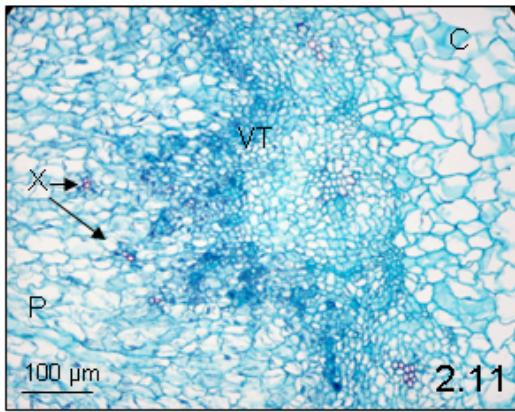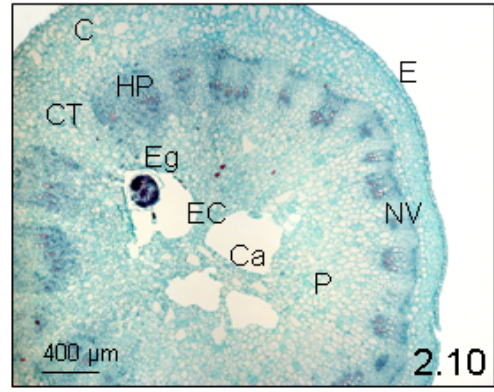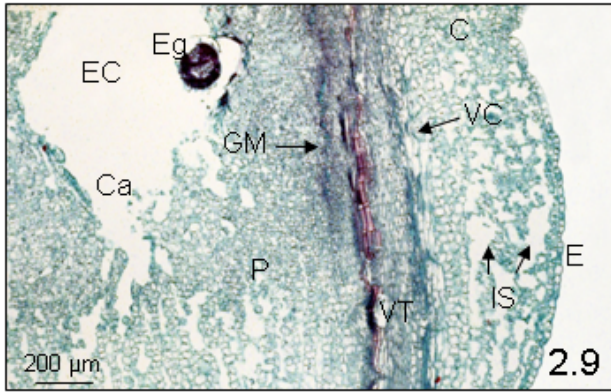
Figures 2.15 – 2.19: Band vascular tissue organization during gall maturation. **2.15**. Photograph of a Canadian gall 16 days after oviposition. **2.16**. Interior of gall in Figure 2.15 showing a second instar larva and narrow vascular band and a large cortex area. **2.17**. Cross-section of gall in Figure 2.15 showing narrow vascular band and cortex with intercellular spaces. **2.18**. Longitudinal-section of a maturing Serbian gall (20 days after oviposition) with double layer of cambium. **2.19**. Cross-section of a mature Canadian gall (16 days after oviposition) with gall meristem and vascular cambium. Band vascular tissue (BVT); cortex (C); epidermis (E); frass (F); gall (G); gall meristem (GM); gall parenchyma (GP); intercellular space (IS); larva (L); larval chamber (LC); vascular cambium (VC); xylem (X).

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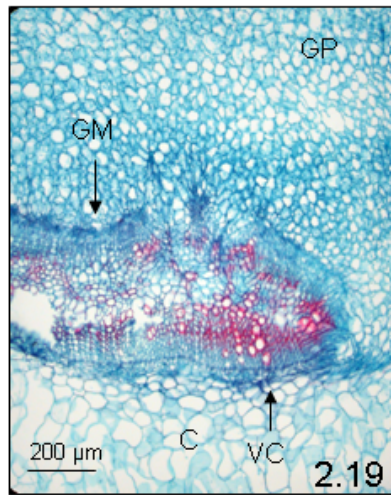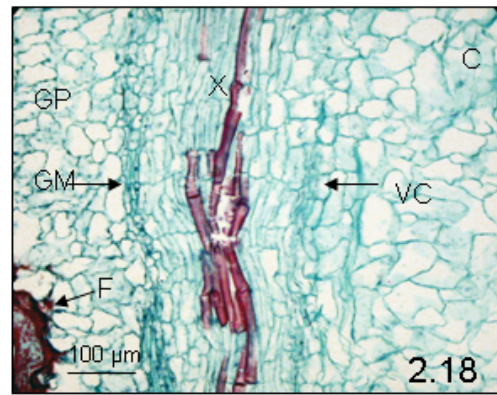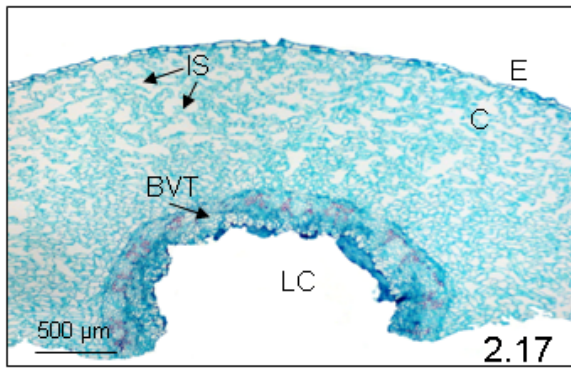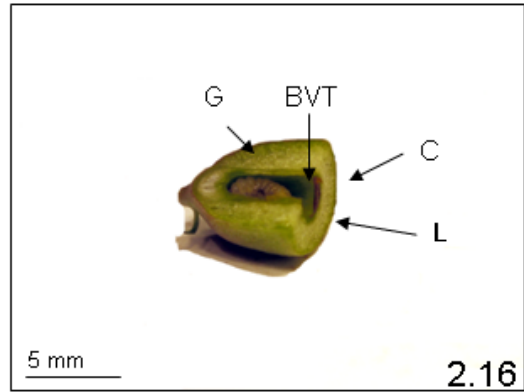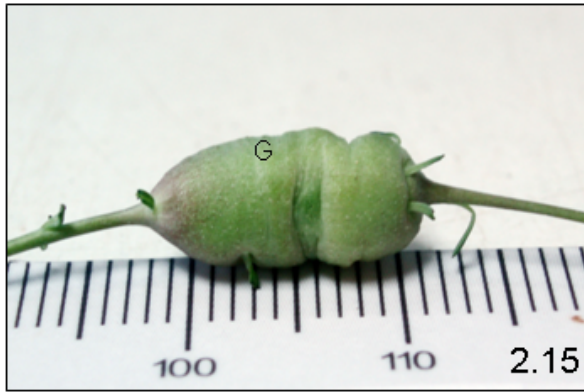
Table 2.1: Mean gall dimensions and number of oviposition marks per gall on *Linaria vulgaris* plants from Serbia and British Columbia, Canada, grown in controlled conditions. Mature galls only (12 to greater than 35 days after oviposition). Dissimilar letters are significantly different between populations (ANOVA, $P < 0.05$).

Origin	Gall width (mm ± SE)	Gall length (mm ± SE)	Gall volume (mm^3 ± SE)	Oviposition marks (n ± SE)
Serbia (n =13)	9.10 ± 0.5^a	18.7 ± 1.7^a	1676 ± 261^a	5.67 ± 0.8^a
Canada (n = 14)	9.30 ± 0.5^a	24.4 ± 2.0^b	2433 ± 346^a	4.21 ± 0.6^a

Figures 2.20 – 2.25: Large bundle vascular tissue organization during gall maturation. **2.20**. Photograph of a Canadian gall 20 days after oviposition. **2.21**. Interior of gall in Fig. 2.20 showing a third instar larvae and large vascular bundles with small cortex area. **2.22**. Cross-section of gall in Fig. 2.20 showing very large vascular bundles. **2.23**. Cross-section of a maturing Canadian gall (28 days after oviposition) showing hypertrophy of vascular parenchyma cells and lignifications of parenchyma cells proximal to the outer portion of the gall. **2.24**. Cross-section of a maturing Serbian gall (20 days after oviposition) showing large vascular bundles composed of hypertrophied vascular parenchyma cells and many small gall meristematic zones. **2.25**. Longitudinal-section of gall from Figure 2.24. Lignification of the vascular parenchyma cells are also visible. Cortex (C); epidermis (E); frass (F); gall (G); gall meristem (GM); gall parenchyma (GP); hyperplasied cells (HP); HT (hypertrophied cells); large vascular bundle tissue (LVT); larva (L); larval chamber (LC); lignin (Li).

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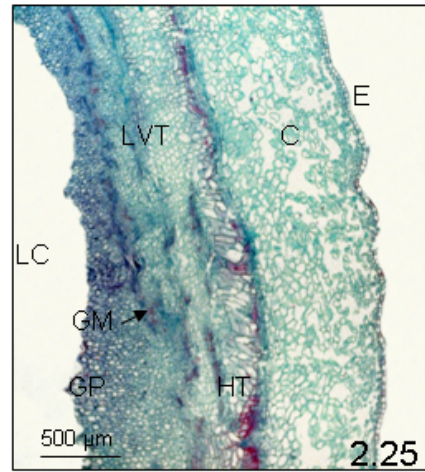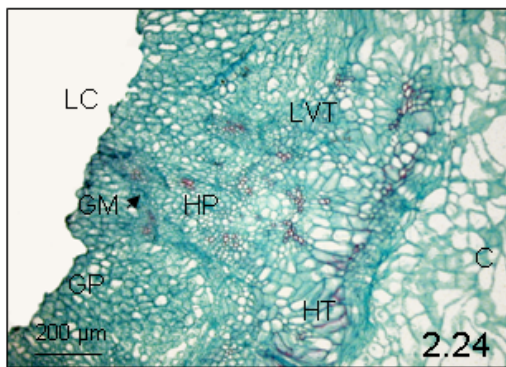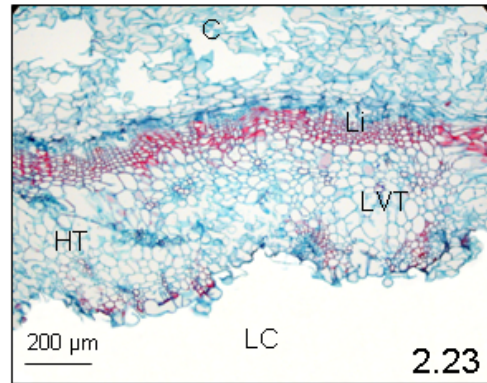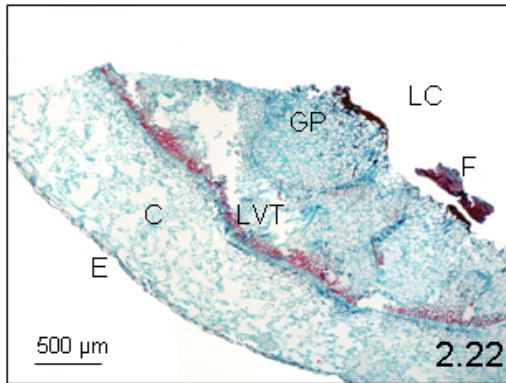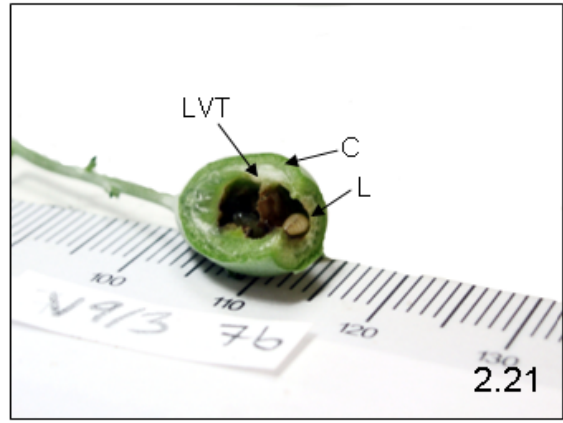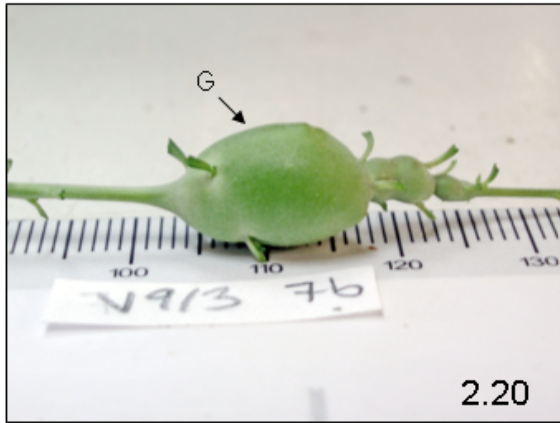
Figures 2.26 – 2.29: Gall maturation phase with increasing lignification of the vascular

tissue **2.26**. Longitudinal-section of a late maturing Serbian pupal chamber (28 days after

oviposition) with increased lignification of the vascular parenchyma cells.

2.27. Cross-section of a late maturation Serbian gall (greater than 35 days after

oviposition) with the majority of cells within the vascular tissue containing lignified

walls. **2.28**. Longitudinal-section of Figure 2.27. **2.29**. Cross-section of a late maturation

Serbian gall (greater than 35 days after oviposition) showing lack of lignin in vascular

cells proximal to an adult chamber. Adult chamber (AC); band vascular tissue (BVT);

cortex (C); large bundle vascular tissue (LVT); gall parenchyma (GP); lignin (Li); pith

(P); pupa (Pu); pupal chamber (PC).

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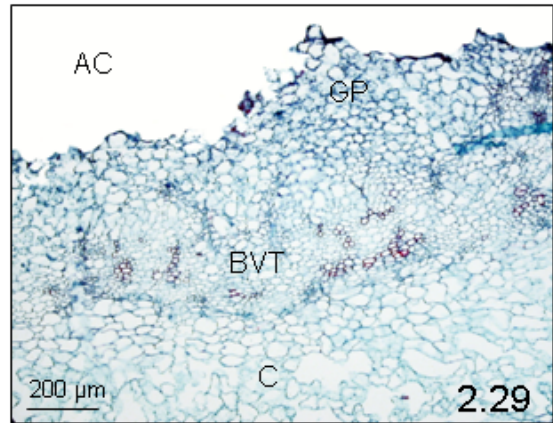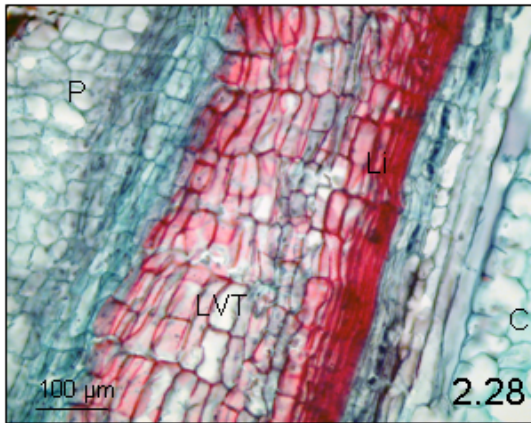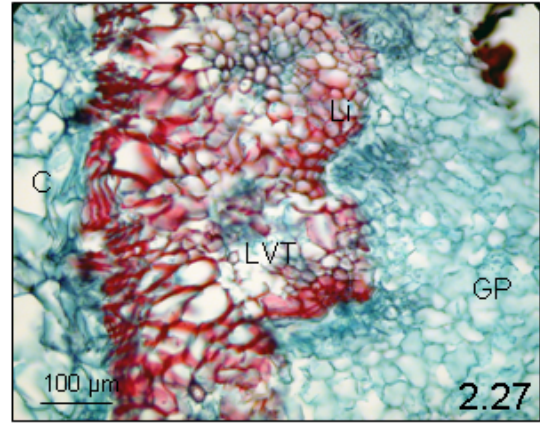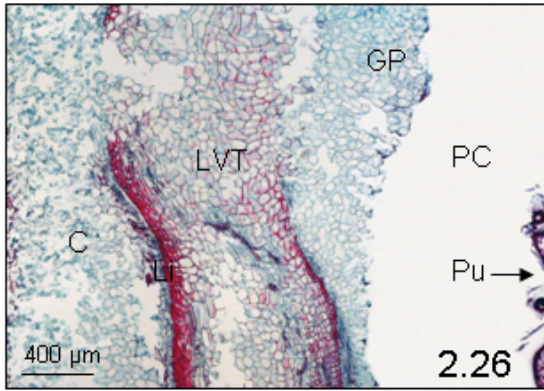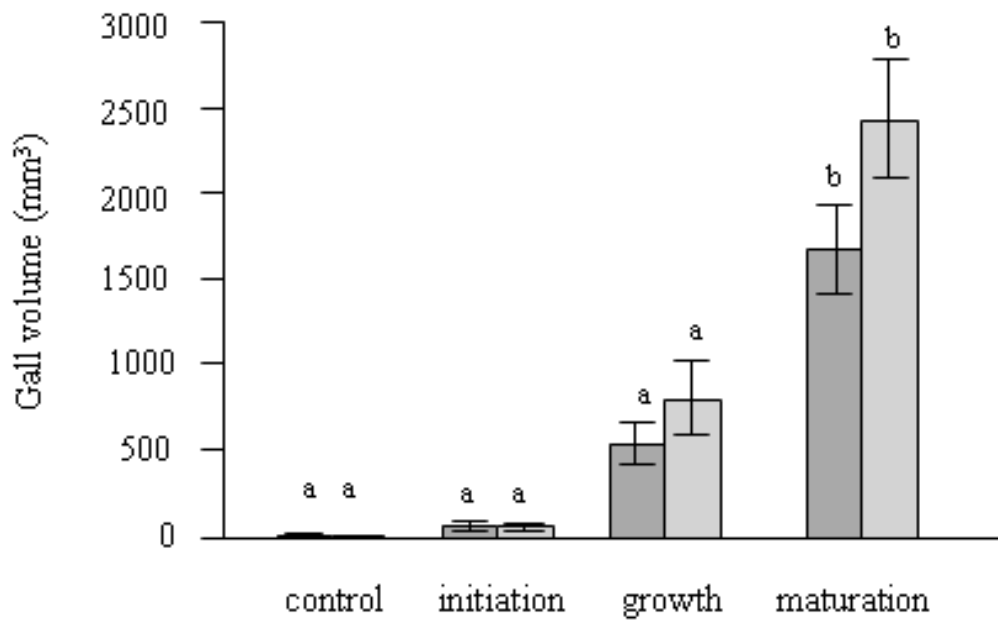
Figure 2.30: Gall volume by gall development stage and plant source. Control = ungalled stems (Serbia n = 3, Canada n = 3), initiation = galls collected less than 24 hours after oviposition to 48 hours after oviposition (Serbia n = 3, Canada n = 4); growth = 4 days to 8 days after oviposition (Serbia n = 4, Canada n = 4); and maturation = 12 days to greater than 35 days after oviposition (Serbia n = 13, Canada n = 14). Dark grey bars = Serbia; light grey bars = Canada. Vertical lines represent standard error of the mean. Bars with dissimilar letters are significantly different between stages (ANOVA, $P < 0.05$).

Figures 2.31 – 2.35: Gall volume in relation to plant response variables measured from mature galls from both Serbian and Canadian populations. **2.31**. Relationship between gall volume and width of stem 1 cm below the gall ($F_{1,22} = 0.06$, $P = 0.80$, $r^2 = 0.00$). **2.32**. Relationship between gall volume and number of oviposition marks per stem ($F_{1,24} = 6.90$, $P = 0.01$, $r^2 = 0.22$). **2.33**. Relationship between gall volume and width of cortex ($\log_{10}$) measured in cross-section ($F_{1,23} = 4.57$, $P = 0.04$, $r^2 = 0.17$). **2.34**. Relationship between gall volume and width of the pith measured in cross-section ($F_{1,13} = 6.6$, $P = 0.02$, $r^2 = 0.34$). **2.35**. Relationship between gall volume and width of the vascular tissue measured in cross-section ($F_{1,23} = 0.00$, $P = 0.99$, $r^2 = 0.00$).

Fig. 2.31
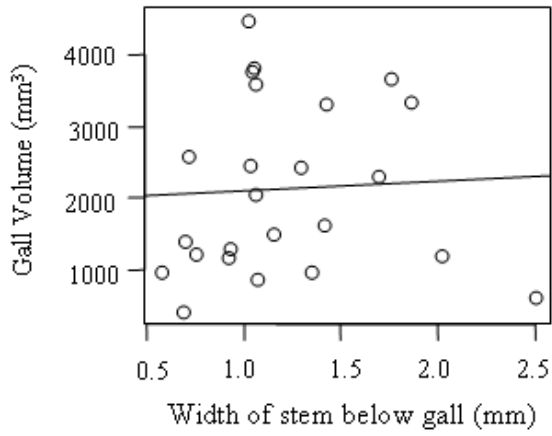
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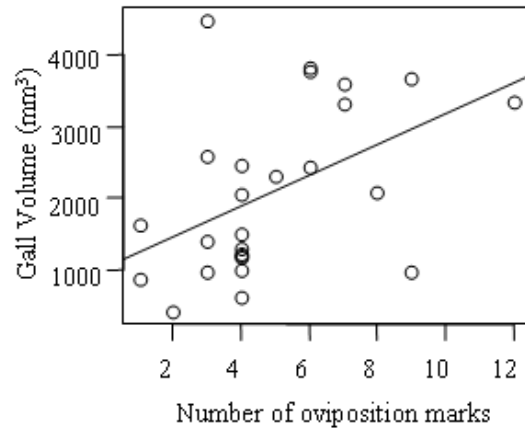
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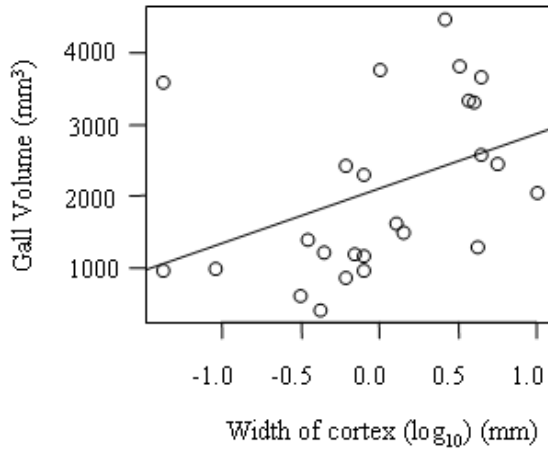
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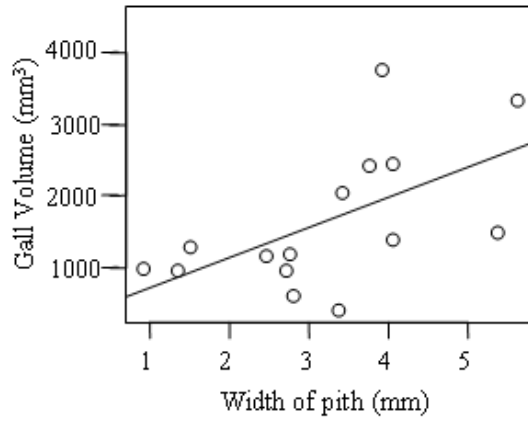
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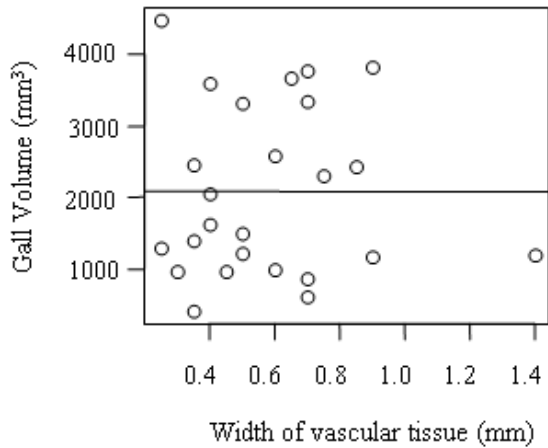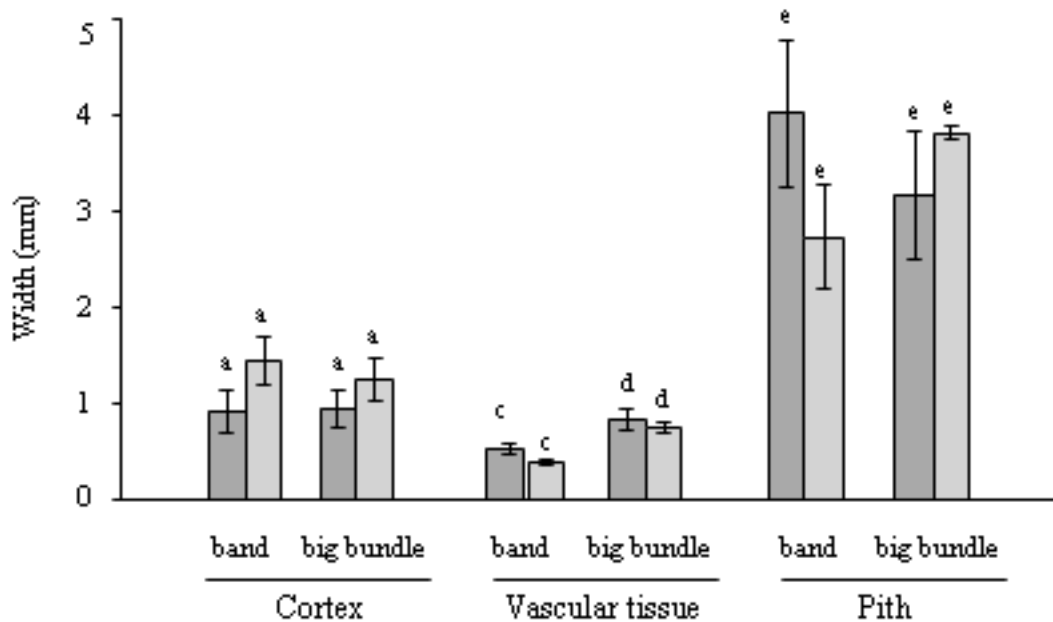
Figure 2.36: Vascular tissue organization by type, gall tissue, and plant source. Vascular tissue types are band and big bundle, while the gall tissues are cortex, vascular tissue, and pith. Measurements from mature galls sectioned (12 to greater than 35 days after oviposition). Dark grey bars = Serbia; light grey bars = Canada. Vertical lines represent standard error of the mean. Bars with dissimilar letters are significantly different between Serbian and Canadian populations (ANOVA, $P < 0.05$). Cortex was $\log_{10}$ transformed for the analysis.

CHAPTER 3: GALL INDUCTION IN THE *RHINUSA PILOSA – LINARIA VULGARIS* SYSTEM

INTRODUCTION

Galls are atypical plant growths induced by host-specific organisms such as bacteria, mites, and insects (Abrahamson & Weis, 1987). In this interaction, the insect provides the stimulus and the plant initiates the growth response (Weis & Abrahamson, 1986). In most gall forming systems, larval feeding is required to induce gall formation (Rohfritsch, 1992). However, gall induction prior to larval feeding has been observed in at least three hymenopteran gall-forming groups, the gall wasps (Cynipidae), the gall-inducing sawflies (Tenthredinidae), and the fig-wasps (Chalcidoidea) (Kjellberg, Jousselin, Hossaert-McKey, & Rasplus, 2005; Rohfritsch, 1992). In these taxa, gall induction is either due to female ovipositional fluid (Kjellberg, et al., 2005; Leggo & Shorthouse, 2006) or secretions from the egg (Rey, 1992) to create a "procecidium" (pre-formed gall). However, larval feeding is generally required to complete gall development in these gall inducers (Dreger-Jauffret & Shorthouse, 1992).

Salivary secretions from other gall formers have been responsible for gall induction, either from larval feeding (Dreger-Jauffret & Shorthouse, 1992; Lalonde & Shorthouse, 1984) or from oral secretions from the female (Lewis & Walton, 1947). For example, *Urophora cardui* L. (Diptera: Tephritidae) larvae tunnel in the Canada thistle, *Cirsium arvense* (L.) Scop. (Asteraceae), stem on route to the procambial tissue where larval feeding induces gall formation. The plant's response to larval feeding is to initiate callus tissue growth (undifferentiated cells that form in response to wounding (Evert,

2006)) in the tunnel (Lalonde & Shorthouse, 1984). Female feeding also has been responsible for gall induction. For the aphid, *Hormaphis hamamelidis* Fitch (Hemiptera: Aphididae), the mother or fundatrix uses her stylets to inject small drops of solution between the cells on Witch Hazel, *Hamamelis virginica* L. (Hamamelidaceae), to induce galls (Lewis & Walton, 1947). Experiments injecting homogenized heads of a Cecidomyiid fly, *Dasyneura urticae* (Perris) (Diptera: Cecidomyiidae), also have shown to stimulate callus tissue production (Leatherdale, 1955).

Ovipositional fluid has been shown to be responsible for gall induction in a number of insect groups. Within the Hymenoptera, two genera of sawflies, *Euura* spp. and *Pontania* spp. (Hymenoptera: Tenthredinidae), ovipositional fluid is important for gall induction (McCalla, Genthe, & Hovanitz, 1962; Price, 1992). Ovipositional fluid also has been associated with gall induction in the Cynipidae (Leggo & Shorthouse, 2006; Rohfritsch, 1992) and the Chalcidae (Kjellberg, et al., 2005). Within the Coleoptera, at least one other beetle has been able to induce gall formation prior to larval feeding, *Ceuthorrhynchus napi* Gyll. (Coleoptera: Curculionidae) (Le Pape & Bronner, 1987). In early studies examining the biology of *C. napi* on *Brassica napus* L. (Brassicaceae), ovipositional fluid was thought to be responsible for gall induction (Deubert, 1955; Günthart, 1949). In a more detailed study, no evidence of ovipositional fluid was detected during oviposition (Le Pape & Bronner, 1987). Le Pape and Bronner (1987) suggested that the ovipositional fluid identified in previous studies was the exochorion that had separated from the egg, and had adhered to the plant cells lining the egg chamber.

Linaria vulgaris (L.) Mill. (Family Plantaginaceae, formerly Scrophulariaceae (Olmstead et al., 2001)) is a non-native, highly invasive plant originating from Eurasia. *Linaria vulgaris* is found across Canada and the United States and is a common weed of dark, loamy soils (Wilson et al., 2005). This plant out competes native plants in undisturbed grasslands and is a problem weed of annual and perennial crops (Parchoma, 2002). *Linaria vulgaris* is difficult to control manually and with herbicides due to its rhizomatous root system (Wilson, et al., 2005). *Linaria vulgaris* reproduces by seed, averaging 30,000 seeds per plant annually, or vegetatively by a creeping root system (Saner, Clements, Hall, Doohan, & Crompton, 1995). Due to the resistance of *L. vulgaris* to herbicides (Saner, et al., 1995) the weed has been an attractive candidate for biological control studies since the 1960's (McClay & De Clerck-Floate, 2002). To date, eight host-specific biocontrol agents have been either intentionally released to control *L. vulgaris* in North America, or were accidentally introduced from Europe and occur adventively on *L. vulgaris* (McClay & De Clerck-Floate, 2002; Wilson, et al., 2005). None of these agents have been successful at controlling *L. vulgaris* in Canada (McClay & De Clerck-Floate, 2002).

Rhinusa pilosa Gyllenhal (Coleoptera: Curculionidae) is one of two insects currently under investigation as a potential biological control agent of *L. vulgaris* in North America. *Rhinusa pilosa* is a stem-galling weevil on *Linaria spp*. but its preferred host is *L. vulgaris* (Caldara, Desančić, Gassmann, & Toševski, 2008). In Serbia, over-wintered adults emerge in early spring to mate, feed, and oviposit. Females oviposit within the upper portions of young, fast-growing stems by chewing an oviposition canal into the stem followed by the deposition of an egg into the canal. Under artificial

conditions, females, on average, oviposit for 26.3 ± 3.44 days, induce 27.7 ± 4.5 galls and produce 51.9 ± 9.7 live adults (n = 32) (author, unpublished data, 2009 rearing results). Multilocular gall development is complete approximately 8 - 10 days after oviposition, which corresponds to larval hatch when reared at a 22˚C/18˚C day/night temperature in artificial conditions (Chapter 2). The developing larvae feed on the gall parenchyma cells within the pith. Third instar larvae pupate within the gall and adults eclose and fed on the remaining gall parenchyma cells (Toševski, Gassmann, & Desančić, 2004). Externally, stem galls are generally green and oblong. Galls can be uni- or multilocular averaging 2.4 adults per gall (Toševski, et al., 2004). Serbian galls average 9.1 ± 0.5 mm wide by 18.7 ± 1.7 mm long (Chapter 2).

Galls induced in the *R. pilosa - L. vulgaris* system were unique in that: 1) the gall-inducing agent was a weevil; and 2) gall development was nearly completed by the time the larvae had hatched from their eggs. The most common gall-inducing organisms belong to the Family Cecidomyiidae (Diptera) and Family Cynipidae (Hymenoptera) and larval feeding is the most common method insects use to induce gall formation (Rohfritsch, 1992). By isolating the response of the host plant to the different stages involved in female oviposition, the key step(s) that cause the plant tissue to induce gall formation may be isolated. The goal of this experiment was to identify the key step(s) involved with gall initiation in the *R. pilosa – L. vulgaris* system. Mated, ovipositing females were removed at different stages during the oviposition sequence in an attempt to experimentally isolate the response of the host plant. This will contribute valuable information towards gall induction by a non-conventional gall former.

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MATERIALS AND METHODS

Gall Induction 2009

The gall induction experiment in 2009 used three artificially over-wintered plants originally collected in the spring of 2008 from a gall susceptible population Mountain View, Alberta, Canada (49° 3'N 113°27'W). Plants were potted in 15 cm clay pots using a 30/30/30 soil mix (1 part sand, 1 part top soil and 1 part Cornell mix (110 L vermiculite, 60 L peat moss, 9 L sand, 1.5 kg osmocote fertilizer, 380 g superphosphate fertilizer, 1 kg calcium flour, 10 g chelated iron, 20 g fritted trace elements (Frit Industries, Ozark, Alabama, USA). Three plants with greater than 10 young, growing stems, less than 11 cm in length, were chosen to reduce plant response variation and the stem length corresponds to the length that females would normally use for oviposition. Plants were grown under artificial conditions in the Insect Microbial Containment Facility at the Lethbridge Research Centre, Agriculture and Agri-food Canada in Lethbridge, Alberta, Canada. Rearing room conditions were set for 14 hours daylight/10 hours dark and temperatures of 22˚C/18˚C day/night with plant grow fluorescent light bulbs (Standard F32T8, Saint-Laurent, Quebec, Canada).

The Institute of Plant Protection and Environment in Zemun, Serbia supplied the insects. Insects arrived on March 19, 2009 and nine females that induced galls successfully were used for the experiment.

Prior to starting the experiment, casual observations were made on three instances of complete oviposition to define the stages of the oviposition sequence. To determine the appropriate time to disrupt the female during her oviposition sequence for the Oviposition

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Fluid treatment, an additional three females where disrupted while ovipositing to isolate when the egg was deposited into the stem. Stems were dissected after the female was removed to confirm presence/absence of an egg. One female deposited an egg inside the stem as quickly as 25 seconds after inserting her ovipositor into the stem, thus females would be removed at or close to 20 seconds for the Ovipositional Fluid treatment.

Six experimental groups were selected to investigate gall induction based on the stages of oviposition: 1) Control, 2) Complete Oviposition Sequence, 3) Oviposition Canal, 4) Ovipositional Fluid, 5) Egg Insertion, and 6) Wounding. The Control treatment consisted of a non-treated stem. A small dot was made with a fine-tipped, non-toxic marker 5 mm below the apical meristem of control stems, the approximate location a female would oviposit. A 3 mm long stem section just above the mark was collected 3 days after application of the mark to coincide with collection of the treatment group stem material.

For the Complete Oviposition Sequence (undisturbed female) treatment, the female was allowed to oviposit normally with no interruptions and removed after she completed her oviposition sequence.

The Oviposition Canal treatment consisted of removing a female after she had chewed an oviposition canal, within 10 mm of the apical meristem, but before she inserted her ovipositor into the stem as if to oviposit. A small dot was made with a fine-tipped non-toxic marker just below the entrance to the oviposition canal for ease in locating the oviposition canal at treatment collection time.

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During the Ovipositional Fluid treatment, the female was removed at 20 seconds after her ovipositor was inserted into the oviposition canal. It is hypothesized that the female would secret an ovipositional fluid into the stem before the egg (Price, 1992). To confirm that no eggs were deposited during this treatment, stems that were not collected for histological analysis were dissected 10 days after treatment. The stems collected for histological analysis were checked for absence of egg during histological processing. A small dot was made beneath the entrance to the oviposition canal for ease in locating this spot when the stem was collected.

The Egg Insertion treatment involved inserting an egg that was dissected from a stem that was oviposited into, 2 to 4 days previously. Before insertion, the egg was washed in distilled water in an attempt to remove any ovipositional or accessory fluid on the exterior of the egg. A small hole, where the egg was inserted into, was created by inserting an insect pin, sized 00, into a fresh stem approximately 5 mm below the apical meristem.

The Wound treatment involved insertion of an insect pin, size 00, into a stem, approximately 5 mm below the apical meristem, to create a small wound. This treatment doubled as a control for the Egg Insertion treatment (described above) and to determine *L. vulgaris*'s response to mechanical wounding.

Stems and females were randomly assigned to treatments on one of the three *L. vulgaris* plants used for the experiment. Stems selected for the experiment were between 7 and 11 cm. Samples were collected 3 days after treatment, which corresponds to the early gall growth stage (Chapter 2). Three to six replicates were collected for histological

analysis from each group. Two additional Oviposition Fluid samples were collected and fixed 10 days post treatment. The remaining samples were assessed 10 days after treatment. For example, Complete Oviposition Sequence treatment samples were dissected to confirm presence of egg/larva, Ovipositional Fluid treatments were dissected to confirm absence of egg and all remaining stems were observed for delayed stem swelling.

Mated and gall-inducing females were placed on a shoot, randomly assigned to a treatment (Oviposition Canal, Complete Ovipositional Sequence, or Ovipositional Fluid) and observed for a maximum of 15 minutes. If the female did not oviposit within the 15 minute observation period, the female was replaced with another female. Of the seven females used for this test, only one female would "oviposit on demand". Two additional females were used for the Egg Insertion Treatment.

Gall Induction 2010

After histologically processing the samples collected in 2009, it was determined that samples collected between days 3 and 10 after treatment would be best to show host-plant response, thus the samples from 2010 were collected 5 days after treatment. Problems during histological processing in 2009 also occurred that resulted in an insufficient number of usable replicates in some treatments. Thus it was decided that this experiment should be repeated. Due to experimental design problems with the Egg Insertion treatment (see Results), it was decided that this and the Wound treatment would not be repeated in the 2010 trial.

Six artificially over-wintered *L. vulgaris* plants collected from the same site as in 2009 (Mountain View, Alberta, Canada) were used. Field collected plants were potted and grown under the same artificial conditions as in the 2009 experiment, except that a mixture of incandescent (GE Ecolux ESP41, Mississauga, Ontario, Canada) and florescent plant grow lights (Standard F32T8, Saint-Laurent, Quebec, Canada) were used.

Rhinusa pilosa used in the experiment were from a laboratory reared colony recently established at the Lethbridge Research Centre. The insects originated from the Institute of Plant Protection and Environment in Zemun, Serbia, shipped in 2009. A small batch of insects (ca. 70) from the colony were taken out of winter diapause conditions on Feb. 3, 2010 and transferred to 10˚C for 2 weeks followed by a week at 15˚C. Insects were transferred to the same conditions as the plants and caged on fresh plants to confirm their ability to induce galls. Twelve females that successfully induced galls were selected for the experiment.

For the 2010 gall induction experiment, there were four groups: 1) Control, 2) Oviposition Canal, 3) Ovipositional Fluid, and 4) Complete Oviposition Sequence. Control stems were treated the same as in 2009. Stems and females were randomly assigned to treatments on one of six short-stemmed *L. vulgaris* plants. Stems were between 7 and 11 cm. Females were observed for up to 2 hours. Five to eight samples from each group were collected 5 days after treatment. The treatments were conducted as in 2009.

Histology 2009/2010

Three to eight replicate samples per group, from each year, were collected and fixed in formalin acetic acid alcohol (FAA), an acid-based fixative (Ruzin, 1999; Schichnes, Nemson, & Ruzin, 2001). FAA was replaced up to three times until the solution remained clear to ensure proper fixation. All collected samples were embedded using a modified microwave protocol (Ruzin, 1999; Schichnes, et al., 2001). In summary, samples were cleared using isopropyl alcohol followed by an ethanol graded dehydration series. Eosin dye was added to the absolute ethanol for better visualization of the samples prior to embedding. The samples were then slowly infiltrated with paraffin and embedded. Three to five replicate samples from each treatment, in each year, were cross-sectioned at 9 μm in 2009 or 10 μm in 2010. One Oviposition Canal sample from 2009 was longitudinally sectioned to better visualize the shape of the oviposition canal. Due to problems with sections not adhering to the slides for the 2009 experiment, microscope slides were coated with Haupt's solution for the 2010 experiment to increase section adhesion to the slide (Johansen, 1940). Slides were left over night on a hot plate to ensure that the sections fused to the slide. Slides were stained using a modified Safranin-Fast Green protocol (Schneider, 1981). Protocol SafeClear II (Fisher Scientific) was substituted for xylene. Coverslips were attached with Poly-Mount(R) (Polysciences, Inc).

RESULTS

Oviposition Sequence

The oviposition sequence was as follows: 1) the female positioned herself near the apical meristem of the stem with her head pointed towards the ground, usually with her head rested against a leaf; 2) She chewed an oviposition canal into the stem pith for

approximately 120 seconds (range 130 to 150 seconds, n = 3); 3) Following creation of

an oviposition canal, the female removed her rostrum from the stem and rotated 90˚; 4)

The ovipositor was inserted into the oviposition canal for 90 – 180 seconds (n = 3) and

remained motionless while she deposited an egg; 5) After oviposition, the female tapped

her antennae on the stem as the ovipositor was removed from the stem.

Control Treatment

Externally, the *L. vulgaris* stems suitable for oviposition are young, succulent, and

vegetative (Fig. 3.1). No external stem swelling was observed in any of the Control stem

samples. Approximately 5 mm beneath the apical meristem, the interior of the stem was

composed of pith, a ring of vascular tissue, cortex, and epidermis (Fig. 3.2). The vascular

bundles consisted of xylem, phloem, and vascular cambium and were separated by

interfascicular parenchyma. The parenchyma cells within the cortex were generally

smaller than those of the pith at this stage. The parenchyma cells contained a large central

vacuole and green-stained plastids (Fig. 3.2). Cross-sections of control stems sampled

were equivalent for both collection periods (3 days after treatment, 2009, and 5 days after

treatment, 2010).

Complete Oviposition Sequence Treatment

When a female was allowed to complete her oviposition sequence without

interruption, external stem swelling was visible in all replicate samples collected (five

samples collected 3 days after treatment in 2009, and five samples collected 5 days after

treatment in 2010) (Fig. 3.3). Internally, three host tissue layers responded (Fig. 3.4). The

pith parenchyma cells, which surrounded the egg chamber, were smaller and more

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densely packed and were more nucleated than pith cells in the control stem. The egg was clearly visible in nine of the 10 replicate samples and the exochorion had adhered to the pith cells in three of the five replicate samples sectioned in 2009, and in four of the five replicate samples sectioned in 2010. Of the remaining samples with an egg, the two samples from 2009 were poorly processed, and upon viewing the sections, it was not clear if the exochorion was present. In both sampling time periods, the vascular cambium had undergone increased hyperplasy, in both the fascicular and interfascicular cambium. The hyperplasy of the vascular cambium pushed the xylem cells towards the pith. The vascular tissue above the entrance to the oviposition canal was replaced with callus tissue (Fig. 3.4). The cells within the cortex were hypertrophied in comparison to the cells from the control (Fig. 3.4). In four of the five replicate samples collected 5 days after treatment in 2010, large intercellular spaces were visible (not shown). Large intercellular spaces were not observed from the replicate samples sectioned 3 days after treatment or in the control replicates.

Oviposition Canal Treatment

To determine the plant's response to the creation of the oviposition canal (i.e., wounding), a female was removed after she chewed an oviposition canal, but before the female inserted her ovipositor into the stem. Replicate samples collected 3 days (four replicates) or 5 days (five replicates) after treatment did not exhibit external stem swelling (Fig. 3.5). Internally, the oviposition canal, chewed by the female, was in the shape of her rostrum, curved into the pith of the stem (Fig. 3.6). Of the samples collected 5 days after treatment, there was a variety of responses to wounding. In two of five samples, the oviposition canal was completely filled by callus tissue, showing a rapid

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wound response (Fig. 3.7). The three remaining samples showed reduced wound responses. In one sample, the canal was filled with callus tissue but the entrance to the canal was not filled with callus tissue (Fig. 3.8). Another sample exhibited the opposite callus tissue growth pattern, where the entrance to the oviposition canal was filled with callus tissue but the oviposition canal within the pith was free of callus tissue (Fig. 3.9). In four of the five samples, cells oriented around the wound and the periclinal cell divisions resembled wound meristem (Fig. 3.7). In two of the five samples, a small growth of callus tissue developed above the entrance to the oviposition canal (Fig. 3.9). Above the entrance to the oviposition canal (ca. 170 - 250 μm), in all samples, the vascular tissue became interconnected creating one wide vascular bundle (Fig. 3.10). Below the entrance to the oviposition canal, the vascular tissue and the pith cells resembled those of the control stem; this was observed in all samples (not shown). Of the samples collected 3 days after treatment in 2009, two of the four samples sectioned were not interpretable. The sample sectioned longitudinally (depicted in Fig. 3.6) and the one interpretable cross-sectioned sample did not show any callus tissue filling the entrance to the oviposition canal or within the oviposition canal (not shown). The discrepancy in the amount of callus tissue production between the two sample periods could be that the plant had more time to respond and initiate the wound response in the samples collected later.

Ovipositional Fluid Treatment

To assess the plant's response to ovipositional fluid during the oviposition sequence, a female was removed 20 seconds after she inserted her ovipositor into the oviposition canal. Replicate samples collected 3 days after treatment in 2009 and 5 days after treatment in 2010 did not exhibit any external growth (Fig. 3.11). The two replicate

samples collected 10 days after treatment in 2009 showed minor stem swelling (not shown). Internally, in six of the 10 replicate samples sectioned, ovipositional fluid was interpreted to be the red, densely stained substance (Fig. 3.12). In the sections where the fluid was visible, there was a variety of plant responses. Of the samples collected 3 and 5 days after treatment with the fluid present (three replicate samples from 2009, one sample 2010), two samples showed some minor cell orientation around the wound (Fig. 3.12). Increased vascular tissue also was observed in the two samples in that de novo xylem cells were located in the pith region (Fig. 3.12). In one replicate sample with ovipositional fluid present, the fluid was at the entrance to the oviposition canal (not shown). In this replicate and the two replicates without oviposition fluid (both collected 5 days after treatment), the pith cells were oriented around the wound and callus tissue filled the oviposition canal as in the rapid wound response of Fig. 3.7. The fourth sample with oviposition fluid was collected 3 days after treatment and was poorly processed, making detailed interpretation of the sample impossible. The two replicate samples collected 10 days after treatment both contained ovipositional fluid (Fig. 3.13). Long rows of periclinal cell divisions were oriented around the red-stained oviposition fluid and the entrance to the oviposition canal was filled with callus tissue. Above the entrance to the oviposition canal, position 2 in Fig. 3.6, the ovipositional fluid was not present and the centre of the pith tissue was filled with densely packed cells (Fig. 3.14). These pith cells contained a large central vacuole with multiple green-stained plastids or gall parenchyma (Chapter 2), and were not observed in Oviposition Fluid samples collected at day 3 or day 5 after treatment. Since no Control or Complete Oviposition Sequence samples were collected 10 days after treatment, no direct comparisons of the plant

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response to Ovipositional Fluid at 10 days after treatment could be made. The reader may refer to Chapter 2 of this thesis for normal gall development patterns that occurred during this time frame.

Egg Insertion Treatment

The Egg Insertion treatment involved piercing a young stem approximately 5 mm below the apical meristem with a size 00 insect pin, and the insertion of an egg into the wound. This treatment was only conducted in 2009 and samples were collected 3 days after treatment. No visible stem swelling was noticed for collected samples (not shown). The wound and egg both desiccated, which resulted in the death of the embryo in all four replicate samples (Fig. 3.15). In three of the four replicate samples, the egg fell out of the wound, most likely during histological processing. A few rows of periclinal cell divisions were noticed around the wound in all samples and there was no callus tissue in the pith (Fig. 3.15). The cortex and vascular tissue resembled that of the controls.

Wound Treatment

The Wound treatment involved piercing a young stem approximately 5 mm below the apical meristem with a size 00 insect pin. This treatment was only conducted in 2009 and samples were collected 3 days after treatment. No external swelling was noticed at time of sample collection (not shown). Internally, periclinal cell divisions were noticed around the wound in two of three replicate samples and no callus tissue was observed to fill in the wound (not shown). All three replicate samples from this treatment experienced histological processing problems, thus detailed interpretation of this treatment was not possible and a representative photo was not included.

DISCUSSION

Isolating the critical stage(s) involved in gall induction in the *R. pilosa – L. vulgaris* system was attempted in this experiment. A dark-staining fluid was isolated that is suspected to be ovipositional fluid and the fluid stimulated periclinal divisions, gall parenchyma differentiation, and de novo xylem formation. Female feeding stimulated the plant to produce callus tissue and wound meristem. Although a variety of plant responses were observed, none of the treatments that isolated the different stages of the oviposition sequence caused the plants to initiate normal gall development.

The treatment hypothesized to be the most critical to gall induction in this system was either the Ovipositional Fluid or the Egg Insertion treatments. Ovipositional fluid from the female was potentially isolated and initiated similar plant tissue responses to that of normal gall induction (i.e., de novo xylem production), but this fluid alone was not strong enough of a stimulus for normal gall induction. Major problems resulted from the experimental design of the Egg Insertion treatment, thus the role of the egg in gall induction was not elucidated from this treatment.

Minor external stem swelling was observed in the Oviposition Fluid treatment samples collected 10 days after treatment, indicating that there was a delayed plant response to the fluid. Internally, the vascular tissue did not respond as dramatically as in the Complete Oviposition Sequence, but there were unique vascular tissue changes such as the de novo xylem development within the pith. The ovipositional fluid could be "conditioning" the plant cells, making them more susceptible to gall induction. The red-stained fluid also was observed in serial sections, both above and below the wound, in all

three sampling time periods, indicating that the fluid can move between the plant cells. If the oviposition fluid was acting as a cell conditioner, being able to move between cells would increase the number of cells the fluid could interact with, and thus facilitate gall induction.

The pith parenchyma cells that contained high densities of green-stained plastids, or gall parenchyma (Chapter 2), as seen in the Oviposition Fluid treatment collected 10 days after treatment, also were present within normally developed galls (Fig. 3.14 and Chapter 2). These cells were very important in that both the larvae and adults fed on this tissue after pupation to aid in their survival during winter diapause (Toševski, personal communication). In other gall formers, nutritive tissue, cytoplasmically dense cells, are stimulated by feeding larvae (Bronner, 1992). Here, the specialized pith parenchyma cells may be initially stimulated by the ovipositional fluid and not in response to larval feeding.

While normal gall development patterns were not observed in stems exposed to the Oviposition Fluid treatment, it is difficult to make conclusions on the role of ovipositional fluid in gall induction in this system. Outside of this experiment, ovipositional fluid has been suggested by I. Toševeski (personal communication) as being a major contributor to gall induction in this system because of normal looking galls that, upon dissection, lacked developing insects (Fig. 3.16). The lack of insects inside the gall could be due to: 1) the female did not deposit an egg; 2) the egg was crushed by the rapidly dividing cells within the pith; or 3) the female deposited a sterile egg. The eggs are quite small, 0.3 mm (Toševski, et al., 2004), and a dead egg could be missed upon

dissection. Empty galls have been observed in other gall induction systems where larval feeding is not required for full gall development to occur (Günthart, 1949; Hovanitz, 1959). In empty *R. pilosa* galls, a variety of gall shapes result. In some cases, a normal unilocular gall forms (spherical, approximately 5 to 10 mm long) (Fig. 3.16), while in others, asymmetrical galls develop (not shown). Also, galls that appeared to be multilocular (contained multiple insect chambers and were greater than 10 mm in length), when dissected, contained one insect. In these large, unilocular galls, the females could be chewing a few oviposition canals and inserting ovipositional fluid into these canals but only depositing one egg into the stem. If ovipositional fluid contributes to gall induction, this can result in the development of a larger gall that would have increased area of nutrients to support the developing insect. The observation of empty galls, as well as long rows of periclinal divisions, de novo xylem production, and gall parenchyma differentiation in response to the Oviposition Treatment has lead to the possibility of gall induction via ovipositional fluids as being associated with gall induction in this system.

Within the Order Coleoptera, at least one other weevil has been able to induce gall formation prior to larval feeding. In early studies examining the biology of *Ceuthorrhynchus napi* Gyll. (Coleoptera: Curculionidae), on *Brassica napus* L. (Brassicaceae), ovipositional fluid was thought to be responsible for gall induction (Deubert, 1955; Günthart, 1949). However, no evidence of ovipositional fluids was detected during oviposition in later studies (Le Pape & Bronner, 1987). Le Pape and Bronner (1987) suggest that it was the exochorion and not the ovipositional fluid that had separated from the egg and had adhered to the plant cells lining the egg chamber. The exochorion from the *R. pilosa* egg appeared to adhere to the plant cells walls as well (Fig.

3.4). The red-staining ovipositional fluid, observed in the Ovipositional Fluid treatment (Fig. 3.12), was not visible in the Complete Oviposition treatment. Potentially, the ovipositional fluid could be undergoing chemical reactions with either the plant cells (causing the fluid to stain red) or the egg (causing the fluid to stain green), or to be absorbed by the egg.

In gall induction experiments involving insects that use ovipositional fluid to induce galls, experiments showed that when a female *Pontania proxima* (Serville) (Hymenoptera: Tenthredinidae) was removed prior to deposition of an egg during oviposition, small callus tissue developed (Hovanitz, 1959). When the female was removed immediately after inserting her ovipositor in the stem (i.e., just wounding), no growth was detected (Hovanitz, 1959). To further explore this observation that ovipositional fluid was responsible for gall induction, a solution of collateral glands (glands associated with oviposition) dissected from *P. proxima* was injected into early galls, producing growths (McCalla, et al., 1962).

When a *R. pilosa* female was removed after she chewed an oviposition canal, we could clearly see that female feeding, in itself, did not cause gall induction, but it appeared that the feeding induced the development of wound meristem and callus tissue. The orientation of cells around the wound and the periclinal cell divisions resembled a similar tissue, called wound periderm, observed due to mechanical wounding in potatoes and eucalyptus (Ginzberg, 2008). Wound periderm consisted of a few cell layers that were specifically designed to prevent desiccation and microbial attack by deposition of suberin and lignin within the cells (Ginzberg, 2008; Hawkins & Boudet, 1996). Periderm

can be identified by the rectangular shape of the cells, periclinal cell divisions, and UV illumination. This wound response has only been studied by mechanical injury and not by insect feeding even though the biochemical defense pathways induced by insect feeding have been examined (Allison & Schultz, 2004; Ginzberg, 2008; Meiners & Hilker, 2000). Insect feeding has initiated biochemical defense pathways that are different from those of mechanical wounding (Allison & Schultz, 2004).

In addition to the wound meristem observed in this system, the production of callus tissue to fill the oviposition canal also was observed. A similar wound response has been observed in Canada thistle stems in response to tunneling by *Urophora cardui* L. (Diptera: Tephritidae) larvae where periclinal cell divisions bordered the callus-filled tunnel (Lalonde & Shorthouse, 1984). Small growths of callus tissue also were found above the entrance to the oviposition canal and an increased area of vasculature was observed above the callus growth. The presence of callus tissue raises some questions: 1) why is the plant producing callus tissue above the wound entrance and not below or to the sides?; and 2) Why are the cells oriented toward the wound, pushing the cells outwards? The increased vascular tissue above the wound may provide increased structural support from increased lignification of the vascular tissue (Evert, 2006) or as a means to reduce the threat of microbial attack as there has been a positive correlation between lignin concentration and microbial resistance (Hawkins & Boudet, 1996). It seemed that within the abaxial portions of the oviposition canal (position 2 in Fig. 3.6), callus tissue production occurred in an attempt to block the wound while at the entrance of the canal (position 1 in Fig. 3.6), a mixture of callus and wound-like periderm was observed. Since the wound periderm was located near the entrance of the ovipositional canal, perhaps the

wound periderm was set-up as a defense mechanism or barrier against invading organisms.

The Wound treatment suffered from some histological processing problems. This treatment could have been used compare the plant's response to female feeding (i.e., did the plant respond differently to female feeding, or insect saliva, as opposed to mechanical wounding). General plant anatomy could be deduced from the sections, but detailed interpretation was not possible. It was decided not to conduct this treatment in 2010 in that there was a desire to simplify this experiment by concentrating on the four main treatments. When the experiment was initially conducted, it was not known that the female created an oviposition canal in the shape of her rostrum, thus the insertion of the pin for the wounding canal was not an equal representation of the Oviposition Canal treatment. The mechanical wounding produced a larger wound within the pith in comparison to the female feeding, thus any attempts at reproducing this treatment should try to imitate the female-feeding pattern more closely. The large wound created and the early sample collection time (3 days after treatment) could explain some of the limited wound response initiated by the plant.

Future directions

To better understand gall induction in the *R. pilosa - L. vulgaris* system, further studies are required. To isolate the ovipositional fluid, it would be ideal to remove the female after set time intervals after she has inserted her ovipositor into the oviposition canal as there was variation in the time necessary for the females to inject ovipositional fluid into the stem. The females used in 2009 injected the oviposition fluid into the

oviposition canal sooner than the females used in 2010. Injections of homogenized collateral glands (glands associated with the females reproductive tract) dissected from gravid females into young stems to study the plant's response to ovipositional fluid would be a logical step in studying gall induction in this system.

As an attempt to elucidate the chemical nature of the ovipositional fluid, the staining characteristics of the fluid could be a starting point. Here, the ovipositional fluid stained red due to the safranin stain used (Fig. 3.12 and 3.13). Safranin binds to acidic, negatively charged cellular components (Ruzin, 1999) and in plant cells, stains lignin and the nuclei red (Schneider, 1981). Using the staining characteristics of safranin as a preliminary starting point, the chemical nature of the ovipositional fluid from the female can start to be deduced.

Attempts at inserting freshly laid eggs into stems resulted in the dehydration of plant cells surrounding the egg and then the dehydration of the egg. The resulting histology showed an orientation of cells around the wound and a dehydrated egg (Fig. 3.15). To isolate the egg's role in gall induction, better attempts at preventing the dehydration of the stem are required. For example, wrapping the stem with Parafilm® where the wound was situated may prevent or delay the desiccation of the wound and egg. Killing freshly laid eggs may also be a method to isolate the role of the egg in gall induction. Producing a scanning electron micrograph (SEM) to determine the egg's structure would be useful to examine the structure of the exochorion (the outer layer of the egg). For instance, some gall-inducing insects use secretions from pores in the egg to

lyse cells as part of host conditioning prior to gall induction (Brooks & Shorthouse, 1998; Rey, 1992).

CONCLUSION

The purpose of this experiment was to isolate the early stages of gall induction in the *R. pilosa – L. vulgaris* system. Galls induced by *R. pilosa* were nearly fully developed by the time the larvae had hatched from their eggs and observations of "empty" galls have indicated that the egg or ovipositional fluid secreted by the female during oviposition may be associated with gall induction in this system. A dark-staining fluid was isolated prior to egg deposition that is suspected to be ovipositional fluid, and this resulted in the differentiation of gall parenchyma, long rows of periclinal divisions, and de novo xylem production. Attempts to isolate the plant's response to the egg were not successful. Female feeding to create the oviposition canal did not result in gall induction, and can be eliminated as a cause of gall induction. Callus tissue production and wound meristem formation was induced in response to female feeding. Future studies should be conducted to focus on the role of the ovipositional fluid and the egg in gall induction.

REFERENCES

Abrahamson, W. G., & Weis, A. E. (1987). Nutritional Ecology of Arthropod Gallmakers. In F. J. Slansky & J. G. Rodriquez (Eds.), *Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates* (pp. 235-258). New York: John Wiley & Sons, Inc.

Allison, S. D., & Schultz, J. C. (2004). Differential activity of peroxidase isozymes in response to wounding, gypsy moth, and plant hormones in northern red oak (*Quercus rubra* L.). *Journal of Chemical Ecology, 30*(7), 1363-1379.

Bronner, R. (1992). The Role of Nutritive Cells in the Nutrition of Cynipids and Cecidomyiids. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 118-140). New York: Oxford University Press.

Brooks, S. E., & Shorthouse, J. D. (1998). Developmental morphology of stem galls of *Diplolepis nodulosa* (Hymenoptera: Cynipidae) and those modified by the inquiline *Periclistus pirata* (Hymenoptera: Cynipidae) on *Rosa blanda* (Rosaceae). *Canadian Journal of Botany, 76*, 365-381.

Caldara, R., Desančić, M., Gassmann, A., & Toševski, I. (2008). On the identity of *Rhinusa hispida* (Brullé) and its current synonyms (Coleoptera: Curculionidae). *Zootaxa, 1805*, 61-68.

Deubert, K. H. (1955). Beiträge zu den beziehungen zwischen *Ceuthorrhynchus napi* Gyll. (Col. Curc.) und winterraps hinsichtlich der gallenbildung mit ovarienuntersuchungen an verschiedenen *Ceuthorrhynchus*-Arten. *Wissenschaftliche Zeitschrift Der Martin-Luther-Universität Halle-Wittenberg, 4*, 909-932.

Dreger-Jauffret, F., & Shorthouse, J. D. (1992). Diversity of Gall-Inducing Insects and their Galls. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 8-33). New York: Oxford University Press.

Evert, R. F. (2006). *Esau's Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function, and Development* (3rd ed.): Wiley-Interscience.

Ginzberg, I. (2008). Wound-Periderm Formation. In A. Schaller (Ed.), *Induced Plant Resistance to Herbivory* (pp. 130-146): Springer.

Günthart, E. (1949). Beiträge zur lebensweise und bekämpfung von *Ceuthorrhynchus quadridens* PANZ. und *Ceuthorrhynchus napi* GYLL. *Mitteilungen der Schweizerischen Entomologischen Gesellschaft, 32*, 489-566.

Hawkins, S., & Boudet, A. (1996). Wound-induced lignin and suberin deposition in a woody angiosperm (*Eucalyptus gunnii* Hook.): histochemistry of early changes in young plants. *Protoplasma, 191*, 96-104.

Hovanitz, W. (1959). Insects and plant galls. *Scientific American, 201*(5), 151-162.

Johansen, D. A. (1940). Reagents. *Plant Microtechnique* (pp. 15-26). New York: McGraw-Hill Book Company.

Kjellberg, F., Jousselin, E., Hossaert-McKey, M., & Rasplus, J.-Y. (2005). Biology, Ecology, and Evolution of Fig-pollinating Wasps (Chalcidoidea, Agaonidae). *Biology, Ecology, and Evolution of Gall-inducing Arthropods* (pp. 539-572).

Lalonde, R. G., & Shorthouse, J. D. (1984). Developmental morphology of the gall of *Urophora cardui* (Diptera, Tephritidae) in the stems of Canada thistle (*Cirsium arvense*). *Canadian Journal of Botany, 62*(1372-1384).

Le Pape, H., & Bronner, R. (1987). The Effects of *Ceuthorrhynchus napi* (Curculionidae, Coleoptera) on Stem Tissues of *Brassica napus* var *oleifera*. In V. Labeyrie, G. Fabres & D. Lachaise (Eds.), <ins>*Insects-Plants: Proceedings of the 6th International Symposium on Insect-Plant Relationships (PAU 1986)*</ins> (pp. 207-212). Dordrecht: Dr W. Junk Publishers.

Leatherdale, D. (1955). Plant hyperplasia induced with a cell-free insect extract. *Nature, 175*, 553-554.

Leggo, J. J., & Shorthouse, J. D. (2006). Development of stem galls induced by *Diplolepis triforma* (Hymenoptera: Cynipidae) on *Rosa acicularis* (Rosaceae). *The Canadian Entomologist, 138*, 661-680.

Lewis, I. F., & Walton, L. (1947). Initiation of the cone gall of witch hazel. *Science, 106*(2757), 419-420.

McCalla, D. R., Genthe, M. K., & Hovanitz, W. (1962). Chemical nature of an insect gall growth-factor. *Plant Physiology*, 98-103.

McClay, A. S., & De Clerck-Floate, R. A. (2002). *Linaria vulgaris* Miller, Yellow Toadflax (Scrophulariaceae). In P. G. Mason & J. T. Huber (Eds.), *Biological Control Programmes in Canada, 1981-2000* (pp. 375-382). New York: CABI Publishing.

Meiners, T., & Hilker, M. (2000). Induction of plant synomoes by oviposition of a phytophagous insect. *Journal of Chemical Ecology, 26*(1), 221-232.

Olmstead, R. G., DePamphilis, C. W., Wolfe, A. D., Young, N. D., Elisons, W. J., & Reeves, P. A. (2001). Disintegration of the Scrophulariaceae. *American Journal of Botany, 88*(2), 348-361.

Parchoma, G. (2002). Yellow Toadflax *Guide to Weeds in British Columbia* (pp. 192-195). Burnaby: Province of British Columbia.

Price, P. W. (1992). Evolution and Ecology of Gall-Inducing Sawflies. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 208-224). Oxford: Oxford University Press.

Rey, L. A. (1992). Developmental Morphology of Two Types of Hymenopterous Galls. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 87-101). New York: Oxford University Press.

Rohfritsch, O. (1992). Patterns in Gall Development. In J. D. Shorthouse & O. Rohfritsch (Eds.), *Biology of Insect-Induced Galls* (pp. 60-86). New York: Oxford University Press.

Ruzin, S. E. (1999). *Plant Microtechnique and Microscopy*. New York: Oxford University Press.

Saner, M. A., Clements, D. R., Hall, M. R., Doohan, D. J., & Crompton, C. W. (1995). The biology of Canadian weeds. 105. *Linaria vulgaris* Mill. *Canadian Journal of Plant Science, 75*, 525-537.

Schichnes, D., Nemson, J. A., & Ruzin, S. E. (2001). Microwave Paraffin Techniques for Botanical Tissues. In R. T. Giberson & R. S. J. Demaree (Eds.), *Microwave Techniques and Protocols* (pp. 181-189). Totowa: Humana Press.

Schneider, H. (1981). Plant Anatomy and General Botany. In G. Clark (Ed.), *Staining Procedures* (pp. 315-333). Baltimore: Williams & Wilkins.

Toševski, I., Gassmann, A., & Desančić, M. (2004). *Annual Report 2003: Biological Control of Dalmatian and Yellow Toadflaxes, Linaria dalmatica and L. vulgaris*. Delemont: CABI Publishing.

Weis, A. E., & Abrahamson, W. G. (1986). Evolution of host-plant manipulation by gall makers: ecological and genetic factors in the *Solidago-Eurosta* system. *American Naturalist, 127*, 681-695.

Wilson, L. M., Sing, S. E., Piper, G. L., Hansen, R. W., De Clerck-Floate, R. A., MacKinnon, D. K., et al. (2005). *Biology and Biological Control of Dalmatian and Yellow Toadflax*: USDA Forest Service, FHTET-05-13.

Figures 3.1 - 3.4: Control and Complete Oviposition Sequence Treatment. **3.1**.

Photograph of a control *Linaria vulgaris* stem that was suitable for oviposition. The

arrow indicates the approximate location of the cross-section shown in Figure 3.2. **3.2**.

Cross-section of the young *L. vulgaris* stem pictured in Figure 3.1 with distinct vascular

bundles separated by interfascicular parenchyma. Note the smaller parenchyma cells in

the cortex in comparison to the large randomly oriented parenchyma cells with large

vacuoles in the pith. **3.3**. A *L. vulgaris* stem 5 days after a *R. pilosa* female completed

oviposition showing normal early, gall development. **3.4**. Cross-section of the gall shown

in Figure 3.3 of the early growth development stage. The cambium underwent

hyperplasia and pushed the xylem cells towards the pith, and the cortex parenchyma were

hyperptrophied in comparison to the control replicate samples. The exochorion of the egg

was visible and appeared to be attached to the pith cells. Callus tissue replaced vascular

tissue above the entrance to the oviposition canal. Callus tissue (CT); cortex (C); egg (E);

exochorion (Ec); interfasicular parenchyma (IP); pith (P); vascular bundle (VB); xylem

(X).

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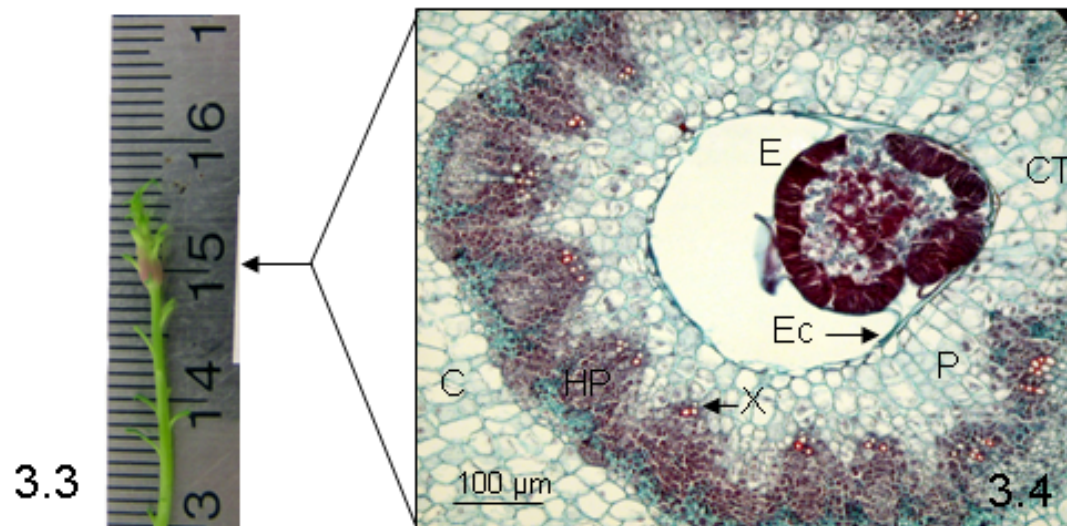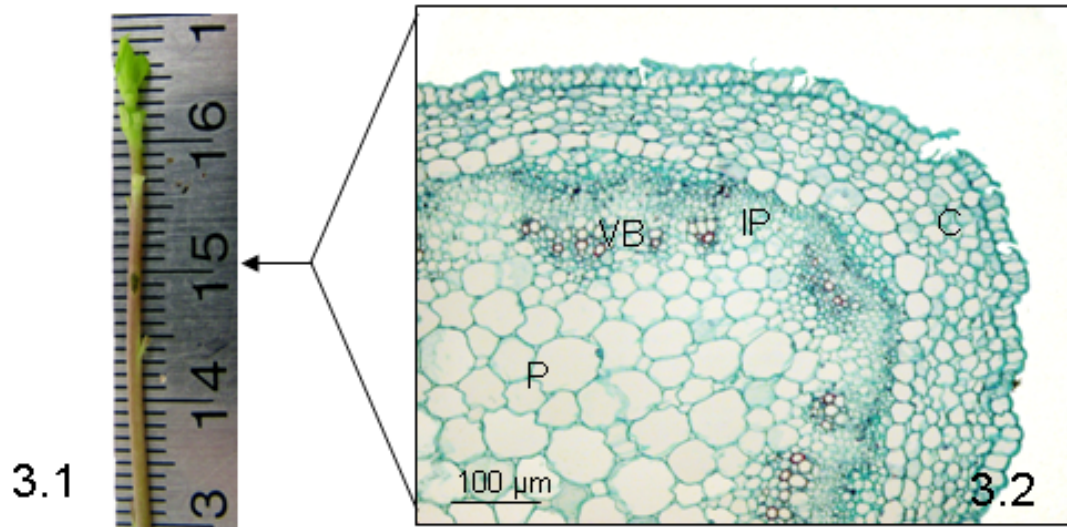
Figures 3.5 - 3.10: Oviposition Canal Treatment. **3.5.** *Linaria vulgaris* stem 5 days after a *Rhinusa pilosa* female chewed an oviposition canal prior to egg deposition. The arrow indicates the approximate location of the cross-sections shown in Figure 3.6. **3.6.** Schematic diagram of a longitudinal section of an oviposition canal based on samples collected 3 - 5 days after treatment. The canal closely resembles the shape of an *R. pilosa* rostrum. Numbers indicate positions of the cross-section made in relation to the oviposition canal. Location of Figure 3.7 and Figure 3.8 = Position 1 (1); Location of Figure 3.9 = Position 2 (2); Location of Figure 3.10 = Position 3 (3). These position numbers are referred to in other figures to aid in orientation. **3.7.** Cross-section of oviposition canal from the stem shown in Figure 3.5 (Position 1). The oviposition canal was filled with callus tissue and surrounded by wound meristem. **3.8.** Cross-section of the entrance to the oviposition canal with reduced wound response (Position 1). The base of the oviposition wound was not completely filled with callus tissue. **3.9.** Cross-section of a stem near the apex of the oviposition canal, above the section shown in Figure 3.8 (Position 2). Note the callus tissue and the absence of vascular tissue above the entrance to the oviposition canal. **3.10.** Cross-section of a stem 125 μm above the entrance to the oviposition canal shown in Figure 3.8 (Position 3). Note the interconnected vascular bundle with increased xylem. Apical meristem (AM); absence of vascular tissue (AV); cortex (C); callus tissue (CT); epidermis (E); interconnected vascular bundle (IVB); oviposition canal (OC); pith (P); vascular bundle (VB); wound meristem (WM).

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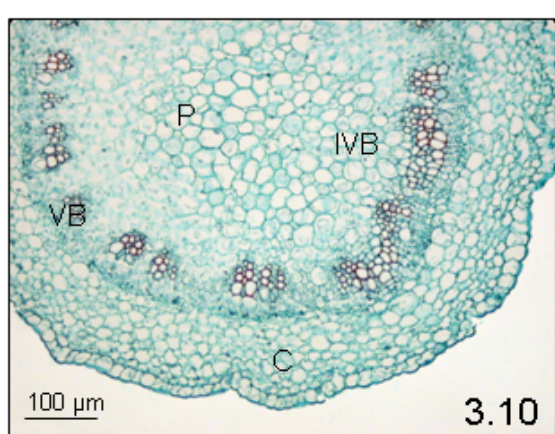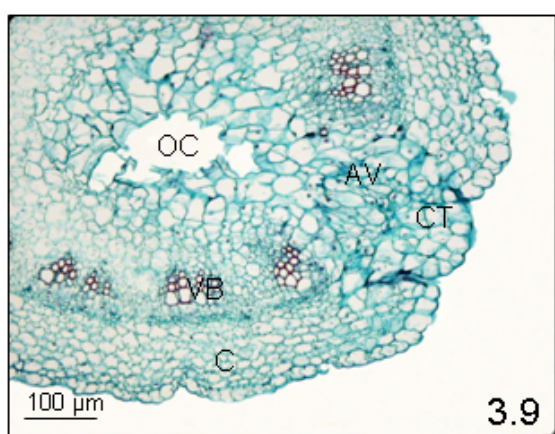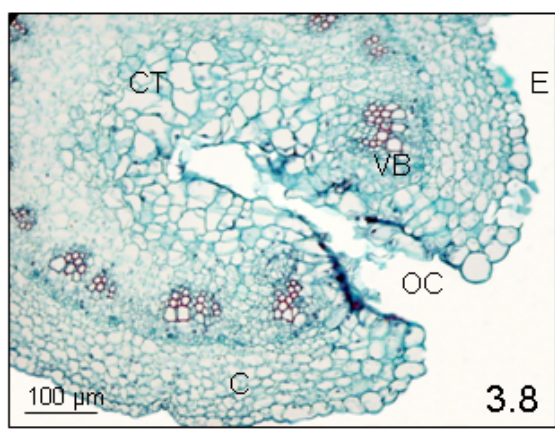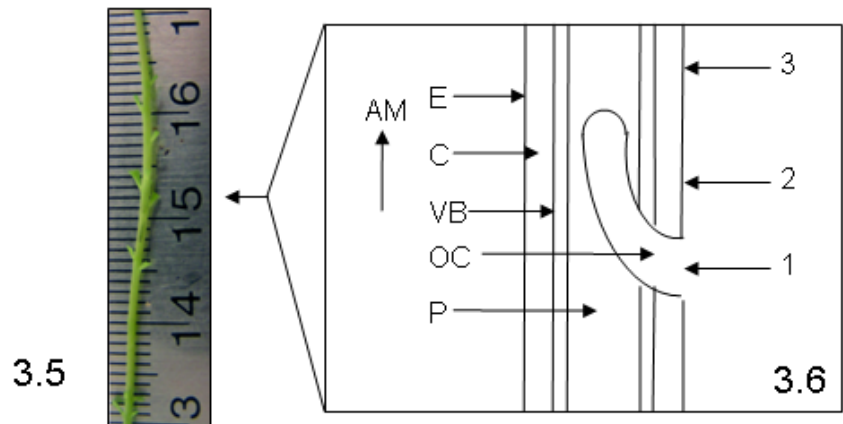
Figures 3.11 - 3.15: Ovipositional Fluid and Egg Insertion Treatment. **3.11**. Photograph of a *Linaria vulgaris* stem 5 days after it has been exposed to an ovipositional fluid treatment. No external stem swelling was observed. The arrow indicates the approximate location of the cross-section shown in Figure 3.12. **3.12**. Cross-section of an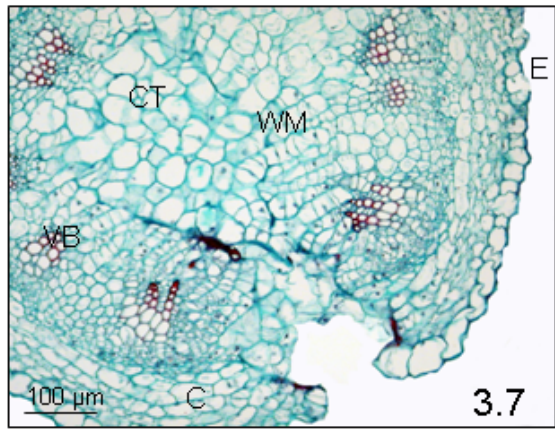 Ovipositional Fluid treatment from the stem shown in Figure 3.11. The ovipositional fluid was interpreted as the red stained, dense substance lining the entrance of the oviposition canal. Note the cellular orientation and the cross-section was approximately located at position 1 (see Figure 3.6). **3.13**. Cross-section of an Ovipositional Fluid treatment sample collected 10 days after treatment. The ovipositional fluid was still visible and long rows of periclinal cell divisions were oriented around the ovipositional fluid within the pith. Cross-section located at position 1 (see Figure 3.6). **3.14** Cross-section above section in Figure 3.13, located at position 2 (see Figure 3.6). The ovipositional fluid is no longer visible. The centre of the pith tissue was composed of densely packed cells with a large central vacuole and green-stained plastids, or gall parenchyma (Chapter 2). **3.15:** Cross-section of an Egg Insertion treatment collected 3 days after treatment. The egg has desiccated and the wound was surrounded by periclinal cell divisions, or wound meristem. Artificial wound (AW); cortex (C); de novo xylem (X); egg (Eg); gall parenchyma (GP); oviposition canal (OC); ovipositional fluid (OF); pith (P); periclinal cell divisions (PD); vascular tissue (VT).

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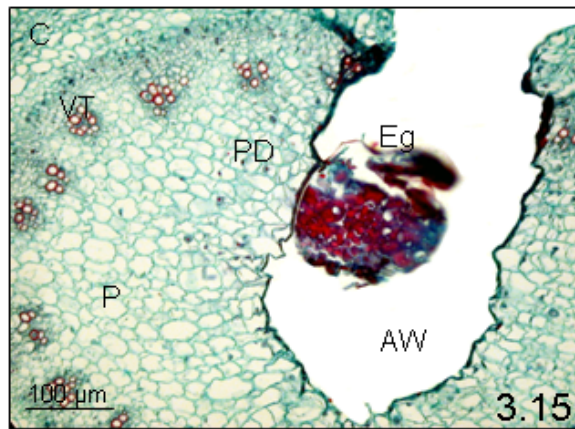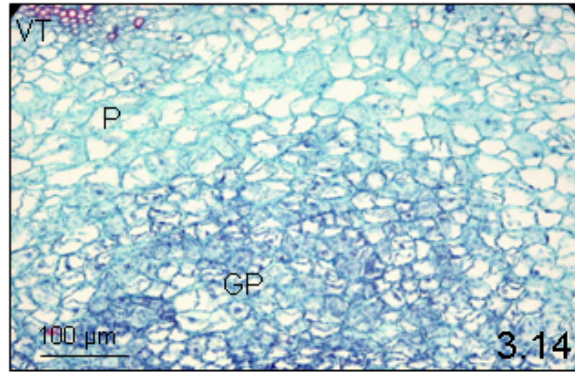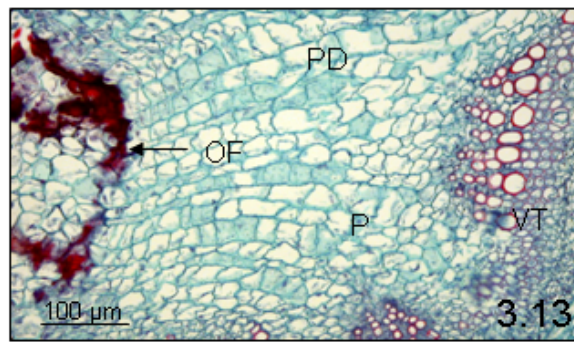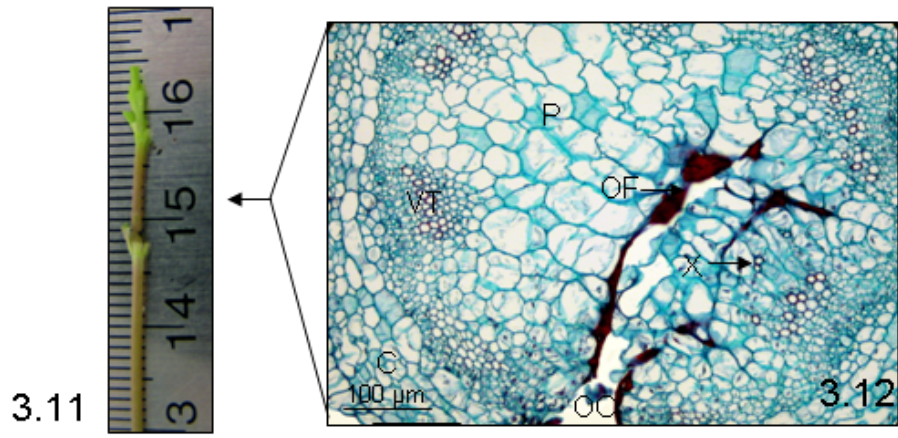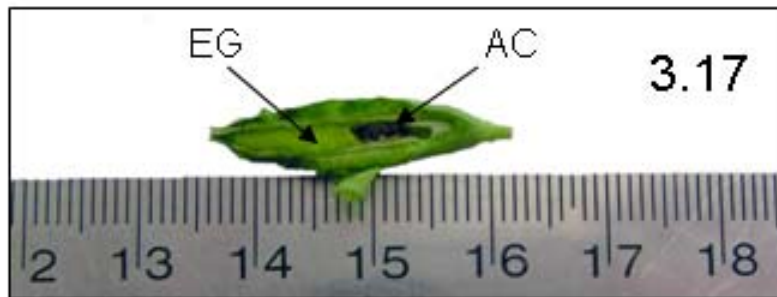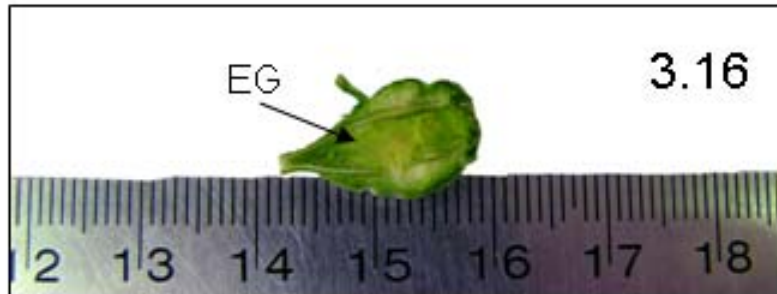
Figures 3.16 - 3.17: Empty galls induced by *Rhinusa pilosa* on *Linaria vulgaris*. **3.16**.

Photograph of a fully developed empty *R. pilosa* gall on *L. vulgaris*. Note lack of insects

within the pith region of the gall. **3.17**. Photograph of a fully developed *R. pilosa* gall on

L. vulgaris that contains a larval chamber and a region with no insects inside the gall pith

area. Adult chamber (AC); empty gall (EG).

CHAPTER 4: PRE-RELEASE IMPACT ASSESSMENT OF A CANDIDATE BIOCONTROL AGENT AGAINST MULTIPLE POPULATIONS OF *LINARIA VULGARIS*

INTRODUCTION

Biological control agent selection is an evolving process with multiple goals, including improved prediction of agent safety and efficacy. The majority of current testing is focused on reducing the potential risk to non-target plant species by determining the host range of candidate biocontrol agents (McFadyen, 1998). While this is mandatory in biocontrol agent screening, there is increasing pressure on biocontrol practitioners to reduce the possibility of "off-target effects" or indirect non-target effects by releasing fewer, more effective agents (Morin et al., 2009; Sheppard & Raghu, 2005). The increased probability of either negative direct or indirect non-target effects occurring can happen when moderately successful agents build up high densities, but are unable to control their target (McClay & Balciunas, 2005; Pearson & Callaway, 2005). Seed head-galling flies, *Urophora* spp., (Diptera: Tephritidae) have been successful at building up high densities on knapweed, *Centaurea* spp. (Asteraceae), but due to the longevity of the seed bank, this agent requires greater than 20 years of continual attack to control the weed (Story, Smith, Corn, & White, 2008). High deer mice (*Peromyscus maniculatus* Wagner) overwintering survival rates due to subsidized feeding on *Urophora*-containing seed heads, has been linked to outbreaks of hantavirus, a human pathogen (Pearson & Callaway, 2005). The availability of this subsidized food source should be a temporary problem as *Urophora*-containing seed heads are reduced due to knapweed control by

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Urophora spp. and other seed head-feeding insects (Story, et al., 2008). Although indirect effects such as this are hard to predict, pre-release efficacy testing is becoming a more important aspect during the screening of potential biocontrol agents to prevent the release of ineffective agents and thus, reduce the risks to non-targets.

Pre-release efficacy assessment's (PREA) main goal is to test the impact of high densities of the candidate agent on the target weed or the "best case scenario" (McClay & Balciunas, 2005). If the candidate agent is not successful in controlling the weed at high densities, this agent could then be considered to be an ineffective agent with a higher risk for off-target effects. PREAs using low densities of the candidate agent also are useful as it will tell us if low densities of the candidate agent can negatively affect the plant. An agent is usually released in low densities and it takes a few years before the agent densities built up to levels sufficient to adequately control the plant. There is always the chance that the agent, once released, for whatever reason, will not reach densities high enough to control the weed. Thus it would be advantageous to assess if low and high levels of the candidate agent are able to control the weed (Conrad & Dhileepan, 2007; Wu, Hacker, Ayres, & Strong, 1999). Ideally, PREAs of candidate biocontrol agents should be done prior to host range testing as a cost saving measure (McClay & Balciunas, 2005). Host range testing can be time consuming and expensive, thus conducting efficacy assessments prior to host range testing can "weed out" ineffective agents early on. Extrapolating results from PREAs to what may happen post-release in the introduced range is not perfect as there is the probability of not releasing an effective agent that performs poorly during testing or releasing an agent that does well in the testing, but not in the introduced range (McClay & Balciunas, 2005; Morin, et al., 2009).

In cases of multiple suspected weed introductions, it would be ideal for PREAs to also assess the ability of the candidate agent to attack and develop on different genetic populations in the invasive range (McFadyen, 2003). Post-release assessments have found that some agents are only successful in attacking certain biotypes of the host weed (Lym & Carlson, 2002; McFadyen, 2003). For example, the feeding preference and development of four species of leafy spurge leaf beetles, *Aphthona* spp. (Coleoptera: Chrysomelidae), were tested against multiple genotypes of leafy spurge, *Euphorbia esula* L. (Euphorbiales), from the invasive and native range. Different agent species show variation in host genotypic feeding preferences and developmental rates (Lym & Carlson, 2002). Therefore, it is important to have some preliminary data on the interaction of the candidate agent with multiple plant populations from the introduced range before release to avoid the release of ineffective agents.

Linaria vulgaris, yellow toadflax (L.) Mill (Plantaginaceae), is a perennial weed introduced to North America from Europe in the 1700s for use as an ornamental (Wilson et al., 2005) and medicinal plant (ILieva, Handjieva, & Popov, 1992). *Linaria vulgaris*, an obligate outcrosser, reproduces with genetically variable seeds (Ward, Fleischmann, Turner, & Sing, 2009) and vegetatively through a rhizomatous, clonal root system (Saner, Clements, Hall, Doohan, & Crompton, 1995). The inflorescence is composed of an indeterminate raceme on the main stem, which can develop lateral inflorescences. The weed is commonly found in disturbed sites such as road sides, but also can invade undisturbed grasslands (Saner, et al., 1995; Sutton, Stohlgren, & Beck, 2007). *Linaria vulgaris* is currently listed as a noxious weed in four Canadian provinces and eight US

states and the weed is distributed across the continental United States and in nine Canadian provinces and two of the three territories (USDA, 2010). *Linaria vulgaris* is difficult to control with herbicides due to its rhizomatous root system (Wilson, et al., 2005). Two adventitious seed feeders, *Brachypterolus pulicarius* L. (Coleoptera: Brachypteridae) and *Rhinusa antirrhini* (Paykull) (Coleoptera: Curculionidae) have been successful at reducing seed output, but have had little impact on *L. vulgaris* populations overall (McClay & De Clerck-Floate, 2002).

Rhinusa pilosa Gyllenhal (Coleoptera: Curculiondiae), a stem-galling weevil, is one of two insects currently being assessed as a potential biocontrol agent for *L. vulgaris*. *Rhinusa pilosa* originates from Europe (Sweden in the north to France in the west and Russia to the east) (Caldara, Desančić, Gassmann, & Toševski, 2008). Overwintered *R. pilosa* adults feed and mate in early spring and females oviposit within young *L. vulgaris* shoots near the apical meristem and induce gall formation. The multilocular gall develops in 8 to 10 days when reared at 22˚C (See chapter 2). Under artificial conditions, females, on average, oviposit for 26.3 ± 3.44 days, induce 27.7 ± 4.5 galls and produce 51.9 ± 9.7 live adults (n = 32) (author, unpublished data, 2009 rearing results). After three larval instars, the insect pupates within the gall. Adults continue to feed on the remaining gall tissues. The adult chews a small exit hole, or "window", prior to emergence from the gall. During the summer, adults remain in the leaf litter, while periodically feeding on *L. vulgaris* stems prior to winter diapause (Toševski, Gassmann, & Desančić, 2004).

The objectives of this Chapter were to: 1) determine if *R. pilosa* can gall and develop on different populations of *L. vulgaris* collected from western Canada and, 2)

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determine the impact of low densities of *R. pilosa* on *L. vulgaris*. Multiple introductions of *L. vulgaris* to North America are suspected (Ward, Reid, Harrington, Sutton, & Beck, 2008), hence, any insects used for biocontrol will be exposed to many populations, and may encounter multiple host genotypes upon release. High levels of genetic diversity were found within and among populations of *L. vulgaris* due to sexual reproduction via seeds, contrary to the common belief that *L. vulgaris* reproduces clonally with rhizomes (Ward, et al., 2008). High levels of genetic diversity indicate that conducting a multi-population PREA with *R. pilosa* and this weed would be beneficial. Four widely separated *L. vulgaris* populations from western Canada (two from Alberta and two from British Columbia) and one population from Serbia were used. Having preliminary plant population responses to galling by *R. pilosa* provides insight into the potential establishment in the field based on galling rates and insect development ability. This provides valuable data for biocontrol practitioners on the suitability of this agent for release and could aid in developing a release strategy for *R. pilosa*.

MATERIALS AND METHODS

Study organisms

Geographically distinct locations were chosen as plant collection sites to increase the probability of collecting genetically diverse populations. Plants for the population impact study were collected in late June of 2009 from Tie Lake, British Columbia (BC), Canada (49° 22'N 115°19'W), and Mountain View, Alberta (AB), Canada (49° 3'N 113°27'W) and in early September from Prince George, BC (53° 56'N 122°45'W), and Edmonton, AB (53°29'N, 113°32'W). Plants were collected as roots and shipped to the

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Insect Microbial Containment Facility at the Lethbridge Research Centre, Agriculture and Agri-food Canada in Lethbridge, Alberta, Canada. On arrival, plants were dipped into a 10 % bleach solution and planted in 15 cm clay pots using 30/30/30 soil mix (1 part sand, 1 part top soil and 1 part Cornell mix (110 L vermiculite, 60 L peat moss, 9 L sand, 1.5 kg osmocote fertilizer, 380 g superphosphate fertilizer, 1 kg calcium flour, 10 g chelated iron, and 20 g fritted trace elements (Frit Industries, Ozark, Alabama, USA)). Each population had from 1 - 2 months to establish in a greenhouse set at 22°C with ambient lighting before undergoing vernalization. Vernalization consisted of at least 4 weeks at 15°C followed by at least 3 weeks at 10°C with a 12-h photoperiod. Plants were trimmed to 3 cm above ground and wrapped in a double layer of black plastic bags. These were placed in a cold room set at 2°C for 3 months starting in December. Plants were ramped up in temperature from 2°C to 10°C with a 12-h photoperiod on March 11, 2010 for 1 week followed by a subsequent week at 15°C under the same light regime. Plants were transferred to a rearing room with temperature controls set for 22 /18°C, with a 12-h photoperiod prior to testing using artificial lighting (florescent plant grow lights (Standard F32T8, Saint-Laurent, Quebec, Canada)).

Plants from Serbia were grown from seed as a control group. Seeds were collected in the fall of 2008 from Zemun, Serbia. Seeds were cold stratified on February 11, 2009 using moist paper towel in glass Petri dishes for eight weeks at 5°C in a standard refrigerator. Petri dishes were brought to 22°C in a greenhouse with ambient lighting. Seedlings were transferred to a root trainer after 1 week and transferred to 15 cm clay pots with 30/30/30 soil mix (see above for recipe) 2 weeks later. Plants were allowed to establish in the greenhouse for 4 months, and then were exposed to the same

vernalization and temperature ramp-up conditions as the Canadian field collected populations (see above).

The insects used for the experiment originated from a laboratory colony recently established at the Lethbridge Research Centre from insects shipped from Serbia in 2009. On March 4, 2010, about 400 artificially overwintered *R. pilosa* adults were taken out of winter diapause conditions and transferred to 10°C for 1 week followed by 1 week at 15°C. Insects were subsequently transferred to the same conditions as the plants. Insects were mated, tested for oviposition, and gall induction. Only females that oviposited and induced galls successfully were selected for the experiment.

Experimental design

Each replicate consisted of one paired treatment and control plant with 10 replicates per population for Tie Lake, Edmonton, and Prince George, while Mountain View had 9 replicates. An additional population (Belgrade, Serbia) was added as a control group (6 replicates). Within populations, plants were paired based on vigor (i.e., growth rates and number of stems), and plants were randomly assigned as either treatment or control. One replicate from each population was set up per day for a total of 10 days. Prior to the start of the experiment, a subset of 10 stems per plant (test stems) was randomly chosen. All stems that were not included in the subsample from the plant were considered non-test stems. The stem length (i.e., soil surface to apical meristem) was measured from each test stem to confirm that control and treatment plants were the same size. A fresh female was used for each treatment plant and females were caged on

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the test plants for 36 hours. Plants were enclosed in a cylindrical mesh cage (20 cm in diameter by 70 cm tall).

Test plants were examined 3 days after removal of the female by visually checking stems for the presence of galls and counting the number of oviposition scars per stem. Galls were checked for windows every 3 days, 9 weeks after the test was set-up. If a window was observed, the test and control plant pair was re-caged with mesh sleeves until the harvest date to prevent *R. pilosa* adults that may have emerged from their galls from escaping.

Each replicate (treatment and control pair) was harvested 72 to 75 days (ca. 10.5 weeks) after the female weevil was removed. Stems were clipped at ground level, and the following was measured from all galled stems from the treatment plants, and the 10 test stems measured at the beginning of the experiment from the control and treatment plants: stem length, number of flowers, and number of lateral shoots. Lateral shoots are shoots branching off the main stem. If an ungalled test stem was missing or dead, an ungalled non-test stem was randomly selected among the remaining living stems within the same plant and measured as a replacement. All galls were clipped from the stems and gall length and widest part of gall were measured. Galls were dissected and life stage of the insects was recorded. Live adults were sexed and weighed on a microbalance (fresh weight). The number of flowers and lateral shoots on the non-test stems was counted and the non-test stems were tallied. "New stems" are the number of above-ground rhizomatous stems that were produced after the initial set-up.

For the above-ground biomass samples, the galls were separated from the stems and weighed independently from the stem samples. Roots were removed from the soil and gently washed as the below-ground biomass sample. Biomass samples were dried in a drying oven set at $60 \pm 5°C$ for 2 weeks prior to being weighed on a top-loading balance.

Statistical analysis

To avoid pseudo-replication, data were analyzed using response variables that were averaged by plant. Proportion data were arcsine square root transformed, and in cases of positive skew, data were log or square root transformed to meet assumptions of normality.

To determine if there was a difference in galling by *R. pilosa* among toadflax populations, a MANOVA was conducted using the gall response variables (proportion of galled stems, gall volume, gall mass, proportion of live adults, average number of adults per gall, and adult mass). One-way ANOVAs for the effect of population followed by Tukey's HSD were performed on populations that were significantly different. Gall volume was determined by using the volume of a prolate sphere

$$4/3\pi(d/2)^2 l \tag{1}$$

where π is 3.14, d is the widest gall diameter, and l is gall length.

The impact of low densities of *R. pilosa* was evaluated by performing a MANOVA, using Pillai's test (Quinn & Keough, 2002), assessing the effect of population and treatment on the final plant response variables (final stem length,

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proportion of flowering stems, proportion of stems with lateral shoots, proportion of dead stems, proportion of new stems, above-ground biomass, and below-ground biomass). Blocking (initial pairing of control and treatment plants) was not included in the final analysis, as this effect was not statistically significant. Since the effect of population and treatment were significant from the MANOVA, two-way ANOVAs were performed using each plant response variable separately on the effect of population and treatment. When populations were found to be significantly different, one-way ANOVAs for the effect of treatment were performed separately on each population.

Since *L. vulgaris* plants are composed of rhizomatous stems, the within plant response to galling also was investigated. This was accomplished by performing a second two-way ANOVA, on the effect of population and galling, where galled plants were subdivided into galled and ungalled stems. Blocking was not included, as this effect was not statistically significant. Since this is an incomplete nested design, i.e., control plants cannot be subdivided into galled and ungalled stems, two separate two-way ANOVAs were performed on the effect of population and galling. Data were analyzed using R version 2.9.2 (R Development Core Team, 2009). Results are stated in untransformed means ± standard error of the mean unless otherwise stated.

RESULTS

Can R. pilosa gall and develop on all tested populations of L. vulgaris?

All populations tested successfully developed galls and produced live F1 adults. Mean gall densities per population ranged from Edmonton with the lowest number of galls per plant at 3.7 ± 0.5 (n = 10) to Tie Lake with 5.6 ± 1.0 (n = 10). The average

number of galls per plant from all populations pooled was 4.5 ± 0.4 (n = 45) galls per plant. The proportion of galled stems averaged 0.21 ± 0.02 (n = 45) and was not significantly different among populations (Table 4.1). Average gall volume per plant was significantly different among populations (Table 4.1). The smaller galls (Serbia, Tie Lake, and Mountain View) were 65 % smaller than the larger galls (Edmonton, Prince George, and Mountain View) (Fig. 4.1). The dry gall mass per plant was not significantly different among populations and averaged 0.29 ± 0.04 g (n = 45) (Table 4.1). The proportion of live adults that were harvested at the termination of the experiment was not significantly different among populations (0.42 ± 0.01, n = 45) nor the average number of live adults per gall (0.94 ± 0.2, n = 45) (Table 4.1). The average adult fresh mass per plant was significantly different among populations (Table 4.1) with Serbia having the smallest adults (2.6 ± 0.8 µg, n = 6) and Prince George with the largest (4.38 ± 0.2 µg, n = 10) (Fig. 4.2).

What is the impact of low densities of R. pilosa on L. vulgaris?

At the set-up of the experiment, stem length was different among populations, but within populations, the control and treatment plants were not significantly different (Table 4.2).

At the termination of the experiment, or harvest, population level differences in the plant response variables measured continued to be significant (MANOVA, $F_{4,80} = 8.59$, $P < 0.000$; Table 4.2). Differences between control and galled plants for the plant response variables measured were now significant (MANOVA, $F_{1,80} = 2.05$, $P = 0.051$; Table 4.2). One of the five populations, Tie Lake, responded to galling (Table 4.3).

Within this population, galled plants produced 39 % fewer flowering stems and produced 58 % fewer new stems between when the experiment was set-up and harvest. The dry above-ground biomass of the galled plants from Tie Lake was 16 % lighter, and below-ground biomass was 41 % lighter than control plants (Table 4.3). All other populations were not significantly different between treatment and control plants for any of the plant response variables measured (Table 4.3).

To determine the intra-plant effect of galling, comparisons between galled and ungalled stems within the same treatment plant was conducted using multiple plant response variables (final stem length, proportion of flowering stems, lateral shoots, and proportion of dead stems). Within treatment plants, population level differences were significant for the plant response variables measured (MANOVA, $F_{4,80} = 8.56$, $P < 0.000$; Table 4.4). There also was a significant difference in the plant response variables measured between galled and ungalled stems within treatment plants (MANOVA, $F_{1,80} = 9.85$, $P < 0.000$; Table 4.4). Galled stems were 21 % smaller than ungalled stems for Mountain View (Table 4.5). The stem length was not significantly different between the galled and ungalled stems within the same plant in the four other populations (Table 4.5). Edmonton had proportionally more galled stems that flowered than ungalled stems within the same plants (Table 4.5). In all other populations, there was no significant difference between the proportion of ungalled and galled flowering stems (Table 4.5). Two of the five populations (Edmonton and Serbia) had proportionally more galled stems with lateral shoots than ungalled stems, while the remaining three populations were not significantly different (Table 4.5). There was a significantly higher proportion of dead ungalled stems than dead galled stems in four of the five populations (Table 4.5).

DISCUSSION

In this study, we found that *R. pilosa* was able to gall and develop successfully on all populations of *L. vulgaris* tested even though the plant response variables measured were different among the populations at the set-up of the experiment. At harvest, the proportion of galled stems, dry gall mass, and proportion of live adults collected were not significantly different among populations. Being able to induce an equal proportion of galls per population, which produce an equal proportion of live adults, suggests that *R. pilosa* can successfully attack multiple plant populations in the field if approved for release.

While there were not considerable differences in the ability of *R. pilosa* to gall and develop on all populations, other biocontrol agents have been shown to be less effective in attacking and developing equally across populations from the invasive range. Post-release studies of the tephritid fly, *Urophora jaculata* Rondani (Diptera: Tephritidae), showed that this agent could only successfully develop on genotypes of yellow starthistle, *Centaurea solstitialis* L. (Asteraceae) from the native range that it has been associated with, which may help explain why this agent failed to establish in California (Clement, 1994). Leafy spurge, *Euphorbia esula* L. (Euphorbiales), in North America is composed of multiple genotypes which resulted in variable establishment rates of different species of spurge leaf beetles, *Aphthona* spp. (Curculionidae: Chrysomelidae) (Lym & Carlson, 2002). Different *Aphthona* species showed variation in host genotypic feeding preferences and developmental rates during post-release testing,

which may help explain the variable establishment rates observed in the field (Lym & Carlson, 2002).

When comparing the effect of galling between treatment and control plants, the *L. vulgaris* populations tested responded differently to low densities of *R. pilosa*, with one invasive population, Tie Lake, BC, negatively affected by galling. The other three invasive populations tested were not significantly different between the treatment and control plants for any of the plant response variables.

When comparing the intra-treatment plant effect to galling by *R. pilosa*, more varied responses were observed. One population, Mountain View, was negatively affected by galling as the galled stems were significantly smaller than the ungalled stems within the same plant. One population, Edmonton, had significantly more galled stems with flowers than ungalled stems. However, there was no significant difference in the proportion of stems with lateral shoots between treatment and control plants from Edmonton. The lateral shoots are related to flowering in that lateral shoots can develop small inflorescence but usually do so after the main stem begins to flower. An increased proportion of shoots with lateral shoots was observed in galled plants from Edmonton and Serbia. It could be that the taller shoots are more likely to produce flowers and lateral shoots, and since the galled stems from Edmonton and Serbia tended to be taller (although not significantly taller) and significantly taller in Mountain View, than the ungalled stems, this higher proportion of lateral shoots may be related to stem length rather than galling. There was a positive relationship between final stem height and proportion of flowering stems (linear regression, $F_{1,88} = 20.2$, $P < 0.000$, $r^2 = 0.18$).

Increased growth has been induced by some biocontrol agents. Galling by *Tetramesa romana* Walker (Hymenoptera: Eurytomidae) caused higher numbers of lateral branching on giant reed, *Arundo donax* L. (Poaceae) (Goolsby, Spencer, & Whitehand, 2009). Herbivory by the cinnabar moth, *Tyria jacobaeae* (L.) (Lepidoptera: Arctiidae), caused the re-growth of new capitulas in tansy ragwort, *Senecio jacobaea* L. (Compositae) (Islam & Crawley, 1983). In some cases, increased growth is advantageous to the gall maker because it can increase the number of oviposition sites for future females (Goolsby, et al., 2009), but it can have direct negative impacts to the plant in terms of delayed seed output (Islam & Crawley, 1983).

Pre-release studies comparing the efficacy of a candidate biocontrol agent on the native host with host plants from the invasive range are rare (McFadyen, 2003). Determining the efficacy on the native host can set base-line data in which to compare the efficacy of the agent against populations or genotypes of the host plant in the invasive range. Here, we found that galls that developed on plants from the native range were smaller and produced smaller adults than galls on the invasive range plants. Serbian galled plants also produced more lateral shoots in response to galling, while three of the four populations from the invasive range did not. This suggests that plants from the native range may have developed ways to compensate for galling. Since the agent may have been associated with the Serbian population for long periods of time, it is possible that the Serbian plants have developed defenses against *R. pilosa* that the invasive populations have lost or never had. This finding supports one part of the evolution of increased competitive ability (EICA) hypothesis where specialist herbivores will perform

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better on plants from the introduced range rather than on plants from the native range (Blossey & Nötzold, 1995).

Few published studies have conducted pre-release efficacy assessments (PREA) on multiple plant populations from the invasive range. The petiole-galling weevil, *Coelocephalapion camarae* Kissinger (Coleoptera: Curculionidae), was tested against two varieties of *Lantana camara* L. (Verbenaceae) from South Africa during a quarantine PREA study (Baars, Hill, Heystek, Neser, & Urban, 2007). The weevil was able to gall both populations successfully. Unfortunately, there was no mention of insect survivorship on the two host varieties, even though an impact assessment was terminated 30 days after set-up, which is when the insect pupates (Baars, et al., 2007). Generally, multi-population testing occurs after the agent has been released in response to inconsistent agent establishment (Lym & Carlson, 2002) or agents that fail to establish in the field (Clement, 1994).

Since *R. pilosa* was able to gall and develop on all populations tested, any of these sites could be suitable release sites. These sites are accessible, have potentially long lived plant populations, and have historic site records. Low densities of *R. pilosa* had a negative impact on plants from Tie Lake, BC; thus this site may be a good candidate site for an initial release. The results from the experiment give decision makers more confidence that this agent is plastic enough to be able to attack multiple plant populations. Re-testing the invasive populations with high densities of the agent would be the logical next step in assessing the impact of galling. Chapter 5 of this thesis tests this hypothesis on one population, Mountain View.

The four western Canadian populations tested were all grown in the same conditions at the same time and they exhibited different characteristics indicating that these populations may be different plant genotypes. Unfortunately, limited *L. vulgaris* population genotypic studies have been conducted in Europe and North America (Ward, et al., 2008). Ward (2008) has found high levels of diversity among *L. vulgaris* populations in a relatively small study area of Intermountain West, USA. Future studies to identify if the populations tested in this study are different genotypes, their relatedness, and potentially their source population in the native range should be investigated. Samples were sent to J. Gaskin (USDA-ARS in Sidney, Montana) for molecular analysis and results have not been fully explored. If the populations tested are genetically distinct, this may help explain the differences in plant response variables measured. Also, the ability of *R. pilosa* to gall and develop on genetically distinct *L. vulgaris* populations would give greater confidence that this agent would be a suitable agent for release since it may be able to attack and develop on a wide range of *L. vulgaris* genotypes.

Gall volume was significantly different between populations yet the dry gall mass was not. Serbian galls had the smallest volume and these galls were generally quite small, and there were usually multiple galls per stem. In contrast, the Edmonton galls had the largest gall volume and were multilocular. The Serbian galls also were woodier than the Edmonton galls. The Edmonton galls had air spaces within the gall tissues and large insect chambers within the pith of the gall. These observations may explain the discrepancy between the gall volume and dry gall mass differences.

CONCLUSION

Few studies test the insect development ability and impact of a candidate biocontrol agent on multiple plant populations from the invasive range prior to release. In this study, we compared low densities of a candidate biocontrol agent, *R. pilosa*, on four geographically distinct populations of *L. vulgaris* from western Canada and one population from the native range, Serbia. We found that *R. pilosa* was able to gall and develop successfully on all populations tested even though the plant response variables measured were different at the set-up of the experiment. The populations tested responded differently to low densities of *R. pilosa*, with one population negatively affected by galling. The other four populations tested were not significantly different between the treatment and control plants for any of the plant response variables tested. Therefore, based on the ability of *R. pilosa* to gall and develop on multiple invasive populations of *L. vulgaris*, *R. pilosa* would make a suitable candidate to control *L. vulgaris* in Canada.

REFERENCES

Baars, J. R., Hill, M. P., Heystek, F., Neser, S., & Urban, A. J. (2007). Biology, oviposition preference and impact in quarantine of the petiole-galling weevil, *Coelocephalapion camarae* Kissinger, a promising candidate agent for biological control of *Lantana camara*. *Biological Control, 40*, 187-195.

Blossey, B., & Nötzold, R. (1995). Evolution of increased competitive ability in invasive nonindigenous plants: a hypothesis. *Journal of Ecology, 83*, 887-889.

Caldara, R., Desančić, M., Gassmann, A., & Toševski, I. (2008). On the identity of *Rhinusa hispida* (Brullé) and its current synonyms (Coleoptera: Curculionidae). *Zootaxa, 1805*, 61-68.

Clement, S. L. (1994). Resistance among populations of yellow starthistle to thistle-head insects: results from garden plots in Italy. *Biological Control, 4*, 149-156.

Conrad, K. A., & Dhileepan, K. (2007). Pre-release evaluation of the efficacy of the leaf-sucking bug *Carvalhotingis visenda* (Heteroptera: Tingidae) as a biological control agent for cat's claw creeper *Macfadyena unguis-cati* (Bignoniaceae). *Biocontrol Science and Technology, 17*(3), 303-311.

Goolsby, J. A., Spencer, D., & Whitehand, L. (2009). Pre-release assessment of impact on *Arundo donax* by the candidate biological control agents *Tetramesa romana* (Hymenoptera: Eurytomidae) and *Rhizaspidiotus donacis* (Hemiptera: Diaspididae) under quarantine conditions. *Southwestern Entomologist, 34*(4), 359-376.

ILieva, E. I., Handjieva, N. V., & Popov, S. S. (1992). Iridoid glucosides from *Linaria vulgaris*. *Phytochemistry, 31*, 1040-1041.

Islam, Z., & Crawley, M. J. (1983). Compensation and regrowth in ragwort (*Senecio jacobaea*) attacked by cinnabar moth (*Tyria jacobaeae*). *Journal of Ecology, 71*(3), 829-843.

Lym, R. G., & Carlson, R. B. (2002). Effect of leafy spurge (*Euphorbia esula*) genotype on feeding damage and reproduction of *Aphthona* spp.: Implications for biological weed control. *Biological Control, 23*, 127-133.

McClay, A. S., & Balciunas, J. K. (2005). The role of pre-release efficacy assessment in selecting classical biological control agents for weeds - applying the Anna Karenina principle. *Biological Control, 35*, 197-207.

McClay, A. S., & De Clerck-Floate, R. A. (2002). *Linaria vulgaris* Miller, Yellow Toadflax (Scrophulariaceae). In P. G. Mason & J. T. Huber (Eds.), *Biological*

Control Programmes in Canada, 1981-2000 (pp. 375-382). New York: CABI Publishing.

McFadyen, R. E. (1998). Biological control of weeds. *Annual Review in Entomology, 43*, 369-393.

McFadyen, R. E. (2003). Does Ecology Help in the Selection of Biocontrol Agents? In H. Spafford Jacob & D. T. Briese (Eds.), *Improving the Selection, Testing and Evaluation of Weed Biological Control Agents* (pp. 5-9). Adelaide: Cooperative Research Centre for Australian Weed Management.

Morin, L., Reid, A. M., Sims-Chilton, N. M., Buckley, Y. M., Dhileepan, K., Hastwell, G. T., et al. (2009). Review of approaches to evaluate the effectiveness of weed biological control agents. *Biological Control, 51*, 1-15.

Pearson, D. E., & Callaway, R. M. (2005). Indirect nontarget effects of host-specific biological control agents: Implications for biological control. *Biological Control, 35*, 288-298.

Quinn, G., & Keough, M. (2002). *Experimental Design and Data Analysis for Biologists*. Cambridge: Cambridge University Press.

R Development Core Team (2009). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Saner, M. A., Clements, D. R., Hall, M. R., Doohan, D. J., & Crompton, C. W. (1995). The biology of Canadian weeds. 105. *Linaria vulgaris* Mill. *Canadian Journal of Plant Science, 75*, 525-537.

Sheppard, A. W., & Raghu, S. (2005). Working at the interface of art and science: How best to select an agent for classical biological control? *Biological Control, 34*(3), 233-235.

Story, J. M., Smith, L., Corn, J. G., & White, L. J. (2008). Influence of seed head-attacking biological control agents on spotted knapweed reproductive potential in western Montana over a 30-year period. *Environmental Entomology, 37*(2), 510-519.

Sutton, J. R., Stohlgren, T. J., & Beck, K. G. (2007). Predicting yellow toadflax infestations in the Flat Tops Wilderness of Colorado. *Biological Invasions, 9*, 783-793.

Toševski, I., Gassmann, A., & Desančić, M. (2004). *Annual Report 2003: Biological Control of Dalmatian and Yellow Toadflaxes, Linaria dalmatica and L. vulgaris*. Delemont: CABI Publishing.

USDA, N. R. C. S. (2010). The PLANTS Database. Retrieved 5 October, 2010, from http://plants.usda.gov/java/profile?symbol=LIVU2

Ward, S. M., Fleischmann, C. E., Turner, M. F., & Sing, S. E. (2009). Hybridization between invasive populations of Dalmatian toadflax (*Linaria dalmatica*) and yellow toadflax (*Linaria vulgaris*). *Invasive Plant Science and Management, 2*, 369-378.

Ward, S. M., Reid, S. D., Harrington, J., Sutton, J., & Beck, K. G. (2008). Genetic variation in invasive populations of yellow toadflax (*Linaria vulgaris*) in the western United States. *Weed Science, 56*, 394-399.

Wilson, L. M., Sing, S. E., Piper, G. L., Hansen, R. W., De Clerck-Floate, R. A., MacKinnon, D. K., et al. (2005). *Biology and Biological Control of Dalmatian and Yellow Toadflax*: USDA Forest Service, FHTET-05-13.

Wu, M.-Y., Hacker, S., Ayres, D., & Strong, D. R. (1999). Potential of *Prokelisia* spp. as biological control agents of English cordgrass, *Spartina anglica*. *Biological Control, 16*, 267-273.

Table 4.1: Results from the 1-way ANOVAs on the effect of population against multiple

gall and insect parameters. Degrees of freedom (DF); *F*-value (F).

Gall Response Variable	DF	F	*P*-value
Proportion of galled stems [a]			
Population	4	1.82	0.14
Residual	40		
Gall volume [b]			
Population	4	5.42	0.001
Residual	40		
Gall mass [b]			
Population	4	1.90	0.13
Residual	40		
Live adults per gall [c]			
Population	4	1.07	0.39
Residual	40		
Proportion of live adults [a]			
Population	4	1.01	0.42
Residual	40		
Live adult mass			
Population	4	2.76	0.051
Residual	24		

[a] data arcsine square root transformed
[b] data square root transformed
[c] data $Log_{10} + 0.5$ transformed

Fig. 4.1
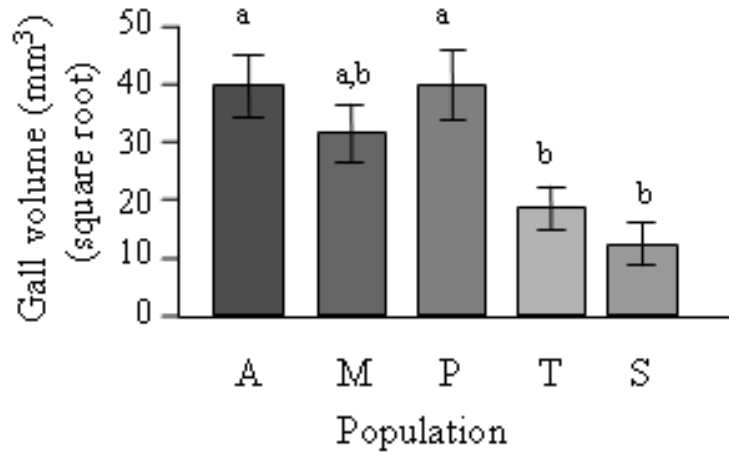
Fig. 4.2
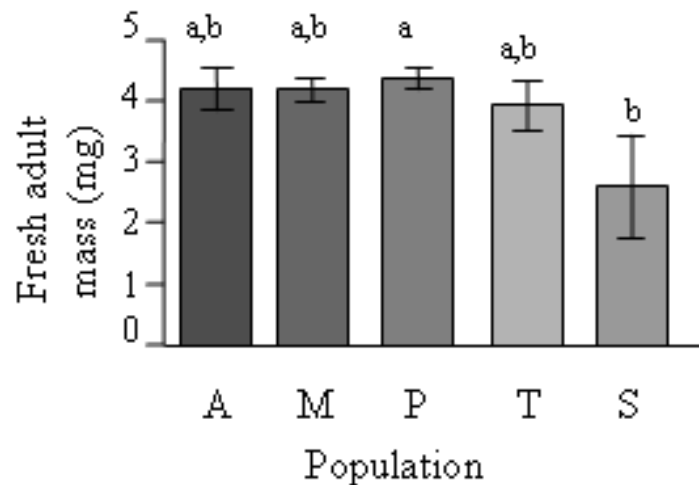
Figures 4.1 – 4.2: Plant response to gall induction by *Rhinusa pilosa* using the response variables gall volume and adult mass on the effect of *Linaria vulgaris* plant population. **4.1.** Mean gall volume from the *L. vulgaris* populations tested. **4.2.** Mean fresh adult mass of *R. pilosa* that were collected from the different *L. vulgaris* populations. Vertical lines represent standard error of the mean. Bars with dissimilar letters are significantly different between populations (ANOVA, $P < 0.05$). Edmonton, AB (A); Mountain View, AB (M); Prince George, BC (P); Serbia (S); Tie Lake, BC (T).

Table. 4.2: Results from 2-way ANOVA comparisons on the effect of *Rhinusa pilosa* and *Linaria vulgaris* population at the initial set-up and at harvest. Degrees of Freedom (DF); F-value (F); *P*-value (P); population (Pop); treatment (Treat).

	Df	Initial set-up		Harvest													
		Initial stem length		Final stem length		Proportion of stems with flowers [a]		Proportion of stems with lateral shoots [a]		Proportion of stems dead [a]		Proportion of new stems [a]		Above-ground biomass [b]		Below-ground biomass [c]	
		F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Pop	4	7.92	0.000	15.07	0.000	9.57	0.000	35.80	0.000	6.07	0.000	0.86	0.492	7.69	0.000	22.00	0.000
Treat	1	0.03	0.870	2.17	0.145	1.73	0.192	0.99	0.322	0.36	0.552	5.40	0.023	4.14	0.045	6.60	0.012
Pop X Treat	4	0.4	0.809	0.17	0.955	0.31	0.869	0.93	0.453	1.16	0.337	1.10	0.361	1.07	0.375	1.44	0.229
Residual	80																

[a] data arcsine square root transformed for 2-way ANOVAs
[b] data square root transformed for 2-way ANOVAs
[c] data $\log_{10}$ transformed for 2-way ANOVAs

Table 4.3: Results from 1-way ANOVA comparisons on the effect of *Rhinusa pilosa* on *Linaria vulgaris* response variables at harvest. Degrees of freedom (DF); F-value (F); standard error of the mean (SE); denominator degrees of freedom used in F-value calculation (x).

Table 4.3

Plant Response Variable	Control Mean ± SE	Treatment Mean ± SE	DF (x)	$F_{1,x}$	*P*-value
Final stem length (cm)					
Edmonton	57.45±2.9	54.04±4.6	18	0.39	0.54
Mountain View	51.35±3.4	51.14±4.2	16	0.00	0.97
Prince George	47.58±3.4	42.06±4.0	18	1.11	0.31
Tie Lake	34.66±2.4	28.99±2.9	18	2.21	0.15
Serbia	60.99±8.1	58.13±4.6	10	0.09	0.77
Proportion of stems flowering [a]					
Edmonton	0.41±0.06	0.35±0.08	18	0.63	0.44
Mountain View	0.29±0.03	0.30±0.06	16	0.02	0.90
Prince George	0.44±0.08	0.38±0.08	18	0.12	0.73
Tie Lake	0.19±0.02	0.11±0.03	18	5.81	0.027
Serbia	0.10±0.05	0.11±0.06	10	0.01	0.93
Proportion of stems with lateral shoots [a]					
Edmonton	0.77±0.03	0.77±0.05	18	0.16	0.69
Mountain View	0.56±0.03	0.57±0.03	16	0.14	0.71
Prince George	0.74±0.03	0.77±0.04	18	0.62	0.44
Tie Lake	0.36±0.05	0.31±0.05	18	0.55	0.47
Serbia	0.34±0.05	0.50±0.06	10	4.04	0.07
Proportion of stems dead [a]					
Edmonton	0.08±0.03	0.17±0.04	18	2.38	0.14
Mountain View	0.24±0.04	0.19±0.04	16	1.07	0.32
Prince George	0.14±0.04	0.13±0.03	18	0.00	1.00
Tie Lake	0.24±0.03	0.30±0.05	18	0.56	0.46
Serbia	0.23±0.04	0.20±0.03	10	0.49	0.50
Proportion of new stems [c]					
Edmonton	0.11±0.03	0.09±0.04	18	0.32	0.58
Mountain View	0.10±0.04	0.07±0.03	16	0.37	0.55
Prince George	0.18±0.03	0.13±0.05	18	1.71	0.21
Tie Lake	0.19±0.05	0.06±0.02	18	8.70	0.009
Serbia	0.95±0.03	0.13±0.05	10	0.17	0.69
Above-ground biomass (g) [a]					
Edmonton	11.44±1.17	10.88±1.25	18	0.13	0.72
Mountain View	10.02±1.31	9.55±0.52	16	0.01	0.93
Prince George	10.64±0.76	10.34±0.86	18	0.08	0.78
Tie Lake	8.80±0.81	6.28±0.84	18	4.50	0.048
Serbia	16.28±2.44	11.70±1.39	10	2.65	0.13
Below-ground biomass (g) [b]					
Edmonton	4.34±0.57	4.64±0.58	18	0.10	0.75
Mountain View	6.09±1.40	4.79±0.67	16	0.14	0.72
Prince George	5.30±0.67	4.05±0.52	18	1.99	0.18
Tie Lake	5.41±0.84	2.76±0.37	18	8.55	0.009
Serbia	27.91±7.22	15.74±3.49	10	1.68	0.22

[a] data square root transformed for 1-way ANOVA
[b] data $\log_{10}$ transformed for 1-way ANOVA
[c] data arcsine square root transformed for 1-way ANOVA

Table. 4.4: Results from 2-way ANOVAs comparing intra-plant effect of *Rhinusa pilosa* on galled *Linaria vulgaris* plants at harvest. Degrees of freedom (DF); F-value (F); galled stems from treatment plants (G); *P*-value (P); population (pop); standard error of the mean (SE); ungalled stems from treatment plants (U).

	Df	Final stem length		Proportion of stems with flowers [a]		Proportion of stems with lateral shoots [a]		Proportion of stems dead [a]	
		F	P	F	P	F	P	F	P
Pop	4	12.34	0.000	5.21	0.001	23.63	0.000	3.37	0.013
G vs. U	1	0.000	0.972	2.41	0.125	22.35	0.000	38.18	0.000
Pop x G vs. U	4	1.33	0.266	1.43	0.231	2.33	0.063	1.18	0.324
Residual	77								

[a] data arcsine square root transformed for 2-way ANOVA

Table 4.5: Results from 1-way ANOVA comparisons on the intra-plant effect of *Rhinusa pilosa* on *Linaria vulgaris* plant response variables at harvest. Degrees of freedom (DF); F-value (F); galled stems from treatment plants (G); standard error of the mean (SE); ungalled stems from treatment plants (U); denominator degrees of freedom used in F calculation (x).

Plant Response Variable	Ungalled Mean ± SE	Galled Mean ± SE	DF (x)	$F_{1,x}$	*P*-value
Final stem length (cm)					
Edmonton	53.58±4.5	57.53±6.6	18	0.25	0.63
Mountain View	54.58±5.0	42.69±2.3	16	4.69	0.046
Prince George	43.44±4.9	42.01±4.1	18	0.05	0.83
Tie Lake	26.99±3.1	31.39±3.0	18	1.03	0.32
Serbia	54.89±4.6	63.94±4.4	10	2.03	0.18
Proportion of stems flowering [a]					
Edmonton	0.25±0.08	0.61±0.13	18	4.74	0.043
Mountain View	0.30±0.06	0.35±0.10	16	0.05	0.82
Prince George	0.37±0.08	0.45±0.12	18	0.04	0.84
Tie Lake	0.08±0.03	0.14±0.05	18	0.33	0.57
Serbia	0.13±0.07	0.10±0.06	10	0.24	0.64
Proportion of stems with lateral shoots [a]					
Edmonton	0.71±0.06	0.98±0.03	18	18.25	0.000
Mountain View	0.58±0.03	0.59±0.07	16	0.15	0.70
Prince George	0.74±0.05	0.86±0.04	18	3.64	0.07
Tie Lake	0.26±0.04	0.41±0.09	18	1.50	0.24
Serbia	0.46±0.06	0.77±0.08	10	7.98	0.018
Proportion of stems dead [a]					
Edmonton	0.20±0.06	0.03±0.03	18	12.90	0.002
Mountain View	0.19±0.04	0.15±0.06	16	1.17	0.30
Prince George	0.15±0.03	0.05±0.04	18	8.50	0.009
Tie Lake	0.33±0.06	0.16±0.06	18	6.03	0.024
Serbia	0.23±0.02	0.00±0.00	10	343.61	0.000

[a] data arcsine square root transformed for 1-way ANOVA

CHAPTER 5: PRE-RELEASE EFFICACY ASSESSMENT USING HIGH DENSITIES OF A CANDIDATE BIOCONTROL AGENT ON *LINARIA VULGARIS*

INTRODUCTION

The current paradigm in selecting candidate biocontrol agents for weeds is shifting to releasing fewer, more effective agents (McFadyen, 1998; Morin et al., 2009). A desirable agent is one that does not attack valued non-target species and can control the target weed (McFadyen, 1998). Earlier biocontrol programs concentrated more on assessing the potential risk to economic plant species during pre-release host range testing. With the shifting public views on the importance of protecting native species biodiversity, non-target feeding by biocontrol agents has resulted in a major criticism of classical weed biocontrol (Louda, Pemberton, Johnson, & Follett, 2003). Testing the efficacy of a candidate biocontrol agent is not mandatory and has resulted in the release of ineffective agents (McClay & Balciunas, 2005). Releasing multiple agents that are not effective in controlling the target weed is expensive and has led to the increased risk of both direct and indirect and non-target effects (Morin, et al., 2009). Thus it is recommended to evaluate the potential biological control agent's impact before release in addition to host-specificity testing to avoid the release of ineffective agents (McClay & Balciunas, 2005).

A pre-release impact assessment (PREA) essentially involves studying the "per-capita effect" of a candidate biocontrol agent on its target weed by assessing relevant plant developmental or reproductive parameters (McClay & Balciunas, 2005). PREAs

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can be conducted in the field, greenhouse, or laboratory (e.g., within quarantine) settings. PREAs are more easily and commonly conducted in the native range of the biocontrol agent and the target weed (Djamankulova, Khamraev, & Schaffner, 2008; Morin, et al., 2009). Densities of the candidate agent can be manipulated through cages or insecticide treatments (Briese, Pettit, & Walker, 2004; Goolsby, Zonneveld, & Bourne, 2004). In some cases, biocontrol workers are unable to conduct field trials in the native range and must rely on quarantine or greenhouse studies (Balciunas & Smith, 2006; Goolsby, Spencer, & Whitehand, 2009).

Harris and Shorthouse (1996) suggested that gall-forming insects are suitable candidates for biological control since gall makers typically have a narrow host range and can be effective in controlling their target weed. Galls are defined as atypical plant growths induced by host-specific organisms such as bacteria, mites, and insects (Abrahamson & Weis, 1987). Gall-inducing insects have been successful in weed biological control campaigns (Harris & Shorthouse, 1996; Muniappan & McFadyen, 2005). For example, a gall inducer to successfully control its target weed was *Trichilogaster acaciaelongifoliae* (Froggatt) (Hymenoptera: Pteromalidae), an inflorescence galler of *Acacia longifolia* (Andr.) Willd. (Fabaceae) in South Africa (Dennill, 1988).

Yellow toadflax, *Linaria vulgaris* (L.) Mill (Plantaginaceae), is a perennial weed introduced to North America from Europe in the 1700s for its use as a medicinal (ILieva, Handjieva, & Popov, 1992) and ornamental plant (Wilson et al., 2005). *Linaria vulgaris*, an obligate outcrosser, reproduces with genetically variable seeds (Ward, Fleischmann,

Turner, & Sing, 2009) and a rhizomatous, clonal root system (Saner, Clements, Hall, Doohan, & Crompton, 1995). The inflorescence has an indeterminate raceme on the main stem with indeterminate lateral inflorescences. The weed is commonly found in disturbed sites such as road sides, but can also invade undisturbed grasslands (Saner, et al., 1995; Sutton, Stohlgren, & Beck, 2007). *Linaria vulgaris* is difficult to control with herbicides due to its rhizomatous root system (Wilson, et al., 2005). Two adventitious seed feeders, *Brachypterolus pulicarius* L. (Coleoptera: Brachypteridae) and *Rhinusa antirrhini* (Paykull) (Coleoptera: Curculionidae) have been successful at reducing seed output, but have had little impact on *L. vulgaris* populations overall (McClay & De Clerck-Floate, 2002).

 Rhinusa pilosa Gyllenhal (Coleoptera: Curculiondiae), a stem-galling weevil, is one of two insects currently being assessed as a potential biocontrol agent for *L. vulgaris*. *Rhinusa pilosa* originates from Europe (Sweden in the north to France in the west and Russia to the east) (Caldara, Desančić, Gassmann, & Toševski, 2008). In early spring, overwintered *R. pilosa* adults mate and females oviposit within growing shoots near the apical meristem and induce gall formation. Under artificial conditions, females, on average, oviposit for 26.3 ± 3.44 days, induce 27.7 ± 4.5 galls and produce 51.9 ± 9.7 live adults (n = 32) (author, unpublished data, 2009 rearing results). The multilocular gall develops in 8 to 10 days when reared at 22˚C (Chapter 2). After three larval instars, the insect pupates within the gall and the adult continues to feed on the remaining gall parenchyma tissue. The adult chews a small hole, or "window", out of the gall prior to emergence, leaving the epidermal layer; windows are an indication that the galls contain

viable adults and are ready to be harvested). During the summer, adults periodically feed on *L. vulgaris* stems before winter diapause (Toševski, Gassmann, & Desančić, 2004).

McClay and Balciunas (2005) suggested that PREA should involve a high density of proposed agents or outbreak levels that would replicate the "best case" scenario. If the agent is not effective in controlling the target plant at high densities, that insect is likely to be an ineffective agent and potentially pose a greater threat of non-target interactions if approved for release. To assess the potential impact of high densities of *R. pilosa* on *L. vulgaris*, mated and ovipositing females were caged on overwintered, previously galled, *L. vulgaris* plants under quarantine conditions. The logic behind selecting previously galled plants was to simulate post-release field conditions. Since *L. vulgaris* is a short-lived perennial plant (4 years), it is highly likely that an individual plant will be exposed to a biological control agent for multiple years after release as the insect population densities increase over time. Toadflax plant growth and reproductive output were compared between treatment and control plants 11 weeks after the start of the experiment. Having preliminary impact data using a high density of *R. pilosa* females provides insight into the potential ability of this agent to control its target weed. This provides valuable data for biocontrol practitioners on the suitability of this agent for release.

MATERIALS AND METHODS

Study organisms

Plants for the high density impact assessment (both control and treatment plants) were randomly chosen from plants that were used for *R. pilosa* rearing in 2009. *Linaria*

vulgaris rearing plants were field collected from Mountain View, Alberta, Canada (49° 3'N 113°27'W) in the summer of 2008 and root pieces with 1 to 2 stems were potted in 15 cm clay pots using a soil mix (1 part sand, 1 part top soil, and 1 part Cornell mix (110 L vermiculite, 60 L peat moss, 9 L sand, 1.5 kg osmocote fertilizer, 380 g superphosphate fertilizer, 1 kg calcium flour, 10 g chelated iron, 20 g fritted trace elements (Frit Industries, Ozark, Alabama, USA)). Plants were grown under artificial conditions in the Insect Microbial Containment Facility at the Lethbridge Research Centre, Agriculture and Agri-Food Canada in Lethbridge, Alberta, Canada. Plants were allowed to establish over the summer and subsequently vernalized prior to being used for insect rearing in the spring of 2009 (Chapter 4). The rearing plants used in this experiment were exposed to a variety of weevil densities (range 1 - 23 average 3.6, n = 10), which produced an average of 16 galls per plant (range 5 - 48, n = 10), which contained an average of 49.6 adults (range 0 - 135, n = 10). After removal of galls, plants remained in the greenhouse for 2 months prior to vernalization during the winter of 2009 – 2010. Twelve plants were taken out of vernalization on Feb. 24, 2010 and transferred to 10˚C with 12 h light/12 h dark photoperiod for 7 days. Ten of the most vigorous vernalized plants were chosen for the experiment. Plants were watered daily as needed and grown in a quarantine rearing room using a mixture of incandescent (GE Ecolux ESP41, Mississauga, Ontario, Canada) and florescent plant grow lights (Standard F32T8, Saint-Laurent, Quebec, Canada) with 12 h light/12 h dark photoperiod at 22˚C day/ 18˚C night.

The insects used for this experiment were a mixture of insects shipped from Serbia (the Institute of Plant Protection and Environment in Zemun, Serbia) and insects from the Lethbridge Research Centre (LRC) colony. On March 7, 2010, 20 female and 15

male *Rhinusa pilosa* arrived at LRC from Serbia. A small batch of insects (ca. 70) from the LRC colony were taken out of winter diapause conditions on Feb. 3, 2010 and transferred to 10˚C for 2 weeks followed by a week at 15˚C. Insects were transferred to the same test conditions as the plants and caged on fresh plants to confirm their ability to induce galls. Once gall development was observed, the insects were combined in preparation for the test.

Experimental design

To assess the impact of *R. pilosa* on *L. vulgaris*, plants were paired based on vigour (i.e., growth rate and number of stems) and individuals within each pair were randomly designated as control or treatment, for a total of five replicate control-treatment pairs. A subset of 10 stems was randomly chosen from each test and control plant. The stem length (base of stem to apical meristem) and width from the base of the stem of each test stem was measured. Five mated and ovipositing females and three males were caged on each of the five test plants for 2 weeks in mesh cages that were 20 cm in diameter by 70 cm tall. Control plants also were caged, but did not include any insects. All replicates were set-up on the same day. Plants were checked every second day for insect mortality, with dead or missing females replaced with fresh females. Dead males were not replaced due to low male availability. After 2 weeks, all plants were uncaged and the insects were removed. Test stems were remeasured for length and width to determine initial growth rate. Test stems also were checked for evidence of oviposition and gall development. Plants were subsequently checked 6 days a week for new flowering stems and the date of the first flower of each stem was recorded. Galls were checked for windows every 3 days, starting 9 weeks after the test was set-up. If a window was observed, the test and the

paired control plant were recaged to prevent the escape of any adults that may emerge from their galls prior to plant harvest.

Plants were harvested 81 days after set-up, which corresponded to adult eclosion feeding prior to adult emergence from the galls. Stems were clipped at ground level and the following was measured from the 10 test stems: stem length and basal stem width, number of galls, number of flowers and number of lateral shoots. If a test stem was missing or dead, a non-test stem was randomly selected among the remaining living stems within the same plant and measured to replace the test stem (i.e., a galled stem replaced a dead or missing galled stem). All non-test stems were clipped at ground level and the number of lateral shoots and galls counted, as well as the presence/absence of flowers noted. For the above-ground biomass samples, the galled tissue was separated from the stem tissue and weighed independently from the stem samples. Roots were removed from the soil and gently washed for the below-ground biomass sample. Biomass samples were dried in a drying oven set at $60 \pm 5˚C$ for 2 weeks prior to being weighed on a flat top balance.

Statistical analysis

For the maximal ANOVA model, a one-way ANOVA was performed on data averaged per plant using treatment as the main factor and blocking as an error term. Blocking (initial pairing of control and treatment plants) was not significant and was removed for further analysis. Since *L. vulgaris* plants are composed of rhizomatous stems, the within plant response to galling also was investigated. This was accomplished by performing a second one-way ANOVA where galled plants were subdivided into

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galled and ungalled stems. Since this is an incomplete nested design, i.e., control plants

cannot be subdivided into galled and ungalled stems, two separate 1-way ANOVAs were

performed. A MANOVA was not performed due to the small sample size (ten plants).

The discrete response variables were checked for normality and square root transformed

when required (only for below-ground biomass) and all proportion data were arcsine

square rooted to meet assumptions of normality for the ANOVAs.

One treatment plant died during the experiment and was only included in the

initial plant assessments and not the final plant assessments. The deceased plant

contained only galled stems, while the other four galled plants contained galled and

ungalled stems, thus there were five galled and four ungalled stem values included in the

initial plant assessments and four galled and four ungalled stem values for the final plant

assessments. Data were analyzed using R version 2.9.2 (R Development Core Team,

2009). Results are stated in untransformed means ± standard error of the mean.

RESULTS

At the initial set-up of the experiment, the average stem length ($F_{1,8} = 1.73$, $P =$

0.22) and stem width ($F_{1,8} = 0.75$, $P = 0.41$) per plant were not significantly different

between the treatment and control plants (Fig. 5.1). When the insects were removed from

the plants 2 weeks later, galls were observed on all treatment plants. During the first 2

weeks, the treatment plants grew significantly slower than the control plants ($F_{1,8} = 21.47$,

$P = 0.002$). Within the galled plants, not all stems were oviposited into and thus, not all

stems developed galls. On average, the females oviposited into 77 % available stems

within the treatment plants (range of the 55 – 100 %). The average number of galls per plant was 29 ± 11.8 (n = 5) for a total of 145 galls pooled from all treatment plants.

Rhinusa pilosa continued to have an effect on treatment plants at the termination of the experiment (81 days after set-up). Galled plants were 55 % shorter than control plants ($F_{1,7} = 22.37$, $P = 0.002$), but stem widths were not significantly different ($F_{1,7} = 0.09$, $P = 0.77$) (Fig. 5.2). Galling significantly reduced the proportion of flowering stems in comparison to the control ($F_{1,7} = 13.37$, $P = 0.008$) (Fig. 5.3). Only one of the five treatment plants produced flowering stems. Control plants began flowering at 55-92 days (average of 72 days, n = 5) after vernalization, while the only flowering galled plant flowered at 98 days after vernalization. Galling did not result in a significantly higher proportion of stems with lateral shoots ($F_{1,7} = 3.04$, $P = 0.12$) (Fig. 5.3). The proportion of dead stems did not differ between control and treatment plants ($F_{1,7} = 0.92$, $P = 0.37$) (Fig. 5.3). Galled plants did not have proportionally more new stems that grew between the initial set-up and end of the experiment than control plants ($F_{1,7} = 1.27$, $P = 0.31$) (Fig. 5.3). Galling by *R. pilosa* did little to alter the above-ground biomass as there was no significant difference between the galled and control plants ($F_{1,7} < 0.00$, $P = 0.98$) (Fig. 5.2). The above-ground biomass consisted of all above-ground plant and galled material. Within the galled plants however, the dried gall biomass constituted 40 % of the dried above-ground biomass (Fig. 5.2). Below-ground biomass was reduced by 75 % in the galled plants ($F_{1,7} = 8.16$, $P = 0.025$) (Fig. 5.2).

Galling by *R. pilosa* also was effective in causing plant mortality. One of the five galled plants died during the experiment (41 days after the insects were caged with the

plants). The weakest living galled plant at the end of the experiment had 10 % of the stems alive in comparison to 91 % within its paired control plant.

Since not all stems developed galls, the galled plants were further subdivided into galled and ungalled stems to determine the intra-plant response to galling. Within the treatment plants, the galled and ungalled stems were the same length ($F_{1,6} = 0.98$, $P = 0.36$) and width ($F_{1,6} = 3.87$, $P = 0.08$) (Fig. 5.4). Only one of the five treatment plants produced flowering stems which was not significant between ungalled or galled stems ($F_{1,6} = 1.00$, $P = 0.36$) (Fig. 5.5). Galling did not result in a significantly higher proportion of stems with lateral shoots between galled and ungalled stems ($F_{1,6} = 0.52$, $P = 0.50$) (Fig. 5.5). The proportion of dead stems did not differ between galled and ungalled stems ($F_{1,6} = 0.02$, $P = 0.89$) (Fig. 5.5).

DISCUSSION

Heavy galling by *R. pilosa* had a negative impact on below- and above-ground plant growth. At the end of the experiment, below-ground biomass, stem length, and the proportion of stems with flowers were negatively affected by *R. pilosa.* The most significant effect of galling was the 75 % reduction of below-ground biomass. This is of particular relevance since the main mode of growth in *L. vulgaris* is through the development of dense patches of rhizomatous, clonal stems (Nadeau, King, & Harker, 1992) and a strong root system is vital for the overwintering survival of this perennial weed (Bakshi & Coupland, 1960). While there was not a significant difference in the above-ground biomass between the control and treatment plants, the galled material

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composed 40 % of the above-ground biomass of the treatment plants. This indicates that resources are being allocated to the gall and away from growth and reproduction.

Galling delays flowering time and reduces the proportion of flowering stems. All five of the control plants produced flowers during the experiment, while only one galled plant flowered. The single flowering galled plant produced flowers 26 days later than the average control plant. High densities of *R. pilosa* galls were effective at reducing the proportion of flowering stems. This can drastically reduce the quantity of flowers and seeds produced per year. Since toadflax can produce a large number of seeds, it may be seed limited; a seed capsule can produce up to 250 seeds and a healthy plant can produce from 15,000 to 20,000 seeds per year (Arnold, 1982; Wilson, et al., 2005). Although reducing seed output will not control established infestations, it may prevent new infestations from occurring.

Females did not oviposit on all available stems within treatment plants; on average 77 % of the stems had oviposition scars and developed galls. The female's reluctance to oviposit on all stems was not due to the female's inability to lay viable eggs or induce gall formation since test females successfully induced galls when they were transferred to fresh *L. vulgaris* plants after the experiment. The female's reluctance to oviposit on all stems could be a mechanism to avoid exceeding the carrying capacity of the plant. The oviposition behaviour of *Coelocephalapion aculeatum* Fall (Coleoptera: Apionidae), a biocontrol agent for *Mimosa pigra* L. (Mimosaceae), was based on the larval carrying capacity of the inflorescence (Heard, 1995). A female *C. aculeatum* laid an average of 18 eggs per inflorescence even if there were a greater number of

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oviposition sites available within the inflorescence. Avoidance of heavily galled sites could enhance the dispersal of the agent if approved for release as has been observed in the root-feeding weevil, *Mogulones cruciger* Herbst (Coleoptera: Curculionidae), on houndstongue, *Cynoglossum officinale* (L.) (Boraginaceae) (De Clerck-Floate & Wikeem, 2009). By not ovipositing on all stems, *R. pilosa* could be avoiding inducing early plant mortality. Within the stem, ovipositing females reduce intraspecific competition by laying eggs at regular intervals (average of 5.67 ± 0.8 eggs per gall, Chapter 2). Within the gall, the larvae avoid intraspecific competition by feeding within smaller chambers, separated by a thin layer of cells (Chapter 3). Adults generally avoided laying eggs in existing galls (Toševski, et al., 2004, and personal observations). However, one plant did have galls that were oviposited into after gall development, in that there were developing insects within the cortex rather than within the pith (Chapter 2). Larvae that develop within the cortex are closer to the epidermis and become more susceptible to attack by generalist parasitoids.

Studies of PREA have shown biological control agents to have a negative effect on their target weed in controlled environments (Wu, Hacker, Ayres, & Strong, 1999). *Tetramesa romana*, a stem-galling wasp, reduces stem and leaf lengths on giant reed in a quarantine greenhouse study (Goolsby, et al., 2009). A petiole-galling weevil, *Coelocephalapion camarae* Kissinger (Coleoptera: Apionidae), was effective at causing petiole desiccation due to disruption of the vascular tissue within the petiole and reducing below-ground biomass of *Lantana camara* L. (Verbenaceae) in a quarantine greenhouse study (Baars, Hill, Heystek, Neser, & Urban, 2007). Two planthoppers, *Prokelisia marginata* (Van Duzea) and *P. dolus* (Wilson) (Homoptera: Delphacidae), were assessed

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at high and low densities against the target weed, cordgrass, *Spartina alterniflora* Loisel. (Poaceae), in a greenhouse study and found that high densities of the insect were sufficient to cause 93 % mortality over a 4 month period (Wu, et al., 1999). *Prokelisia marginata* was approved for release in 2000 and a field release was conducted in Washington State, USA that same year. Preliminary post-release assessments indicate that this agent was successful in reducing the target weed biomass (Grevstad, Strong, Garcia-Rossi, Switzer, & Wecker, 2003). This suggests that negative effects of galling by *R. pilosa* on *L. vulgaris* exhibited during the PREA may forecast similar field efficacy to that of *P. marginata*. However, follow-up monitoring post-release will be required to verify the results from the PREA.

While attempts were made to maximize the impact of *R. pilosa* during this study, the field impact of *R. pilosa* on *L. vulgaris* can be hard to predict. For example, we do not know if *R. pilosa* will be able to establish and reach outbreak or damaging densities once released in the field. The density used in the study (5 females for 2 weeks) is rather small in comparison to other high density impact experiments. The weevil, *C. camarae* was used in densities of 10 or 20 pairs for 5 days in a moderate and high density impact study (Baars, et al., 2007). A gall fly, *Parafreutreta regalis* Munro (Diptera: Tephritidae), was exposed to its target weed, Cape Ivy, *Delairea odorata* Lemaire (Asteraceae), for 6 weeks in densities of 20 pairs (Balciunas & Smith, 2006). It was initially believed that the major impact of *Mecinus janthinus* Germar (Coleoptera: Curculionidae), a stem-boring weevil on Dalmatian toadflax, *L. dalmatica* (L.) Mill (Plantaginaceae), was the larval stage (Jeanneret & Schroeder, 1992). However, during post-release field evaluations of *M. janthinus*, it was found that early adult feeding on newly emerging

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Dalmatian toadflax shoots had a greater impact than larval feeding (Carney, 2003). As with *M. janthinus*, *R. pilosa* breaks winter diapause when *L. vulgaris* plants are emerging in the spring in their native habitat (Toševski, et al., 2004). The effect of *R. pilosa* adults after eclosion was not fully assessed during this study as this study was terminated prior to F1 adult emergence. After eclosion, the adults continue to feed on the gall parenchyma tissue for approximately 2 weeks, potentially disrupting the vascular tissue, and causing further damage to the plant. The newly emerged adults continue to feed on *L. vulgaris* throughout the summer prior to winter diapause. The emerging adults chew a small hole out of the gall. This emergence hole could provide an opening for saprophytic fungus and generalist herbivores to enter the plant as has been observed in other empty gall systems (Araújo, Lara, & Fernandes, 1995; Wilson, 1995). The endo- and exophagous feeding behaviour of post-eclosion adults and the emergence hole as a gateway for damaging organisms to enter *L. vulgaris* may contribute to *R. pilosa's* effectiveness as a biocontrol agent once field released.

CONCLUSION

By using both non-target host range testing and pre-release efficacy assessments as part of the biocontrol agent screening process, the results from these tests will greatly reduce the probability of releasing an ineffective agent. Based on the results from the pre-release efficacy assessment from this study, high densities of *R. pilosa* have been shown to cause negative effects to *L. vulgaris* growth and reproduction. These results will add valuable information to the petition for the release of *R. pilosa* as a biocontrol agent against *L. vulgaris* in Canada.

REFERENCES

Abrahamson, W. G., & Weis, A. E. (1987). Nutritional Ecology of Arthropod Gallmakers. In F. J. Slansky & J. G. Rodriquez (Eds.), *Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates* (pp. 235-258). New York: John Wiley & Sons, Inc.

Araújo, L. M., Lara, A. C. F., & Fernandes, G. W. (1995). Utilization of *Apion* sp. (Coleoptera Apionidae) galls by an ant community in southeastern Brazil. *Tropical Zoology, 8*, 319-324.

Arnold, R. M. (1982). Pollination, predation and seed set in *Linaria vulgaris* (Scrophulariaceae). *American Midland Naturalist, 107*(2), 360-369.

Baars, J. R., Hill, M. P., Heystek, F., Neser, S., & Urban, A. J. (2007). Biology, oviposition preference and impact in quarantine of the petiole-galling weevil, *Coelocephalapion camarae* Kissinger, a promising candidate agent for biological control of *Lantana camara*. *Biological Control, 40*, 187-195.

Bakshi, T. S., & Coupland, R. T. (1960). Vegetative propagation in *Linaria vulgaris*. *Canadian Journal of Botany, 38*, 243-249.

Balciunas, J., & Smith, L. (2006). Prerelease efficacy assessment, in quarantine, of a tephritid gall fly being considered as a biological control agent for Cape-ivy (*Delairea odorata*). *Biological Control, 39*, 516-524.

Briese, D. T., Pettit, W. J., & Walker, A. (2004). Evaluation of the biological control agent, *Lixus cardui*, on *Onopordum* thistles: experimental studies on agent demography and impact. *Biological Control, 31*, 165-171.

Caldara, R., Desančić, M., Gassmann, A., & Toševski, I. (2008). On the identity of *Rhinusa hispida* (Brullé) and its current synonyms (Coleoptera: Curculionidae). *Zootaxa, 1805*, 61-68.

Carney, V. A. (2003). *Ecological Interactions of Biological Control Agent, Mecinus janthinus Germar, and its Target Host, Linaria vulgaris (L.) Mill.*, University of Lethbridge, Lethbridge, AB.

De Clerck-Floate, R. A., & Wikeem, B. (2009). Influence of release size on establishment and impact of a root weevil for the biocontrol of houndstongue (*Cynoglossum officinale*). *Biocontrol Science and Technology, 19*(2), 169-183.

Dennill, G. B. (1988). Why a gall former can be a good biocontrol agent: The gall wasp *Trichilogaster acaciaelongifoliae* and the weed *Acacia longifolia*. *Ecological Entomology, 13*, 1-9.

Djamankulova, G., Khamraev, A., & Schaffner, U. (2008). Impact of two shoot-galling biological control candidates on Russian knapweed, *Acroptilon repens*. *Biological Control, 46*, 101-106.

Goolsby, J. A., Spencer, D., & Whitehand, L. (2009). Pre-release assessment of impact on *Arundo donax* by the candidate biological control agents *Tetramesa romana* (Hymenoptera: Eurytomidae) and *Rhizaspidiotus donacis* (Hemiptera: Diaspididae) under quarantine conditions. *Southwestern Entomologist, 34*(4), 359-376.

Goolsby, J. A., Zonneveld, R., & Bourne, A. (2004). Prerelease assessment of impact on biomass production of an invasive weed, *Lygodium microphyllum* (Lygodiaceae: Pteridophyta), by a potential biological control agent, *Floracarus perrepae* (Acariformes: Eriophyidae). *Environmental Entomology, 33*(4), 997-1002.

Grevstad, F. S., Strong, D. R., Garcia-Rossi, D., Switzer, R. W., & Wecker, M. S. (2003). Biological control of *Spartina alterniflora* in Willapa Bay, Washington using the planthopper *Prokelisia marginata*: agent specificity and early results. *Biological Control, 27*, 32-42.

Harris, P., & Shorthouse, J. D. (1996). Effectiveness of gall inducers in weed biological control. *The Canadian Entomologist, 128*, 1021-1055.

Heard, T. A. (1995). Oviposition and feeding preferences of a flower-feeding weevil, *Coelocephalapion aculeatum*, in relation to conspecific damage to its host-plant. *Entomologia Experimentalis et Applicata, 76*, 203-209.

ILieva, E. I., Handjieva, N. V., & Popov, S. S. (1992). Iridoid glucosides from *Linaria vulgaris*. *Phytochemistry, 31*, 1040-1041.

Jeanneret, P., & Schroeder, D. (1992). Biology and host specificity of *Mecinus janthinus* Germar (Col.: Curculionidae), a candidate for the biological control of yellow and Dalmatian toadflax, *Linaria vulgaris* (L.) Mill. and *Linaria dalmatica* (L.) Mill. (Scrophulariaceae) in North America. *Biocontrol Science and Technology, 2*(1), 25-34.

Louda, S. M., Pemberton, R. W., Johnson, M. T., & Follett, P. A. (2003). Nontarget effects-the achilles' heel of biological control? Retrospective analyses to reduce risk associated with biocontrol introductions. *Annual Review in Entomology, 48*, 365-396.

McClay, A. S., & Balciunas, J. K. (2005). The role of pre-release efficacy assessment in selecting classical biological control agents for weeds - applying the Anna Karenina principle. *Biological Control, 35*, 197-207.

McClay, A. S., & De Clerck-Floate, R. A. (2002). *Linaria vulgaris* Miller, Yellow Toadflax (Scrophulariaceae). In P. G. Mason & J. T. Huber (Eds.), *Biological Control Programmes in Canada, 1981-2000* (pp. 375-382). New York: CABI Publishing.

McFadyen, R. E. (1998). Biological control of weeds. *Annual Review in Entomology, 43*, 369-393.

Morin, L., Reid, A. M., Sims-Chilton, N. M., Buckley, Y. M., Dhileepan, K., Hastwell, G. T., et al. (2009). Review of approaches to evaluate the effectiveness of weed biological control agents. *Biological Control, 51*, 1-15.

Muniappan, R., & McFadyen, R. E. (2005). Gall-inducing Arthropods Used in the Biological Control of Weeds. In A. Raman, C. W. Schaefer & T. M. Withers (Eds.), *Biology, Ecology, and Evolution of Gall-inducing Arthropods* (Vol. 2, pp. 761-766). Plymouth, UK: Science Publishers.

Nadeau, L. B., King, J. R., & Harker, K. N. (1992). Comparison of growth of seedlings and plants grown from root pieces of yellow toadflax (*Linaria vulgaris*). *Weed Science, 41*(1), 43-47.

R Development Core Team (2009). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Saner, M. A., Clements, D. R., Hall, M. R., Doohan, D. J., & Crompton, C. W. (1995). The biology of Canadian weeds. 105. *Linaria vulgaris* Mill. *Canadian Journal of Plant Science, 75*, 525-537.

Sutton, J. R., Stohlgren, T. J., & Beck, K. G. (2007). Predicting yellow toadflax infestations in the Flat Tops Wilderness of Colorado. *Biological Invasions, 9*, 783-793.

Toševski, I., Gassmann, A., & Desančić, M. (2004). *Annual Report 2003: Biological Control of Dalmatian and Yellow Toadflaxes, Linaria dalmatica and L. vulgaris*. Delemont: CABI Publishing.

Ward, S. M., Fleischmann, C. E., Turner, M. F., & Sing, S. E. (2009). Hybridization between invasive populations of Dalmatian toadflax (*Linaria dalmatica*) and yellow toadflax (*Linaria vulgaris*). *Invasive Plant Science and Management, 2*, 369-378.

Wilson, D. (1995). Fungal endophytes which invade insect galls: insect pathogens, benign saprophytes, or fungal inquilines? *Oecologia, 103*(2), 255-260.

Wilson, L. M., Sing, S. E., Piper, G. L., Hansen, R. W., De Clerck-Floate, R. A., MacKinnon, D. K., et al. (2005). *Biology and Biological Control of Dalmatian and Yellow Toadflax*: USDA Forest Service, FHTET-05-13.

Wu, M.-Y., Hacker, S., Ayres, D., & Strong, D. R. (1999). Potential of *Prokelisia* spp. as biological control agents of English cordgrass, *Spartina anglica*. *Biological Control, 16*, 267-273.
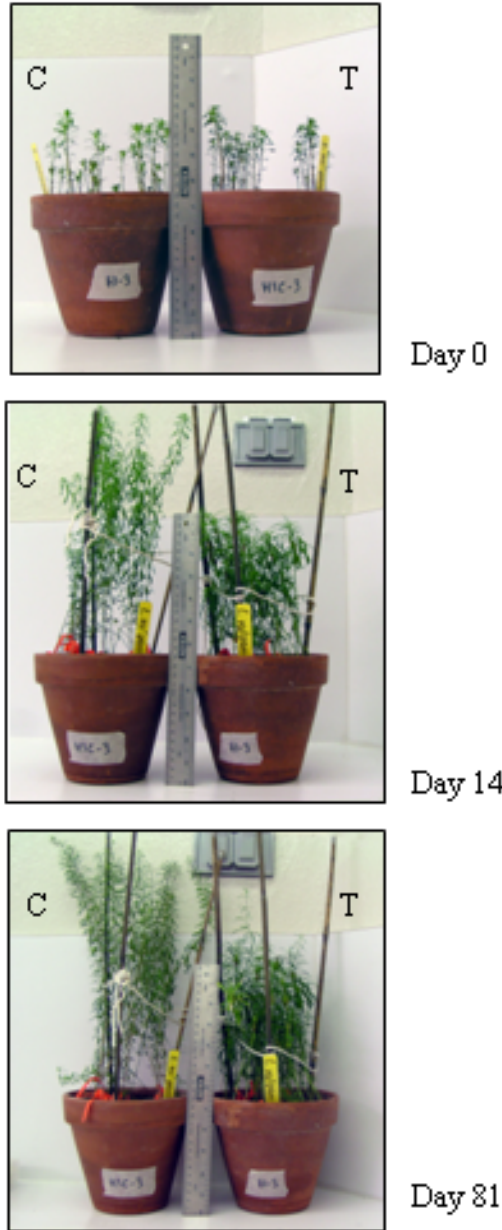
Figure 5.1: Appearance of a representative pair of *Linaria vulgaris* plants at days 0, 14 and 81 after the start of the experiment. The plants were paired ahead of experiment set-up based on similar size and number of stems. The treatment plants (T) (on right) were caged with five *Rhinusa pilosa* females for 2 weeks and the control plants (C) (on left) also were caged but unexposed to the gall former. Notice the small size of the galled plants at days 14 and 81 after set-up.
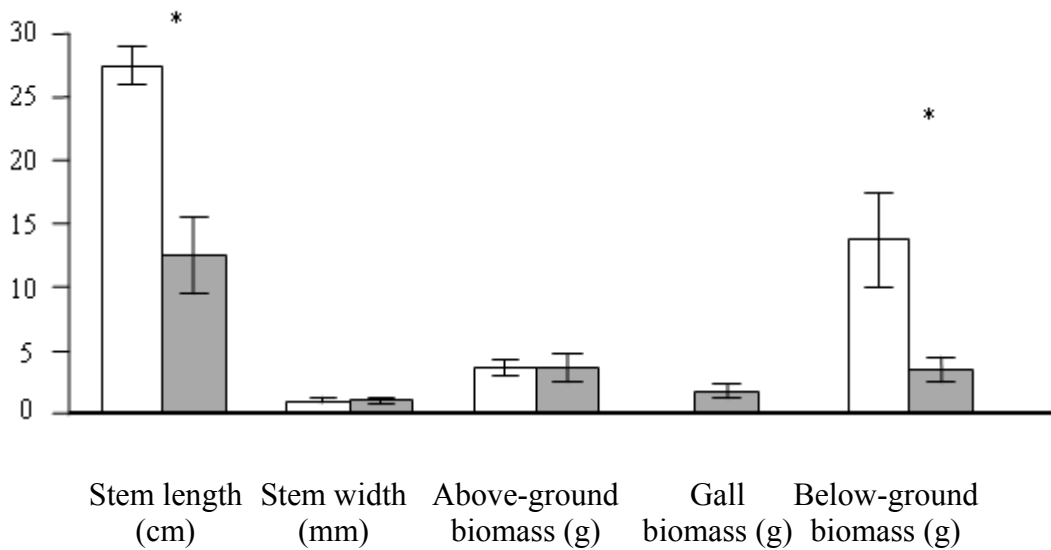
Figure 5.2: Impact of *Rhinusa pilosa* galling on multiple plant response variables at termination of the experiment. Vertical lines represent standard error of the mean. White bars are ungalled, control plants and dark grey bars are galled, treatment plants. Bars with * are significantly different between treatments (ANOVA, *P* < 0.05).

Figure 5.3: Impact of *Rhinusa pilosa* galling on multiple plant response variables from the end of the experiment. Vertical lines represent standard error of the mean. White bars are ungalled, control plants and dark grey bars are galled, treatment plants. Bars with * are significantly different between treatments (ANOVA, $P < 0.05$).
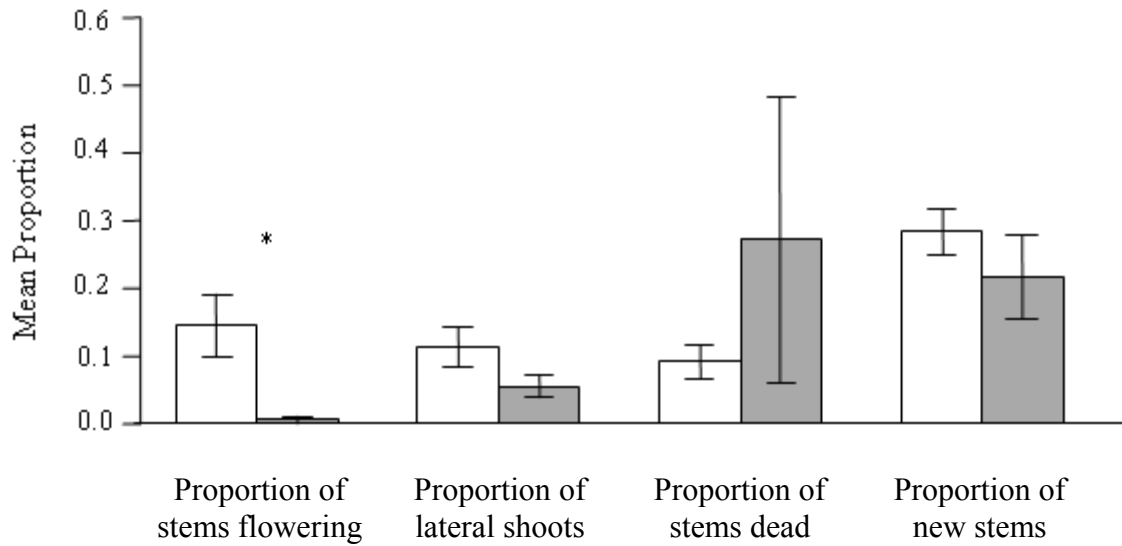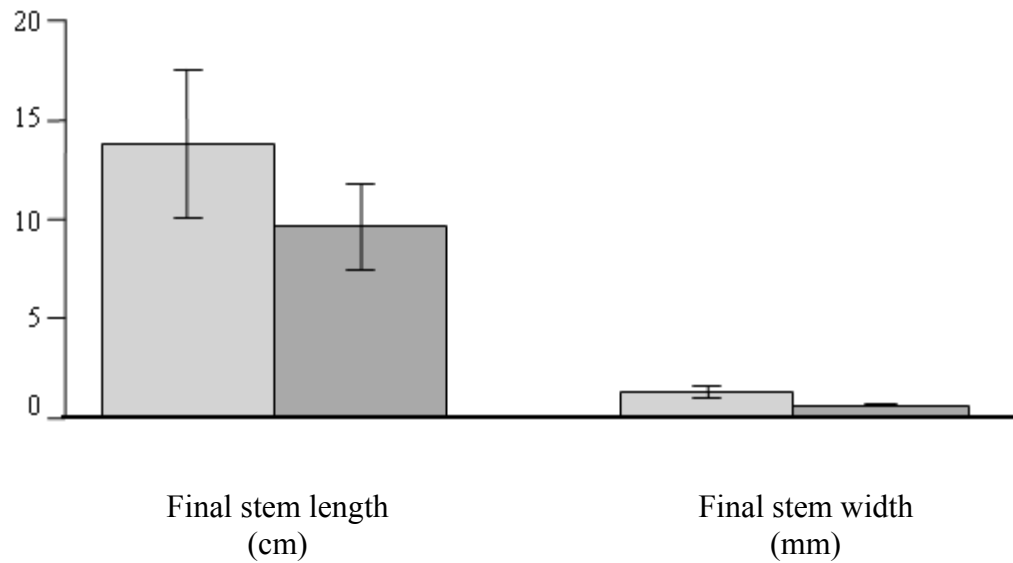
Figure 5.4: Impact of *Rhinusa pilosa* galling on average stem length and average stem width of galled (light grey bars) and ungalled (dark grey bars) stems within treatment plants at termination of experiment. Vertical lines represent standard error of the mean. There were no significant differences between galled and ungalled stems (ANOVA, *P* > 0.05).

Figure 5.5: Impact of *Rhinusa pilosa* galling on proportion of flowering stems, lateral shoots, and dead stems of galled (light grey bars) and ungalled (dark grey bars) within treatment plants at termination of experiment. Vertical lines represent standard error of the mean. There were no significant differences between galled and ungalled stems (ANOVA, $P > 0.05$).
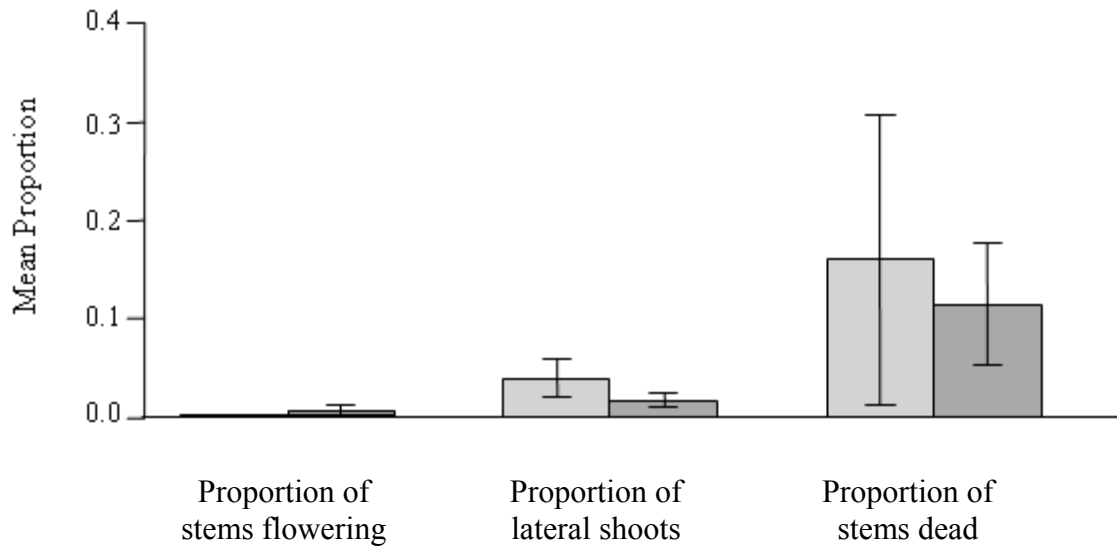
CHAPTER 6: GENERAL CONCLUSIONS

Gall development

Galls induced by *Rhinusa pilosa* on *Linaria vulgaris* were novel in that: 1) Gall development begins after egg deposition where as larval feeding is generally required for gall induction; 2) Multiple vascular tissue organizations (small band of vascular tissue or large vascular bundles composed of hypertrophied cells) developed; 3) Gall parenchyma cells contained increased numbers of plastids and a large central vacuole; 4) Adults eclosed within the gall and fed on the pith parenchyma cells when the pith cells generally form a sclerenchyma sheath in other complex insect galls.

The three stages of gall development in this system were documented using histological methods and included: 1) gall induction, 2) gall growth and development, and 3) gall maturation. Gall induction consisted of increased cell divisions of the vascular cambium, while the cells of the cortex underwent hypertrophy, with some initial separation of parenchyma cells to produce intercellular spaces within the cortex. Gall growth was due to a combination of hyperplasy and large intercellular space development in the cortex tissue, hypertrophy and hyperplasy of the vascular tissue, and hypertrophy of pith parenchyma cells. Gall maturation consisted of lignification of the cells within the vascular tissue. Gall dehiscence consisted of the adult chewing a hole through the gall but this stage was not examined histologically.

Gall development patterns were compared between galls collected from plants from the invasive range to that of gall development patterns on host plants from the native range. Gall development patterns were similar between the two regions.

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Gall induction

Gall induction in the *L. vulgaris* – *R. pilosa* system is unique in that gall development is almost complete by the time the larvae have hatched from their eggs. Normally, larval feeding is required to induce galls. Observations of "empty" galls have indicated that the ovipositional fluid secreted by the female during oviposition may be associated with gall induction in this system. A dark-staining fluid was isolated prior to egg deposition and is suspected to be ovipositional fluid, and long rows of periclinal divisions, de novo xylem production, and gall parenchyma differentiation was observed. Attempts to isolate the role of the egg in gall induction were not successful. Female feeding to create the oviposition canal did not result in gall induction, and can be eliminated as a cause of gall induction. Callus tissue production and wound meristem formation was induced in response to female feeding. Although a variety of plant responses was observed, none of the treatments tested resulted in normal gall development. Future studies should be conducted to improve isolation of the ovipositional fluid and the egg to determine their role in gall induction in this system.

Rhinusa pilosa development on different populations and low impact

Rhinusa pilosa was able to gall and develop successfully on all populations of *L. vulgaris* tested. At the termination of the experiment, the proportion of galled stems, dry gall mass, and proportion of live adults collected at the end of the experiment was not significantly different among populations. The induction of an equal proportion of galls per population, which produce an equal proportion of live adults, suggests that *R. pilosa*

can successfully attack and develop on multiple plant populations. This will contribute to the efficacy of *R. pilosa* in the field if approved for release.

Few studies test the impact of a candidate biocontrol agent on multiple plant populations from the invasive range prior to release. In this study, we compared low densities of a candidate biocontrol agent, *R. pilosa*, on four geographically distinct populations of *L. vulgaris* from western Canada and one population from the native range. One Canadian population, Tie Lake, Alberta, responded negatively to galling by producing a lower proportion of flowering stems, new stems, and smaller above- and below-ground biomass in comparison to the control plants. The other four populations used in the experiment were not significantly different between the treatment and control plants for any of the plant response variables measured. Therefore, based on the ability of *R. pilosa* to gall and develop on multiple invasive populations of *L. vulgaris*, *R. pilosa* would make a suitable candidate to control *L. vulgaris* in Canada.

Heavy impact

Heavy galling by *R. pilosa* had a negative impact on below- and above-ground plant growth. Below-ground biomass, stem length, and the proportion of stems with flowers were negatively affected by high densities of *R. pilosa.* The most significant effect of galling was the 75 % reduction of below-ground biomass. This is of particular relevance since the main mode of growth in *L. vulgaris* is through the development of dense patches of rhizomatous, clonal stems (Nadeau, King, & Harker, 1992) and a strong root system is vital for the overwintering survival of this perennial weed (Bakshi & Coupland, 1960). Heavy galling delayed flowering time and reduced the proportion of

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flowering stems. In the above-ground biomass of the galled plants, 40 % was galled material.

By using both non-target host range testing and pre-release efficacy assessments as part of the biocontrol agent screening process, these tests will greatly reduce the probability of releasing an ineffective agent. Based on the results of this study, high densities of *R. pilosa* have been shown to cause negative effects to *L. vulgaris* growth and reproduction. These results will add valuable information to the petition for the release of *R. pilosa* as a biocontrol agent against *L. vulgaris* in Canada.

Suitability of R. pilosa as a successful biological control agent

Coleopterans and gall-inducing insects have been successful in weed biological control campaigns (Harris, 1991; Harris & Shorthouse, 1996; Muniappan & McFadyen, 2005). Coleopterans have been candidates for weed biocontrol because they are hardy, can have a narrow host range, and can produce sufficient feeding damage to weaken the host plant (Harris, 1991). Gall inducers have been important in weed biological control programs because; 1) they have a narrow host range, and 2) galls are resource sinks, re-routing nutrients towards gall growth and maintenance and away from plant growth and reproduction (Harris & Shorthouse, 1996). For example, *Rhinocyllus conicus* (Froelich) (Coleoptera: Curculionidae), which forms simple callus galls in the flower heads of *Carduus nutans* L. (Asteraceae), has been touted as the most successful gall inducer to control its weed and has been recognized as being the fourth most successful weed biocontrol project internationally (Crawley, 1989). The gall wasp *Trichilogaster acaciaelongifoliae* (Froggatt) (Hymenoptera: Pteromalidae) has been successful in

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controlling its target weed, *Acacia longifolia* (Andr.) Willd. (Fabaceae), in South Africa (Dennill, 1988). This gall wasp reduces the reproductive potential of its host by 89 % and vegetative growth by 53 % (Dennill, 1988).

Based on the results of this thesis and the work of Ivo Toševski through the toadflax consortium, *R. pilosa* has the potential to be an effective agent if approved for release. Harris and Shorthouse (1996) provide a list of four major characteristics (vulnerability to parasitism, vulnerability to moisture stress, the gall as metabolic sink, and agent resource exploitation) that are needed to be assessed to determine the efficacy of a gall-inducing biocontrol agents and *R. pilosa* fulfils most of the required traits. The first characteristic is vulnerability to parasitism. It is expected that *R. pilosa* will be able to build up sufficient numbers required to control *L. vulgaris*. In Serbia, there is an inquiline weevil, *Rhinusa thapsicola* Germar, which utilizes the galls induced by *R. pilosa*. The inquiline larvae have been observed to consume other inquiline larvae and *R. pilosa* larvae within the gall (Toševski, Gassmann, & Desančić, 2004). Since the inquiline is not found in North America, *R. pilosa* will potentially have increased survivorship rates. Attack by generalist parasitoids from North America may occur.

No studies have directly assessed the vulnerability of the *R. pilosa* gall to moisture stress. Most gall formers studied respond negatively to drought. The *Urophora cardui* L. (Diptera: Tephritidae) stem gall on Canada thistle has large stomata which is a contributing factor to the failure of this galler in dry areas (Harris & Shorthouse, 1996). Since there are few stomata observed on the exterior of the *R. pilosa* gall, water loss through transpiration should be minimized, potentially increasing the galling success in

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dry areas. *Linaria vulgaris* can be found in both moist and dry sites (Wilson et al., 2005), making vulnerability to moisture stress potentially less of a concern.

Harris and Shorthouse (1996) recommended gall formers with long gall development periods, which increases the time the gall acts as a resource sink. Here, *R. pilosa* gall growth occurs in a little over 8 days after oviposition (Chapter 2). Continual cellular modifications occurred after gall development ceased (Chapter 2) indicating that the galls of *R. pilosa* may continue to act as resource sinks even after gall development had ceased. Also, gall mass constituted 40 % of the above-ground biomass (Chapter 5) indicating that resources were allocated towards the gall and not to plant growth and reproduction. Histological investigations into gall development indicate that there was increased vasculature within the gall (Chapter 2). Frass that lined the larval chamber had been observed to contain fragments of xylem, indicating that the larvae consumed the vascular tissue in addition to gall parenchyma cells. Feeding on the vascular tissues disrupts the flow of nutrients in the stem. Death of galled stems has been observed due to wilting, which indicates that *R. pilosa*'s damage to the vascular tissue can cause stem mortality. Larval feeding of *Coelocephalapion camarae* Kissinger (Coleoptera: Curculionidae) on the vascular tissue within its galls has resulted in the desiccation of *Lantana camara* L. (Verbenaceae) petioles, making this insect a more effective biocontrol agent (Baars, Hill, Heystek, Neser, & Urban, 2007). Continued cellular modifications are occurring after gall growth has ceased, mainly through cell expansion, cell division of the gall parenchyma cells, and lignin deposition in the walls of the vascular tissue (Chapter 2). Harris and Shorthouse (1996) suggest that the degree of lignification may be related to the degree in which the gall acts as a metabolic sink. Galls

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with the large bundle vascular tissue organization may then be higher metabolic sinks due to the large amount of lignin that is deposited (Chapter 2). Cellular modifications (such as extensive lignin deposition), in addition with the feeding action of the developing larvae disrupting the vascular tissue, could be contributing to the altered resource allocation and damage to galled plants.

Rhinusa pilosa is effective in exploiting host resources by attacking the most vulnerable host stage; when the young *L. vulgaris* shoots are growing from the soil in early spring. It has been suggested that the earlier the gall initiation during the growth or reproductive period of the plant, the greater the biomass allocation (Dennill, 1988). The weevil also has a relatively long oviposition time (ca. 1 month, author unpublished rearing results) and can produce more than one gall per stem, further increasing the resource allocation. The impact of post-diapause feeding by the adults was not assessed in this thesis but could be a major factor contributing the control of *L. vulgaris* in outbreak years. For example, it was initially believed that the major impact of *Mecinus janthinus* Germar (Coleoptera: Curculionidae), a stem-boring weevil on Dalmatian toadflax, *L. dalmatica* (L.) Mill (Plantaginaceae), was the larval stage feeding within the stems of its host plant (Jeanneret & Schroeder, 1992). However, during post-release field evaluations of *M. janthinus*, it was found that early adult feeding on newly emerging Dalmatian toadflax shoots had a greater impact than larval feeding (Carney, 2003). As with *M. janthinus*, *R. pilosa* breaks winter diapause when *L. vulgaris* plants are emerging in the spring in their native habitat (Toševski, et al., 2004). Post-eclosion feeding also could contribute to resource exploitation of this agent. The adults continue to feed on the gall parenchyma tissue for approximately 2 weeks, potentially disrupting the vascular

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tissue, causing further damage to the plant. The emerging adults chew a small hole out of the gall. This emergence hole could provide a opening for saprophytic fungus and generalist herbivores to enter the plant as has been observed in other empty gall systems (Araújo, Lara, & Fernandes, 1995; Wilson, 1995). The newly emerged adults continue to feed on *L. vulgaris* throughout the summer prior to winter diapause. The resource exploitation of *R. pilosa* due to host synchronization to a vulnerable host life stage, the endo- and exophagous feeding behaviour of post-eclosion adults and the emergence hole as a gateway for damaging organisms to enter *L. vulgaris* may contribute to *R. pilosa's* effectiveness as a biocontrol agent once field released.

Rhinusa pilosa has a narrow host range, generally confined to plants within the genus *Linaria*. In non-target testing conducted by Ivo Toševski and Rose De Clerck-Floate (from 2003, 2007-2009), they have found that *R. pilosa* can gall and develop on 11 different species within the genus *Linaria* (*L. alpina* (L.) Mill., *L. angustissima* (Loisel.) Borbás, *L. concolor* Griseb, *L. dalmatica* (L.) Mill., *L. dalmatica* spp. *macedonica* (Griseb.) Sutton, *L. genistifolia* (L.) Mill., *L. purpea* (L.) Mill., *L. rubioides* spp. *nissana* Vis. and Pančić, *L. saxatilis* (L.) Chaz. Lugo., *L. supina* (L.) Chaz., and *L. vulgaris*). *Rhinusa pilosa* also was found to gall and develop on *Sairocarpus virga* (Gray) Sutton, a non-threatened native species to North America although galls only produced adults in one of the four years tested (2007) at a rate of 3 % (Toševski, Gassmann, Desančić, & Jović, 2008). There are no native *Linaria* species in North America (McClay & De Clerck-Floate, 2002; USDA, 2010). *Rhinusa pilosa* can induce galls, but did not complete development on three other species during non-target testing; *Nuttallanthus canadensis* (L.) Sutton (North American native), *L. kurdica* ssp *kurdica* Boiss and Hohen, and

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Sairocarpus nuttallianus (Benth. ex. A. DC.) Sutton (North American native) (Toševski, et al., 2004; Toševski, et al., 2008; Toševski, Gassmann, Desančić, & Jović, 2009, 2010).

Three other non-native *Rhinusa* spp. are present in North America, and are being used as classical biological control agents against yellow and Dalmatian toadflax (Wilson, et al., 2005). *Rhinusa antirrhini* (Paykull) and *R. neta* (Germar) are both seed feeders and were not formally released, but arrived here from Europe adventively. *Rhinusa antirrhini* is a gall former in the fruit capsules of *L. vulgaris* and is distributed across North America (McClay & De Clerck-Floate, 2002). *Rhinusa antirrhini* has been successful in reducing the seed output of *L. vulgaris* at many sites within North America. *Rhinusa neta* does not form galls but feeds on the seeds within *L. vulgaris* fruit capsules and *R. neta*'s establishment has been less successful. *Rhinusa linariae* (Panzer) induces galls on the roots of *L. vulgaris* and has not been well established in North America. It is important to note that none of the *Rhinusa* spp. listed above have been found on non-*Linaria* species in North America (Wilson, et al., 2005). Due to the host-specificity of these three *Rhinusa* spp. in the field, this supports that *R. pilosa* also may have a narrow host range if introduced into North America.

REFERENCES

Araújo, L. M., Lara, A. C. F., & Fernandes, G. W. (1995). Utilization of *Apion* sp. (Coleoptera Apionidae) galls by an ant community in southeastern Brazil. *Tropical Zoology, 8*, 319-324.

Baars, J. R., Hill, M. P., Heystek, F., Neser, S., & Urban, A. J. (2007). Biology, oviposition preference and impact in quarantine of the petiole-galling weevil, *Coelocephalapion camarae* Kissinger, a promising candidate agent for biological control of *Lantana camara*. *Biological Control, 40*, 187-195.

Bakshi, T. S., & Coupland, R. T. (1960). Vegetative propagation in *Linaria vulgaris*. *Canadian Journal of Botany, 38*, 243-249.

Carney, V. A. (2003). *Ecological Interactions of Biological Control Agent, Mecinus janthinus Germar, and its Target Host, Linaria vulgaris (L.) Mill.*, University of Lethbridge, Lethbridge, AB.

Crawley, M. J. (1989). The successes and failures of weed biocontrol using insects. *Biocontrol News and Information, 10*, 213-223.

Dennill, G. B. (1988). Why a gall former can be a good biocontrol agent: The gall wasp *Trichilogaster acaciaelongifoliae* and the weed *Acacia longifolia*. *Ecological Entomology, 13*, 1-9.

Harris, P. (1991). Classical biocontrol of weeds: Its definitions, selection of effective agents, and administrative-political problems. *The Canadian Entomologist, 123*, 827-849.

Harris, P., & Shorthouse, J. D. (1996). Effectiveness of gall inducers in weed biological control. *The Canadian Entomologist, 128*, 1021-1055.

Jeanneret, P., & Schroeder, D. (1992). Biology and host specificity of *Mecinus janthinus* Germar (Col.: Curculionidae), a candidate for the biological control of yellow and Dalmatian toadflax, *Linaria vulgaris* (L.) Mill. and *Linaria dalmatica* (L.) Mill. (Scrophulariaceae) in North America. *Biocontrol Science and Technology, 2*(1), 25-34.

McClay, A. S., & De Clerck-Floate, R. A. (2002). *Linaria vulgaris* Miller, Yellow Toadflax (Scrophulariaceae). In P. G. Mason & J. T. Huber (Eds.), *Biological Control Programmes in Canada, 1981-2000* (pp. 375-382). New York: CABI Publishing.

Muniappan, R., & McFadyen, R. E. (2005). Gall-inducing Arthropods Used in the Biological Control of Weeds. In A. Raman, C. W. Schaefer & T. M. Withers (Eds.), *Biology, Ecology, and Evolution of Gall-inducing Arthropods* (Vol. 2, pp. 761-766). Plymouth, UK: Science Publishers.

Nadeau, L. B., King, J. R., & Harker, K. N. (1992). Comparison of growth of seedlings and plants grown from root pieces of yellow toadflax (*Linaria vulgaris*). *Weed Science, 41*(1), 43-47.

Toševski, I., Gassmann, A., & Desančić, M. (2004). *Annual Report 2003: Biological Control of Dalmatian and Yellow Toadflaxes, Linaria dalmatica and L. vulgaris*. Delemont: CABI Publishing.

Toševski, I., Gassmann, A., Desančić, M., & Jović, J. (2008). *Annual Report 2007: Biological Control of Dalmatian and YellowToadflaxes (Linaria dalmatica and L. vulgaris)*. Delemont: CABI Publishing.

Toševski, I., Gassmann, A., Desančić, M., & Jović, J. (2009). *Annual Report 2009: Biological Control of Dalmatian and Yellow Toadflaxes, Linaria dalmatica and Linaria vulgaris*. Delemont: CABI Publishing.

Toševski, I., Gassmann, A., Desančić, M., & Jović, J. (2010). *Annual Report 2009: Biological Control of Dalmatian and Yellow Toadflaxes, Linaria dalmatica and Linaria vulgaris*. Delemont: CABI Publishing.

USDA, N. R. C. S. (2010). The PLANTS Database. Retrieved 5 November, 2010, from http://plants.usda.gov/java/profile?symbol=LINAR

Wilson, D. (1995). Fungal endophytes which invade insect galls: insect pathogens, benign saprophytes, or fungal inquilines? *Oecologia, 103*(2), 255-260.

Wilson, L. M., Sing, S. E., Piper, G. L., Hansen, R. W., De Clerck-Floate, R. A., MacKinnon, D. K., et al. (2005). *Biology and Biological Control of Dalmatian and Yellow Toadflax*: USDA Forest Service, FHTET-05-13.