INTEGRATION OF *PUCCINIA PUNCTIFORMIS* INTO ORGANIC MANAGEMENT OF *CIRSIUM ARVENSE*

by

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ABSTRACT

Cirsium arvense is a perennial weed that causes significant economic losses in agriculture. An extensive rhizomatous root system makes *C. arvense* difficult to manage, particularly in organic cropping systems that use tillage as a primary management tool. To improve organic management of *C.* arvense, there is a need for the development of alternative and integrated weed management toolsets that include *C. arvense* biological controls. *Puccinia punctiformis* is a fungal pathogen that systemically infects *C. arvense*, with the potential to reduce host vigor. The goal of this research was to assess the impacts of *P. punctiformis* within organic cropping systems, using a greenhouse and a field study that examined integration of the biocontrol with cultural and mechanical management tools. In the greenhouse, *P. punctiformis* was integrated with a competitive annual cropping sequence, where *C. arvense's* biomass production and competitive ability was assessed. *Cirsium arvense* biomass production was significantly reduced when *P. punctiformis* was integrated with the cultural management tactic, more than individual use of the biocontrol or cultural management alone. Additionally, *P. punctiformis* reduced the competitive ability of *C. arvense* over time. In the field, *P. punctiformis* was integrated with mechanical management, where reduced and standard tillage treatments were evaluated to determine the effects on *P. punctiformis* and *C. arvense* abundance. The reduced tillage treatment caused a greater increase in *P. punctiformis* infected *C. arvense* stems compared to standard tillage, however there was no impact to asymptomatic *C. arvense* stem density from either tillage treatment. In both tillage treatments, there was a reduction in asymptomatic *C. arvense* stem density in samples where *P. punctiformis* infection was present. Integration of *P. punctiformis* with cultural and mechanical tools can be an effective way to reduce *C. arvense* vigor. However, successful integration of the biocontrol can be dependent on a combination of environmental factors and deliberate cropping system management. While *P. punctiformis* is not a singular management solution, it has potential to be integrated into reduced disturbance cropping systems for long-term and sustainable *C. arvense* management.

CHAPTER ONE

PROJECT BACKGROUND AND OBJECTIVES

Introduction

The spread of weedy plant species is a major challenge in agricultural production, that must be sustainably addressed in order to meet the shifting demands of a growing world population. Weeds can decrease overall ecosystem biodiversity, cause significant crop yield losses, and reduce land use efficiency (Tiley 2010; Jacobs et al., 2006). They have impacted agricultural production for thousands of years, and despite modern management techniques, weeds remain a primary management challenge. The most problematic weeds are often well adapted to a wide range of habitats, are highly competitive for resources, exhibit rapid growth, and have long-lived seed banks (Baker, 1974). Aggressive weedy plants can quickly displace standing crop and flora, change community composition, and decrease overall biodiversity (Jacobs et al. 2006). The combination of these qualities makes control efforts incredibly challenging and economically taxing. In the United States alone, the estimated economic cost of weed management totals $27 billion annually in cropping systems and $6 billion annually in pastures (Pimental et al., 2005). In order to preserve ecosystem integrity and agricultural production, there will need to be improved integrated weed management techniques for long-term and sustainable weed management.

One of the most problematic weed species in agricultural production is the perennial rhizomatous weed, *Cirsium arvense* L. *Scop.* (Canada thistle, California thistle, Creeping thistle). *Cirsium arvense* is well adapted to a wide range of temperate habitats, where it can aggressively

invade agricultural systems. Management of *C. arvense*, has proven difficult because of its extensive rhizomatous root system, and its resilience to disturbance (Tiley 2010). Despite ongoing research effort and development of management tools, *C. arvense* has persisted as a formidable agricultural pest (Pimentel, 2001; Skinner et al., 2000; Tiley 2010). Thus, there is a need for alternative and ecologically-based management approaches (Liebman et al., 2001) that focus on integrated weed management tactics (Liebman et al., 2001; Orloff et al., 2018; Davis et al., 2018).

Integrated weed management is the practice of using various physical, ecological, chemical, or genetic tactics to manage weeds (Liebman et al., 2001; Tautges et al., 2016). This multifaceted approach to weed management attempts to systematically combine two or more practices so that their effects are complementary, with greater impact and sustainability than stand-alone use (Swanton et al., 2008). In organic agriculture, where synthetic chemicals and genetic modification tools are excluded, integrated weed management includes various cultural tools (i.e., competitive crops, diversified rotations, seeding rates, row spacing, etc.), mechanical tools (i.e., tillage implements, mowing, mulching, etc.), and biological tools (i.e., herbivores, insects, pathogens) (Tautges et al., 2016). Many mechanical and cultural tools are commonly integrated into agricultural systems, but the integration of biocontrols can be challenging due to limitations with host specificity, climate, availability, and lack of research. However, there is opportunity for further investigation into biocontrols that have potential to be integrated into common management tactics for *C. arvense*.

The fungal biocontrol agent, *Puccinia punctiformis* (Str.) Rohl. (thistle rust), is a selective *C. arvense* pathogen that has shown potential as an alternative management tool.

Puccinia punctiformis has been used to effectively manage *C. arvense* in rangeland and non-cropping systems (French et al., 1990; Thomas et al., 1994; Berner et al., 2013; Cripps et al., 2011), but there is a need to explore the pathogen's impact within organic cropping systems. The purpose of this study was to evaluate the potential to integrate the fungal biocontrol into *C. arvense* management tactics that are common to semi-arid organic cropping systems in the Northern Great Plains region of North America. Successful integration of cultural, mechanical, and biological *C. arvense* management has the potential to enhance the efficiency of organic cropping systems, and potentially improve the sustainability of agricultural production in the Northern Great Plains.

History and Biology of *Cirsium arvense*

Cirsium arvense is native to Eurasia, and is now found throughout temperate regions of the world (Hodgson, 1968; Preston and Hill 1997). The weed was first introduced to North America in the 17th century through contaminated grain (Atwater, 1902; Moore, 1975). Since its first recorded introduction, *C. arvense* has spread to most states and territories in the United States and Canada (Moore, 1975; Tiley, 2010), where it has become one the most frequently listed noxious weed (Skinner et al., 2000). By the 20th century, it was reported that *C. arvense* infested more acreage than any other weed throughout the Northern Great Plains regions of Montana, Idaho, Oregon, and Washington, with approximately 253,000 hectares of land infested in Montana by 1956 (Hodgson, 1968).

Cirsium arvense is a perennial polycarpic herb from the Asteraceae family, that produces taproots up to depths of 5 vertical meters and rhizomes that can grow over 5 meters horizontally (Tiley, 2010). Stems grow erect with paniculate inflorescence, irregularly lobed spiny leaves, and

imperfectly dioecious flowers (Tiley, 2010). Flowers are typically pink or purple, with smaller sized globular male heads and larger flask-shaped female heads (Moore, 1975). Reproduction occurs asexually through clonal rhizomes, and sexually, with the potential to produce up to 5,000 seeds per stem (Jacobs et al., 2006). Maturing flowers develop an abundance of grey-white pappus that are easily separated from brown colored achenes (Moore, 1975). Seeds typically germinate during spring climates; however, seed germination rarely leads to population increase due to high abortion rates (Lalonde & Roitberg, 1994). The majority of reproductive energy goes to the development of clonal shoots, which continually develop through the horizontal rhizome throughout the growing season (Lalonde & Roitberg, 1994). Cold temperatures of autumn and winter cause above ground vegetation to senesce, while portions of the below-ground rhizome remain dormant until spring (Berner et al., 2013; Lalonde & Roitberg, 1994). *Cirsium arvense* grows best in regions with long photoperiods, in temperatures of 0°C to 32°C (Moore, 1975; Tiley, 2010), and with a total annual rainfall of 400 mm to 750 mm (Hodgson 1968; Moore 1975). This weed can be found in various temperate habitats, but most often occurs in disturbed areas (Tiley, 2010), making agricultural systems especially vulnerable to invasion (Guggisberg et al., 2012).

Organic Management of *Cirsium arvense*

Organic agriculture is a well-established practice, having been developed out of necessity for the last 4,000 years (Meyers, 2005). Today, there are approximately 71 million hectares of organically certified land throughout the globe, with continued growth from a 2018 estimated value $115 billion USD, where the United States and Western Europe dominated organic imports (Willer and Sahota, 2020). The United States Department of Agriculture (USDA), the regulating

body for organic certification in the United States, aims to "support the cycling of on-farm resources, promote ecological balance, and conserve biodiversity". In response to these goals, the USDA has set guidelines for organic production that restrict the use of synthetic fertilizers, pesticides, and genetic modification techniques (Meyers, 2005). Therefore, modern organic producers generally utilize sustainable practices including crop rotation and diversification, cover cropping, and integrated pest management. These practices are enhanced through the use of modern equipment, improved crop varieties, water conservation practices, and systematic livestock management (Reganold & Wachter, 2016).

Research and management of *C. arvense* has been extensively focused on chemical herbicides since the 1960's (Wyse, 1992; Davis et al., 2018), and as a result, *C. arvense* continues to cause significant yield losses and efficiency reductions in organic agriculture. Without access to chemical controls, organic weed management is reliant on various cultural, mechanical, and biological tools that can be used individually or by integrating two or more management tactics (Melander et al., 2005; Liebman et al., 2009). However, organic weed management is often labor intensive, costly, and sometimes results in little to no effect. Therefore, there is a need for alternative and integrated management approaches that combine cultural, mechanical and biological management techniques (Liebman et al., 2009; Orloff et al., 2018).

Cultural management

Crop competition and diversified crop rotations are cultural management practice that have been frequently used in organic agriculture to help disrupt weed growth and reduce niche dominance of weed species (Liebman and Dyck, 1993; Liebman and Davis, 2009). For example,

Hodgson (1958) and Derscheid et al. (1961) found that multiple years of competition from the forage crop, *Medicago sativa* (alfalfa), resulted in near complete eradication of *C. arvense*. Crop rotations that include multi-year perennial forages, followed by annual cash crops, have also proven to effectively manage *C. arvense* (McKay et al., 1959; Ominski et al., 1999). While cultural management can be effective, Orloff et al.'s (2018) meta-analysis on organic management of *C. arvense* reported that singular use of crop diversification and competition generally resulted in low to moderate success, stressing the importance of integrating cultural management with other tactics.

Mechanical management

One of the most common tools used for suppression of weeds in organic systems is mechanical tillage (Liebman and Davis, 2009; Orloff et al; 2018). Tillage can give crops a competitive advantage by reducing weed vigor and preventing weed seed production (Bowman and Halvorson, 1997). Frequent seasonal plowing and cultivations can successfully manage *C. arvense* (Stevens 1846; Tiley, 2010) with potential to eradicated *C. arvense* with at least one cultivation every 28 days throughout a growing season (Alley, 1981; Tiley, 2010). However, long term and intensive tillage regimes can cause a breakdown of soil aggregates, wind and water erosion, reductions in organic matter, and loss of water through infiltration and evaporation. Additionally, in a meta-analysis of published research focused on organic management of perennial weeds, Orloff et al. (2018) showed that singular use of mechanical weed management, while the most studied management approach, did not outperform other individual approaches. Instead, mechanical management appeared to be most effective when integrated with two or more management techniques.

Biological management

Biocontrol of weeds is an alternative management option, often used in agricultural systems where chemical pesticides are either limited or banned (Guske et al., 2004). Biocontrol agents can be fungi, insects, or microorganisms that are native or exotic enemies that can weaken or kill plant species (Guske et al., 2004; Tiley, 2010). There are many *C. arvense* biocontrol agents that have been considered for their potential impact (Guske et al., 2004; Bond et al., 2006; Cripps et al., 2011). However, most biocontrol agents appear to lack either host selectivity, scalability, or cause insufficient damage to *C. arvense.* The limited impact of *C. arvense* biocontrol agents have highlighted the need for an integrated approach, where successful use of biocontrol agents may be more feasible when combined with various mechanical or cultural management tools (Kluth 2005; Reed et al., 2006).

Puccinia punctiformis

Puccinia punctiformis (thistle rust) is an obligate rust pathogen that is highly selective to *C. arvense* (Guske et al., 2004; Berner et al., 2013). This pathogen was first introduced to North America in the 17th century (Olive, 1913) and has since become naturalized throughout temperate regions of the United States and Canada, where *C. arvense* exists (Far and Rossman, 2023). *Cirsium arvense* acts as host to the pathogen by providing shelter and nutrients. *Puccinia punctiformis* establishes and overwinters in *C. arvense* rhizomes, where it parasitizes resources from the host, and eventually emerges as spores on thistle ramets. The pathogen morphs through a five-stage macrocyclic life cycle:

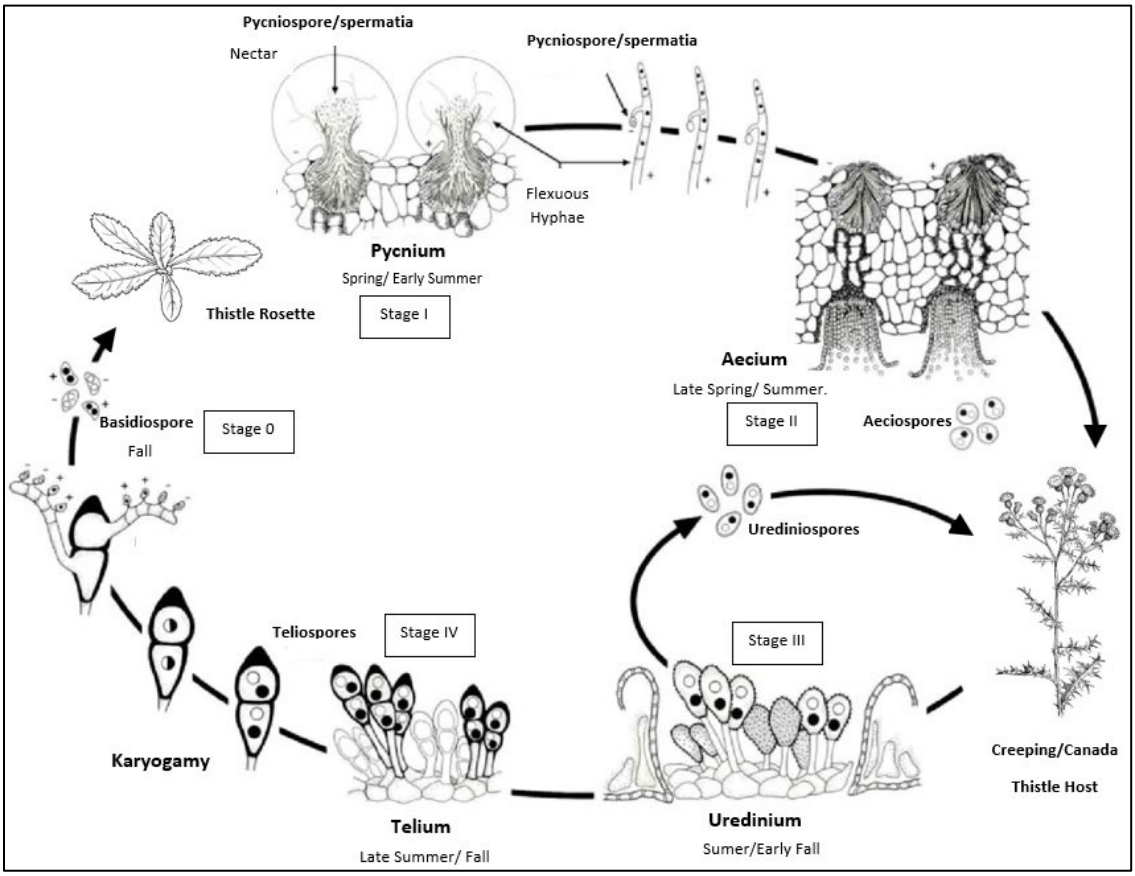

Figure 1: Puccinia punctiformis life cycle: Stage 0: Basidiospores (2n), Stage I: Spermatia (1n), Stage II: Aeciospores (n + n), Stage III: Urediniospores (n + n), and Stage IV: Teliospores (n + n)

The infectious sage of *P. punctiformis* starts as a basidium containing four basidiospores (Stage 0; Figure 1). Haploid basidiospores are produced when ideal climate conditions occur, requiring temperatures between 8℃ and 25℃, adequate moisture, and when stimuled by *C. arvense* volatile organic compounds (French and Lightfield, 1990). The basidiospores penetrate the cell walls of *C. arvense* by producing filamentous haustoria, which develop a systemic network of intra- and intercellular parasitic mycelium that exist within *C. arvense* rhizomes and stems until the host dies (Menzies, 1953; Baka and Losel, 1992).

After one to two seasons of systemic infection in *C. arvense*, orange colored spermatia (Stage I; Figure 1) appear on the underside of leaves. Spermatia production represents the reproductive

phase in the *P. punctiformis* life cycle. These haploid spores either exist as sticky, positive (+) mating types that emit a sweet aroma (Connick and French, 1991; Stephanie et al., 2001), or as negative (-) mating types that act as receptive hyphae (Berner et al., 2013). Genetic outcrossing and fusion occur when the two mating types come in contact, as a result of insect vectors that transmit the sticky aromatic (+) spermatia (Stephanie et al., 2001).

Next, plasmogamy occurs, where the parent spermatia cells combine without fusing the nuclei. The result is a dikaryotic (n + n), cell called an aeciospore (Stage II; Figure 1; Berner et al, 2013; Kentjens et al., 2023). Aeciospores are dark red colored, friable spores which appear on the underside of *C. arvense* leaves in early summer (Thomas et al.,1994). These spores are wind, animal, or mechanically dispersed and they can attach to stem and leaf tissue of neighboring *C. arvense* (Berner et al., 2013). Aeciospores cause localized infection of *C. arvense* leaves and stems; however, they are not known to cause long-term and systemic infection in *C. arvense* (Thomas et al., 1994; Berner et al., 2015; Kentjens et al., 2023). As the seasonal climate shifts to warmer and dryer conditions, aeciospores give rise to dikaryotic (n +n) urediniospores (Stage III; Figure 1). Urediniospores are dark brown, friable pustules on the stems, upper and lower leaf surfaces of *C. arvense* that can cause localized infection on neighboring *C. arvense*. Like aeciospores, urediniospores are not know to be responsible long-term and systemic infection in *C. arvense* (Berner et al., 2015; Kentjens et al., 2023).

As climates become cooler and drier in the fall, *P. punctiformis* begins to produce overwintering teliospores (Stage IV; Figure 1). Teliospores are dikaryotic (n + n), di-cellular spores that appear as small black freckles on senescing leaves of infected *C. arvense*. They have thick melanized cell walls that protect them from UV damage and winter climates. Infected *C.*

arvense leaves and stems become litter on the soil surface as temperatures drop and the photoperiod shortens, where debris containing teliospores has the potential to contact *C. arvense* rosettes through wind, animal, or mechanical dispersal. If conditions are suitable, teliospores undergo karyogamy where the two haploid nuclei in the dikaryotic cells fuse to create diploid cells. These cells then begin the process of meiosis, producing four haploid basidiospore cells, that start the infection life cycle in a new host.

Research Objectives

Puccinia punctiformis has shown potential as an effective *C. arvense* biocontrol (French, 1990; Thomas et al., 1994; Berner et al., 2013; Cripps et al., 2014; Kentjens et al., 2023; Chichinsky et al., 2023), however little is known about the impact or feasibility of using the pathogen as a management tool within cropping systems. This purpose of this study is to evaluate the impact of *P. punctiformis* when it is integrated with other weed management practices that are common to organic cropping systems in the Northern Great Plains region of North America. The main objectives are:

1. Assess the integration of *P. punctiformis* with a competitive crop rotation, and the impact on *C. arvense* growth.
2. Assess the integration of *P. punctiformis* with mechanical management tactics, and the impact on *C. arvense* growth.

Successful integration of *P. punctiformis* into *C. arvense* management tactics can benefit organic agriculture as an integrated weed management approach that sustainably limits crop yield losses due to the *C. arvense* pressure. This work serves as a foundational exploration of the potential for incorporating *P. punctiformis* as part of an integrated weed management toolset that

combines cultural, mechanical, and biological management approaches. With these research findings, we hope to improve the effectiveness of *C. arvense* management so that organic cropping systems in the Northern Great Plains remain a steward of sustainable agriculture.

CHAPTER TWO

IMPACT OF PUCCINIA PUNCTIFORMIS ON *CIRSIUM ARVENSE* PERFORMANCE IN A SIMULATED CROP SEQUENCE

Introduction

Cirsium arvense (L.) Scop. (Canada thistle) is a problematic weed that causes large economic losses in agriculture, driving the need for integrated weed management tools that include biological control agents (Orloff et al., 2018). *Cirsium arvense* can be found throughout temperate climates of the world, where it exists as a perennial herb that reproduces through an extensive rhizomatous root system and wind dispersed seeds (Tiley, 2010). Clonal rhizomes make *C. arvense* resilient to disturbance, particularly in tilled organic cropping systems that do not use synthetic herbicides for weed management (Moore, 1975). Organic producers in the Northern Great Plains region of the United States generally depend on tillage as a primary weed management tool, however this practice increases soils erosions due to wind and water, and depletes soil organic matter over time (Lenhoff et al., 2017). Additionally, tillage can disperse vigorous *C. arvense* rhizomes, causing a rapid increase of the weed's population (Tiley 2010). As a result, *C. arvense* has become a serious management problem within organic cropping systems, where alternative management tools need to be explored (Tautges et al., 2017; Orloff et al., 2018).

The use of competitive annual crops is another common approach used to manage weeds in organic cropping systems (Bullock, 1992; Liebman and Dyck, 1993). Competitive crops can disrupt weed growth by reducing resource availability and niche dominance of weed species

(Liebman and Dyck, 1993). However, the difficult nature of reducing *C. arvense* rhizomes, particularly in organic agriculture (Tautges et al., 2017; Orloff et al., 2018), has led to a search for alternative and integrated tactics, including biocontrol agents that inhibit root development (Berner et al., 2013; Cripps et al., 2011). The use of biocontrol agents can be challenging due to a lack of host specificity, varied responses to environmental conditions, and mismanagement. However, continued exploration of biocontrols for *C. arvense* has the potential to yield low-cost, long-term, host-specific options that can be integrated into existing weed management toolsets (Berner et al., 2013).

Puccinia punctiformis (F. Strauss) Rohl. (thistle rust) is a heterotrophic fungal pathogen of *C. arvens*e that acts as a long-term systemic parasite (Buller, 1950; Menzies, 1953; Berner et al., 2013; Kentjens et al., 2023). As a parasite that consumes resources and weakens the root structure (Buller, 1950; Menzies, 1953), *P. punctiformis* is specific to *C. arvense* (Berner et al., 2013; Kentjens et al., 2023) and has been identified in temperate habitats around the globe (Berner et al., 2013; Kentjens et al., 2023). Once established in the roots, infected *C. arvense* can develop chlorotic leaf tissue with lesions, elongated stems, and growth irregularities which can reduce fitness and cause death (Buller, 1950; Berner et al., 2013). Diseased stems act as aboveground carriers for *P. punctiformis* spores, appearing as orange to dark-red pustules on leaves, where the fungus completes most of its five-stage heterothallic life cycle during summer months, eventually producing transmissible teliospores (Buller, 1950; Menzies, 1953; Kentjens et al., 2023). Teliospore-bearing thistle leaves senesce and abscise as precipitation and temperatures decline, where they can contact healthy *C. arvense* rosettes through wind or

mechanical dispersion, leading to long-term systemic infection in new *C. arvense* hosts under ideal environmental conditions (French and Lightfield, 1990; Berner et al., 2013).

Puccinia punctiformis's impact on *C. arvense* abundance has been well documented (French et al., 1990; Thomas et al., 1994; Berner et al., 2013; Cripps et al., 2016; Kentjens et al., 2023). However, to our knowledge, the effects of integrating the *P. punctiformis* biocontrol with a competitive crop sequence on *C. arvense* growth have not been studied. We addressed this gap in knowledge using greenhouse experiments, which assessed the impact of *P. punctiformis* on *C. arvense* growth and competitiveness. Specifically, our questions were: 1) What is the probability of observing *P. punctiformis* infected *C. arvense* over time, and does the density of infected *C. arvense* stems increase over time? 2) How does *P. punctiformis* affect *C. arvense* above- and belowground biomass, and does crop competition interact with the effects? 3) Using a relative competition intensity, is the competitive ability of *C. arvense* reduced when *P. punctiformis* is integrated into a sequence of competitive annual crops? We hypothesized that the integration of *P. punctiformis* with a competitive crop sequence would lead to a significant reduction in above- and belowground *C. arvense* biomass, compared to individual effects from *P. punctiformis* or a competitive crop sequence when used alone.

Materials and Methods

Experimental Design

A greenhouse study with three independent trials was conducted at the Montana State University Plant Growth Center in Bozeman, Montana, between 2020 and 2022. A nested full factorial (2 x 2) design was used to assess the integration of *P. punctiformis* and crop competition. The primary treatment was *P. punctiformis* inoculation, with two levels: *C. arvense*

inoculated with *P. punctiformis* (n = 20) and non-inoculated *C. arvense* grown as a control (n = 20). Nested within each level of the inoculation treatment was a competition treatment, split into two levels: *C. arvense* grown in monoculture (n = 10) and *C. arvense* grown in competition with a common crop species (n = 10; Supplementary Figure 1).

The competition treatment was a four-phase crop sequence that incorporated common crops used by organic farmers in the dryland areas of the Northern Great Plains. The sequence included the following four phases, with seeding depths and seeding rates scaled for greenhouse pots: 1) Fallow: 1-gram *C. arvense* rhizome planted at ~10 cm deep; 2) spring wheat: 100 kg/hectare planted at ~ 5 cm deep (18 plants/pot); 3) forage pea: 89 kg/hectare planted at ~ 5 cm deep (8 plants/pot); and 4) safflower: 33 kg/hectare planted at ~ 3 cm deep (2 plants/pot). *Cirsium arvense* rhizomes were planted in the approximate center of each pot during the first phase. Crops were planted in a manner that provided approximately equal space between individuals, with at least 5 cm of distance from pot edges.

Two separate greenhouse spaces were used to prevent movement of *P. punctiformis* spores between the *P. punctiformis* inoculated treatment and the non-inoculated (control) treatment. Internal greenhouse temperatures for both spaces were set at a range of 18°C to 26.5℃ during the day, and 10℃ to 24°C at night. To ensure consistent lighting, passive solar lighting with supplemental 1000-watt metal halide lamps, set to 12-hour intervals, were used throughout the course of the study.

Cirsium arvense and *Puccinia punctiformis* Establishment

Cirsium arvense rhizomes were acquired from naturally occurring populations in Gallatin County and Hill County, Montana during the summer of 2019. Rhizomes were maintained in

greenhouse pots, and used as the source of rhizome transplants for the study. Pots (25.4 cm diameter x 20.3 cm deep) were sown with 1-gram cuttings of *C. arvense* rhizome and randomly assigned to a treatment. Rhizomes were planted into a pasteurized soil mixture consisting of equal parts (by volume) of loam soil, washed sand, and Canadian sphagnum peat moss. Pots were watered every two days or as needed, for ten seconds per pot using the shower setting on a conventional garden hose wand. A soluble all-purpose fertilizer (20-20-20 NPK) was added to pots bi-weekly, by mixing 2.5 ml of fertilizer with 3.8 L of water in a watering can, and hand watering for ten seconds per pot. *Cirsium arvense* was grown for an average of 4.5 months during the first phase (fallow) in all treatments, which was approximately timed with the development of flower buds in all pots. In subsequent phases of each trial, *C. arvense* was allowed to grow until harvest at the maturity stage of the crop within each crop phase.

Puccinia punctiformis inoculum was collected from naturally occurring populations of infected *C. arvense* in Gallatin County, Montana and prepared as described by Berner et al. (2013). Systemically infected *C. arvense* stems were harvested in the autumns of 2020 and 2021, and dried in paper bags in a dark room at ambient temperatures. From the dried stems, leaf tissue containing signs of teliospores were gathered, and ground into a coarse powder inoculum using a household blender. The ground teliospore-bearing inoculum was immediately used, or stored for future use in a -80°C freezer. Inoculation methodology followed Berner et al. (2013), where 5 ml of dry rust inoculum was dispersed evenly on the crowns of *C. arvense* rosettes at the beginning of each phase, for a total of four inoculations per pot in each trial. The inoculated rosettes were misted with deionized water once a day for three days post inoculation to maintain humidity for

spore germination. This method was repeated after the harvest of each phase and subsequent regrowth of *C. arvense*, for a total of four inoculations per pot in each trial.

Data Collection

To address our first question, the density of *C. arvense* stems with signs of systemic *P. punctiformis* infection was recorded from each pot at the termination of each crop phase. *Cirsium arvense* stems were identified as systemically infected when spore structures were observed on leaves and stems. To address our second and third questions, *C. arvense* and crop stems were counted and cut at soil level at the termination of each crop phase. To obtain dry weight, the harvested biomass was oven dried for 72 hours at ~40.5℃ and weighed to the nearest 0.01g. After each harvest, pots containing thistle rhizomes were left undisturbed and the next crop phase was seeded into pots assigned to the mixed competition treatment. After the aboveground harvest of final the crop phase (safflower) of each trial, *C. arvense* rhizome biomass was removed from the soil of each pot, cleaned of soil and residue with cool water, dried for 72 hours at ~40.5℃, and weighed to the nearest 0.01g *Cirsium arvense* pots assigned to the monoculture level of the competition treatment were harvested using the same methodology and at the same time as the mixed pots.

Data Analysis

The probability of observing systemic *P. punctiformis* infection in pots was calculated at each phase in the crop sequence, and was modeled using a generalized linear mixed effects model with a binomial distribution ("glmer" function in the R-Package "lmerTest"; Kuznetsova et al., 2022). The fixed effect in this model was crop phase, and pot ID was included as a random effect to account for repeated observations within each pot over the three trials. Model selection

followed a backwards selection from a full model containing all potential explanatory variables using a 'Drop in Deviance' test (Ramsey & Schafer, 2012). Model overdispersion was checked by calculating the sum of squared Pearson residuals and comparing it to the residual degrees of freedom, and assumptions homoscedasticity, normality, or influential observations were visually assessed (Ramsey & Schafer, 2012).

The percentage of *C. arvense* stems with signs of systemic *P. punctiformis* infection within the inoculated treatment was calculated out of the total density of *C. arvense* stems per pot, and was modeled using a linear mixed effects model ("lmer" function in the R-Package "lmerTest"; Kuznetsova et al., 2022). The fixed effects and random effects in this model were the same as previously described. Explanatory variables were backwards selected from a full model containing all potential explanatory variables ("step" function in the R-Package "lmerTest"; Ramsey & Schafer, 2012). Model assumptions of homoscedasticity, normality, and influential observations were visually assessed (Ramsey & Schafer, 2012).

Differences in *C arvense* above- and belowground biomass was evaluated using separate linear mixed effects models. In the model for aboveground biomass, the fixed effects were inoculation treatment, competition treatment, and crop phase, with pot ID as a random effect. In the model for belowground biomass, the fixed effects were inoculation treatment and competition treatment, with trial as a random effect to account for repeated observations within each trial. In both models, explanatory terms were selected and assumptions were checked using methods described previously.

To assess the competitive ability of *C. arvense*, a relative competition intensity (RCI; Weigelt & Jolliffe, 2003) was used to evaluate the impacts of competition between the *P. punctiformis* inoculated and non-inoculated (control) treatments was calculated as:

$$\text{RCI} = \frac{monoculture\_ mixed}{monoculture} \text{ x100}$$

Where "monoculture" was the aboveground biomass of *C. arvense* from the non-inoculated (control) monoculture treatment, and "mixed" was the aboveground biomass of the mixed pots for either the *P. punctiformis* inoculated or non-inoculated (control) treatment. $RCI^{control}$ was calculated using aboveground biomass from the control monoculture and mixed pots that were not inoculated with *P. punctiformis*. $RCI^{inoculated}$ was calculated using aboveground biomass from the non-inoculated (control) monoculture and the aboveground biomass from the mixed pots in the *P. punctiformis* inoculated treatment. An RCI value ≤ 0 indicates that *C. arvense* grown in mixed pots produced as much or more aboveground biomass compared to *C. arvense* grown in a monoculture. In contrast, RCI > 0 indicates that aboveground biomass of *C. arvense* was reduced when grown in mixed pots, and RCI = 100 indicates that no aboveground *C. arvense* biomass was produced in the mixed treatment.

The relationship between $RCI^{control}$ and $RCI^{inoculated}$ was evaluated using a linear mixed effects model, with fixed effects of inoculation treatment and crop phase, and pot ID included as a random effect. Model selection was completed by comparing all potential models with an Extra Sums of Squares F-Test. All model assumptions were visually assessed.

Results

Puccinia punctiformis Establishment

The overall frequency of *P. punctiformis* inoculated pots with systemically infected *C. arvense* stems over the three trials was 52% with no infection observed in the non-inoculated control treatment. Systemically infected *C. arvense* stems were observed in 15% of pots in the fallow phase, 65% of pots in the wheat phase, 60% of pots in the pea phase, and 67% of pots in the safflower phase ($F = 14.159$; $p < 0.001$; Figure 1 (A)). The percentage of *P. punctiformis* infected stems in the inoculated treatment, out of all *C. arvense* stems produced per pot, increased as the crop sequence progressed, with the largest increase occurring after the fallow phase ($F = 8.58$; $p < 0.001$). The overall mean percentage of *P. punctiformis* infected stems per pot was 12%. Out of all stems produced per pot, 4% were systemically infected in the fallow phase, 14% were systemically infected in the wheat phase, 16% were systemically infected in the pea phase, and 14% were systemically infected in the safflower phase (Figure 1 (B)).

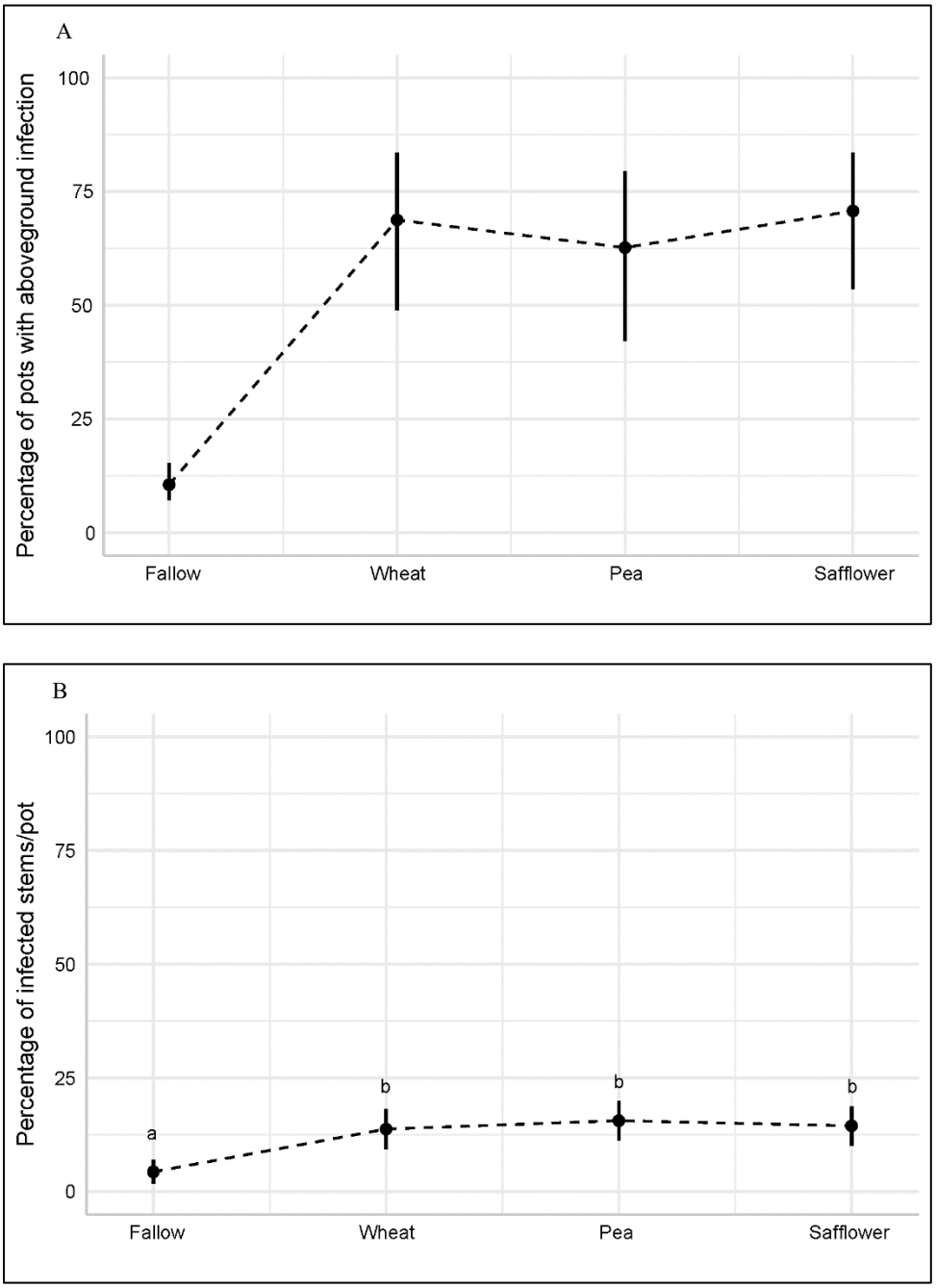

Figure 1: (A**)** Percentage of greenhouse pots with signs of systemically infected *C. arvense* stems throughout the simulated crop sequence in the *P. punctiformis* inoculated treatment. (B) Percentage of systemically infected stems, out of the total *C. arvense* stems produced per pot, in the *P. punctiformis* inoculated treatment throughout the simulated crop sequence.

Cirsium arvense Above-and Belowground Biomass

Cirsium arvense that was inoculated with *P. punctiformis* had (±SE) 1.6 (± 0.52) grams/pot less aboveground biomass compared to non-inoculated (control) *C. arvense* ($F = 9.965$; $p = 0.0020$). *Cirsium arvense* grown with crop competition produced (±SE) 3.1 ± 0.52 grams/pot less aboveground biomass than *C. arvense* grown in monoculture ($F = 36.396$; $p < 0.001$). *Cirsium arvense* biomass in the integrated *P. punctiformis* inoculated and crop competition treatment was (±SE) 4.8 ± 0.74 grams/pot less than *C. arvense* biomass in the monoculture, non-inoculated treatment ($t = 6.506$; $p < 0.001$; Figure 2; Table 1).

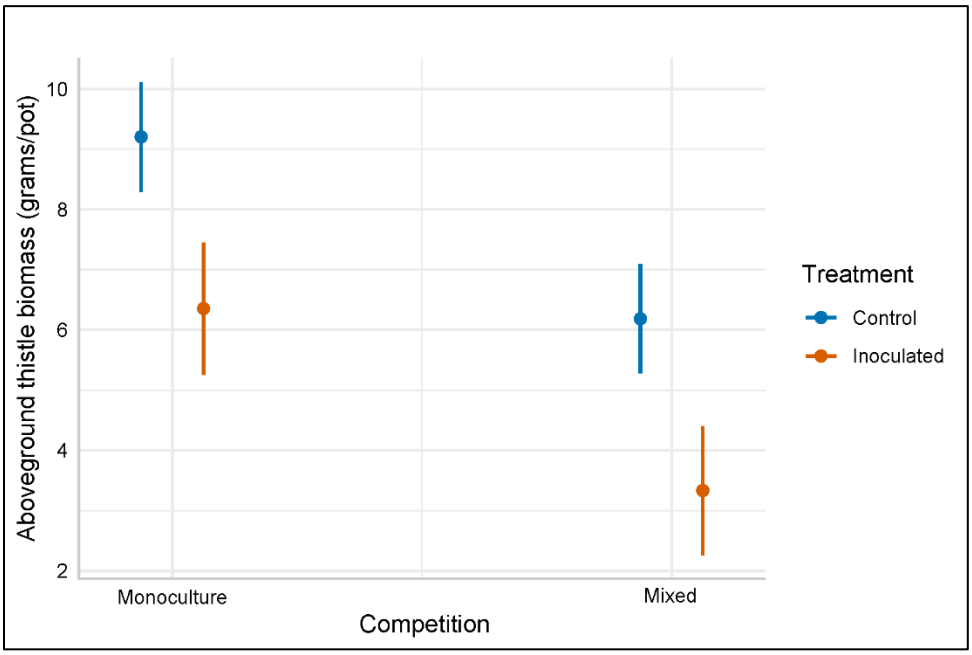

Figure 2: Predicted aboveground *C. arvense* biomass (grams/pot) between the inoculated and non-inoculated (control). Inoculated and non-inoculated (control) *C. arvense* was either grown in a monoculture or grown with interspecific competition where *C. arvense* was mixed with a sequence of annual crops.

C. arvense rhizome biomass was 6.9 grams/pot in the *P. punctiformis* inoculated treatment and 12.5 grams/pot in the non-inoculated (control) treatment, after an average of 12.9 months of growth. Rhizome biomass in the *P. punctiformis* inoculated treatment was less than rhizome biomass in the non-inoculated (control) treatment ($F = 25.791$; $p < 0.001$). The estimated biomass of *C. arvense* rhizome in the inoculated treatment was (±SE) 5.6 ± 1.1 grams/pot less than in the control treatment. *Cirsium arvense* grown with crop competition produced (±SE) 2.7 ± 1.1 grams/pot less rhizome biomass than *C. arvense* grown in monoculture ($F = 6.211$; $p\text{-value} = 0.0141$). Rhizome biomass in the integrated *P. punctiformis* inoculated and crop competition treatment was (±SE) 8.3 ± 1.6 grams/pot less than rhizome biomass in the monoculture, non-inoculated (control) treatment ($t = 5.353$; $p < 0.0001$; Figure 3; Table 2).

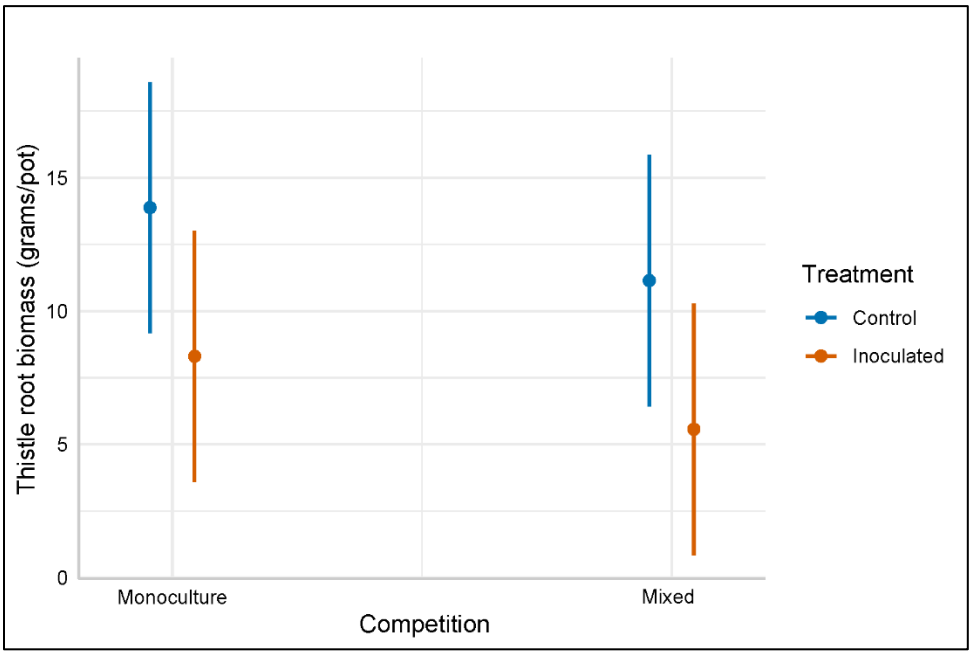

Figure 3: Predicted belowground *C. arvense* biomass (grams/pot) between the inoculated and non-inoculated (control). Inoculated and non-inoculated (control) *C. arvense* was either grown in a monoculture or grown with interspecific competition where *C. arvense* was mixed with a sequence of annual crops.

Puccinia punctiformis Impact on *Cirsium arvense* Competition

Crop competition reduced aboveground biomass, with (±SE) 49.2% ± 5.9 biomass loss in the inoculated treatment, and (±SE) 39.2% ± 5.9 biomass loss in the non-inoculated (control) treatment, when compared against the monoculture index for growth in the non-inoculated (control) treatment. There was some evidence for a difference in RCI between the inoculated treatment and the non-inoculated (control) ($F = 2.816$, $p\text{-value} = 0.0987$). The relative competition of *C. arvense* varied between crop phases (wheat, pea, and safflower) in both the inoculated and control treatments ($F = 63.669$; $p < 0.001$). Crop competition reduced aboveground biomass by (±SE) 48% ± 5.9 in the wheat phase, (±SE) 71% ± 5.9 in the pea phase, and (±SE) 14% ± 5.9 in the safflower phase, when compared against the monoculture index for growth in the non-inoculated (control) treatment. Additionally, there was an interaction between the inoculation treatments and crop phases ($F = 3.329$; $p = 0.0393$). The RCI between the inoculation treatments increasingly separated as the crop sequence progressed, where the inoculated treatment lost (±SE) 24% ± 8.3 more biomass than the non-inoculated (control) treatment by the final safflower phase in the crop sequence (Figure 4; Table 3).

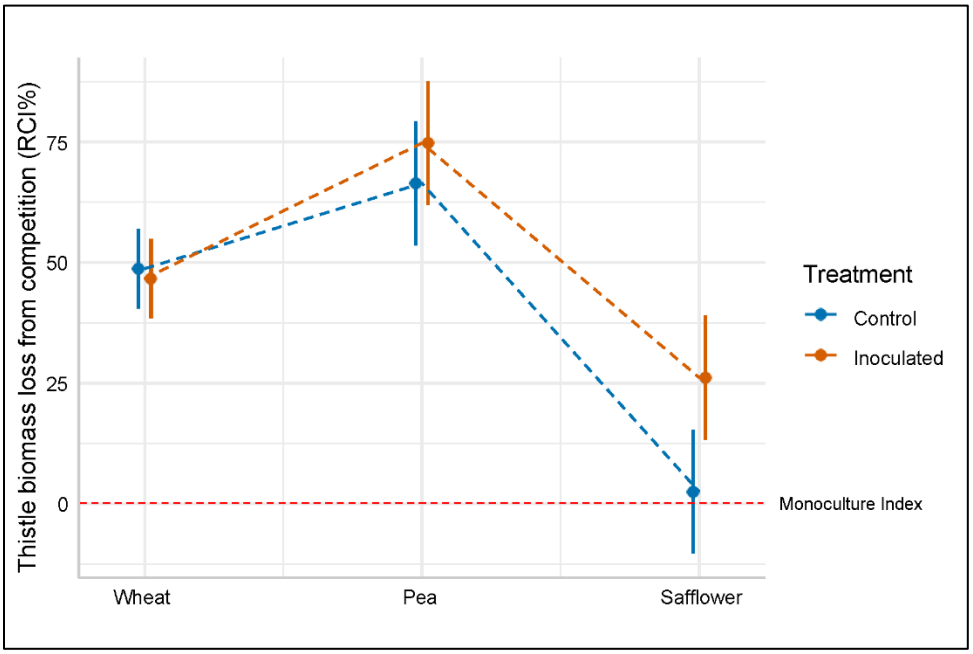

Figure 4: The relationship in aboveground *C. arvense* biomass loss in competition (RCI%) between the *P. punctiformis* inoculated and non-inoculated (control) treatments for the three crop phases for all three trials. There was no difference in RCI between the treatments or the crop phases. An RCI value ≤ 0 indicates that *C. arvense* grown in mixed pots produced as much or more aboveground biomass compared to *C. arvense* grown in a monoculture. In contrast, RCI > 0 indicates that aboveground biomass of *C. arvense* was reduced when grown in mixed pots, and RCI = 100 indicates that no aboveground *C. arvense* biomass was produced in the mixed treatment.

Discussion

Sustainable *C. arvense* management in organic cropping systems is a primary challenge in temperate regions around the globe. Integrated weed management strategies are needed to reduce the abundance, slow the spread, and minimize the impact of *C. arvense* in cropping systems over a long term (Liebman et al., 2001; Liebman et al., 2009; Davis et al., 2018; Orloff et al., 2018). In this study we found that the integration of *P. punctiformis* and crop competition interacted to impact *C. arvense* biomass and competitive ability. Integrated weed management of *C. arvense*

that combines the *P. punctiformis* biocontrol with crop competition can reduce *C. arvense* vigor, but requires careful consideration for effective use within complex cropping systems.

Repeated inoculations of *C. arvense* rosettes with *P. punctiformis* yielded systemically infected *C. arvense* stems in all phases of the crop sequence. Inoculation of rosettes resulted in few systemically infected *C. arvense* stems in the first phase (3-4 months of growth) of the crop sequence, but incidence of infection increased over time. The slow development of systemically infected stems is consistent with the general development of plant pathogens, which often require an incubation period before infected plants develop symptoms (Agrios, 2005). Our findings are consistent with literature that suggests that *P. punctiformis* mostly resides asymptomatically within *C. arvense* rhizomes (Bailiss and Wilson, 1967), especially during the initial stages of infection. In a study testing asymptomatic *C. arvense* rosettes in proximity to *P. punctiformis* inoculations, Berner et al. (2015) discovered that up to 60% of asymptomatic rosettes were positive hosts for *P. punctiformis*. Therefore, the success of our inoculations was likely greater than what was observed aboveground.

While systemically infected stems were observed in most inoculated greenhouse pots, the majority of stems produced were asymptomatic. This supports the conclusion that *P. punctiformis* is primarily a root pathogen (Berner et al., 2015; Kentjens et al., 2023) that remains latent until adequate resources are gathered from the host and environmental conditions are suitable for the emergence of spore bearing *C. arvense* stems (Mendgen and Hahn, 2002). The stabilization of infected *C. arvense* stems after the fallow phase reflects the host's capacity to support *P. punctiformis,* given the limitations of plant growth in greenhouse pots. Berner et al. (2015) and Watson and Koegh (1980) suggested that the robustness of infected *C. arvense* can be

a factor that influences the development of systemically infected *C. arvense* stems, where a robust host of *P. punctiformis* is more likely to produce a relatively high abundance of infected stems, and systemic infection in a weaker host could produce fewer infected stems. It was concluded that systemic infection in a less robust host remains mostly asymptomatic and caused death more quickly than systemic infection in a robust host.

Cirsium arvense that was inoculated with the *P. punctiformis* biocontrol produced less belowground biomass compared to *C. arvense* that was not inoculated. Our results agree with the findings of Thomas et al.'s (1994) greenhouse experiment, where *P. punctiformis* inoculated *C. arvense* produced less root biomass than non-inoculated *C. arvense*. A weakened root system can directly impact aboveground biomass production, where root resources that would otherwise promote stem growth, are instead allocated to costly defense compounds, or become parasitized by *P. punctiformis* (Herms & Mattson, 1992; Thomas et al., 1993; Monson et al., 2021). This was demonstrated in our findings, where *P. punctiformis* inoculations yielded less aboveground biomass compared to *C. arvense* that was not inoculated, confirming that *P. punctiformis* inoculations can effectively impact the overall growth of *C. arvense.*

Competition with annual crops affected *C. arvense* aboveground growth, although the effects differed between crop species. Unexpectedly, peas were the most competitive annual crop species in the sequence, despite their relatively slow germination, shallow rooting depth, and open canopy (McKay et al., 2003). It is possible that wheat, a moderately competitive cereal species (Mason and Spaner, 2006), had a lasting impact on *C. arvense* vigor that wasn't evident until the following pea phase. The weak competitive qualities of peas may have facilitated a recovery in *C. arvense* vigor, becoming evident in the following phase, where safflower had the

lowest relative competition intensity. However, safflower, known to be a weak competitor in the early stages of growth (Emonger and Oagile, 2017), was disadvantaged as the last crop in the sequence. It is possible that greenhouse pots with fully developed roots gave *C. arvense* a strong competitive advantage by the final phase of the crop sequence. Seeding safflower directly into a dense and confined *C. arvense* root network likely impacted optimal safflower development.

When inoculated *C. arvense* was grown in mixed pots with interspecific crop competition, the biocontrol interacted additively with crop competition to further reduce above-and belowground biomass, more than individual impacts from the biocontrol or crop competition alone. Although *C. arvense* was never eradicated by the combination of *P. punctiformis* and crop competition, there was an interaction between the crop phases and the inoculation treatments, where the difference between the *P. punctiformis* inoculated and the non-inoculated (control) relative competition intensities gradually increased as the crop sequence progressed. As *P. punctiformis* inoculations did not immediately affect *C. arvense's* competitive ability, but increased through time, the effects appear to be associated with the establishment of infected *C. arvense* stems. The greatest impact on *C. arvense* competition emerged after aboveground disease incidence stabilized and persisted through time. There is potential to accelerate disease establishment and increase the severity of *P. punctifomris* infection by simulating an herbivory response with foliar applications of jasmonic acid, as discovered by Clark et al. (2020), thus enhancing future integrations of the biocontrol. Overall, these results support our hypothesis and provide evidence in favor of integrated weed management as an effective strategy for *C. arvense* control (Demers et al., 2006; Liebman and Davis, 2009; Sciegienka et al., 2011; Davis et al., 2018; Orloff et al. 2018).

While crop competition is already a common integrated weed management practice (Pavlychenko & Harrington, 1934; Bullock, 1992; Liebman and Dyck, 1993; Liebman and Davis, 2009), there remain practical challenges to the integration of the *P. punctiformis* biocontrol in field settings. Inoculum sourcing and mass production is limited by the inability to culture transmissible teliospores (Kentjens et al., 2023), creating a reliance on the harvest of teliospore bearing *C. arvense*. Limitations in inoculum ultimately reduce the scalability of the biocontrol under current sourcing methods. Most transmissions of *P. punctiformis* are limited to 12 meters from the source plant, with no transmissions occurring beyond 17 meters (Berner et al. 2015). Insect vectors or mowing have shown potential to transmit *P. punctiformis* and increase infection levels across fields (Demers et al., 2006; Wandeler and Bacher, 2006), however, careful cropping system management is required to facilitate effective spore distributions. The greenhouse environment simplifies biocontrol manipulations, however, successful integration of *P. punctiformis* in a field setting could also be dependent on variable environmental conditions and cropping system management that can influence survivability and germination of the biocontrol (French and Lightfield, 1990; Berner et al., 2013; Kentjens et al., 2023). Additionally, Thomas et al. (1994) found that P. punctiformis inoculations did not impact aboveground biomass production compared to non-inoculated *C. arvense*, suggesting inconsistent performance of the pathogen. Inconsistencies in the biocontrol's impact on *C. arvense* aboveground growth may be an indication of genetic variability within the host and pathogen populations, where disease severity can be determined by a range of resistance mechanisms in *C. arvense* or virulence factors in *P. punctiformis*. Regardless of inconsistent findings, it is evident that the *P.*

punctiformis has the potential to affect *C. arvense* biomass production and competitive ability, ultimately increasing *C. arvense's* vulnerability to integrated weed management tactics.

Conclusion

The fungal biocontrol, *P. punctiformis* can be successfully integrated with crop competition as a *C. arvense* management tool. In this greenhouse study, inoculation of *C. arvense* rosettes with *P. punctiformis* teliospores caused an increase of symptomatically infected *C. arvense* stems over time, impacting above- and belowground *C. arvense* biomass production. Furthermore, *P. punctiformis* intensified the effects of crop competition when the biocontrol was integrated into a simulated crop sequence. While the use of *P. punctiformis* is possible in a greenhouse, successful integration of the biocontrol into a field setting will be dependent on a combination of environmental factors and deliberate cropping system management. *Puccinia punctiformis* is not a singular management solution for *C. arvense*, however it has strong potential to be integrated as a low-cost, low-input, and long-term biocontrol agent that can improve sustainable management of *C. arvense.*

CHAPTER THREE

INTEGRATION OF *PUCCINIA PUNCTIFORMIS* INTO MECHANICAL MANAGEMENT FOR *CIRSIUM ARVENSE*

Introduction

Management of perennial weeds is a primary challenge in organic cropping systems, due to characteristics that make them resistant to common management techniques, and their competitive advantage over many annual crop species (Mohler et al., 2001; Tautges et al., 2016). *Cirsium arvense (L.) Scop.* (Canada, California, or creeping thistle) has been identified as one of the most problematic perennial weeds in temperate organic cropping systems, where it has potential to cause large economic losses (Tautges et al., 2016; Orloff et al., 2018). *Cirsium arvense* can be found throughout temperate climates of the world, where it produces extensive underground rhizomes and wind dispersed seeds (Tiley, 2010). Clonal rhizomes make *C. arvense* resilient to mechanical disturbance, particularly in organic cropping systems that rely on tillage as the primary tool for weed management (Moore, 1975; Lenhoff et al., 2017). Tillage is generally ineffective as a *C. arvense* management tool, where infrequent tillage can disperse rhizomes that have the ability to produce new shoots, causing a rapid increase in the weed's population (Blackshaw 2001; Tiley, 2010). In contrast, high frequency tillage can effectively deplete *C. arvense's* energy reserves (Mohler, 2001b), but at the risk of negatively impacting soil properties (Hakansson, 2003; Tiley, 2010; Lenhoff et al., 2017). *Cirsium arvense's* resilience to organic management has highlighted a need for improved *C. arvense* management tactics that can be effectively integrated into organic cropping systems.

The semi-arid Northern Great Plains region of North America is especially sensitive to tillage, where frequent soil disturbances can cause wind and water erosion, reductions in soil organic matter, and increasing moisture loss through evaporation (Triplett and Dick, 2008; Lenhoff et al., 2017). To reduce the reliance on frequent tillage as a weed management tool, organic producers in the region attempt to integrate cultural, mechanical, and biological tools. In this context, using competitive and diversified crop rotations, that are combined with reduced mechanical tillage practices, can be an effective way to conserve water, enhance soil quality, and improve management of weeds within organically managed cropping systems (Lenhoff et al., 2017). However, perennial rhizomatous weeds, including *C. arvense*, remain a primary management challenge. Management of *C. arvense* could be improved by integration of biocontrol agents that inhibit weed growth and subsequently reduce crop yield losses (Cripps et al., 2011; Berner et al.; 2013; Chichinsky et al., 2023). Biocontrol agents that are specific to *C. arvense* may benefit organic cropping systems as low-cost, long-term, host-specific options that can be integrated into existing weed management toolsets (Berner et al., 2013).

Puccinia punctiformis (F. Strauss) Rohl. (thistle rust) is a heterotrophic fungal pathogen of *C. arvens*e that acts as a long-term systemic parasite (Buller, 1950; Menzies, 1953; Berner et al.,2013; Kentjens et al., 2023). *Puccinia punctiformis* is specific to *C. arvense* and has been identified in habitats around the globe (Berner et al., 2013; Kentjens et al., 2023). This pathogen primarily exists as a root parasite where the fungus consumes resources and weakens the root structure (Buller, 1950; Menzies, 1953; Berner et al. 2013). Once established in the roots, infected *C. arvense* can develop chlorotic leaf tissue with lesions, elongated stems, and growth irregularities which can reduce fitness and cause death (Buller, 1950; Berner et al., 2013).

Diseased stems act as above-ground carriers for *P. punctiformis* spores, appearing as orange to dark-red pustules on leaves. The fungus completes the majority of its five-stage heterothallic life cycle on *C. arvense* stems during summer months, eventually producing transmissible spores called teliospores (Buller, 1950; Menzies, 1953). Teliospore-bearing leaves senesce and abscise in the fall, where they can contact healthy *C. arvense* rosettes through wind or mechanical dispersion, leading to long-term systemic infection in new hosts under ideal environmental conditions (French and Lightfield, 1990; Berner et al., 2013).

In rangeland and non-cropping systems, *P. punctiformis*'s impact on *C. arvense* abundance has been well documented (French et al., 1990; Thomas et al., 1994; Berner et al., 2013; Cripps et al., 2011). While *P. punctiformis* has shown potential as a biocontrol agent in cropping systems (Chichinsky et al., 2023), the effects of integrating *P. punctiformis* into agricultural tillage practices have not been evaluated. This knowledge gap was addressed in a three-year agricultural field experiment which assessed the relationship between tillage, and the abundance of asymptomatic *C. arvense* stems and *P. punctiformis* infected stems. Our main research questions were: 1) Do standard tillage practices and reduced tillage practices affect *P. punctiformis* infected stem density and growth rate through time, and 2) Do these tillage practices interact with *P. punctiformis* infection to impact *C. arvense* stem density and cover through time? We hypothesized that reduced tillage practices would promote a larger *P. punctiformis* infected stem density compared to standard tillage practices, and that reduced tillage would interact with *P. punctiformis* to reduce *C. arvense* density and cover over time, more than standard tillage.

Methods

Experimental Design

The study took place at the Montana State University Ft. Ellis Research Farm from 2020 to 2022 (LatLon: 45.667137, -110.977948). Fort Ellis is approximately 6-km east of Bozeman, Montana where the mean annual precipitation during the study period was 530-mm and the mean annual temperature was 6°C (PRISM Climate Group, 2022; Supplementary Figure 1; Supplementary Table 1). The site is not irrigated, and has a 1.5-m deep Blackmore silt loam soil profile (USDA NRCS, 2019). Experimental plots at Ft. Ellis were 5.5-m wide and approximately 15.2-m long (Supplementary Figure 2). At the beginning of the study, each plot contained three to four discrete *C. arvense* patches of varying sizes and with varying degrees of naturally established *C. arvense* and *P. punctiformis* infected stems. Patches were considered discrete when there was at least 2 meters of distance between patch boundaries, where no *C. arvense* stems could be observed. The plots at Ft. Ellis were USDA organically certified in 2015, where they hosted reduced-till livestock grazing studies until 2017 (Lehnhoff et al., 2017; Larson et al., 2021). The plots remained fallow in 2018, followed by one year of a small-plot crop rotation and tillage study in 2019. All plots were uniformly cultivated with a chisel plow in the fall of 2019.

A randomized complete block design was used to evaluate the effects of tillage on *C. arvense* and *P. punctiformis* over three growing seasons. There were two levels of tillage that were randomly assigned to plots and replicated four times each: standard tillage and reduced tillage (Supplementary Figure 2). The standard tillage treatment level was mechanically disturbed with a tandem disc harrow (25-cm depth) and with a chisel plow (25-cm depth). The

reduced tillage treatment level was mechanically disturbed with a flail mower, with grazing sheep (10 individuals), and with light surface disturbance using a chisel plow (1.5-cm depth). Tillage treatments were performed one to two times per growing season (Supplementary Figure 3). To help reduce weed pressure, all plots were uniformly drill-seeded into a three-year green manure sequence that included: 1) 90-kgs/hectare of a spring barley in 2020; 2) 91- kgs/hectare of winter pea intercropped with 84- kgs/hectare winter triticale in 2021; and 3) 90- kgs/hectare of spring wheat in 2022.

The 2020 crop was seeded in early April, and terminated with assigned tillage treatments prior to grain maturity in early July. The plots remained in fallow until mid-September, when the assigned tillage treatments were repeated to prepare seedbeds for the winter crop, which was immediately seeded after seedbed preparation. In July 2021, the crop was terminated using the respective tillage treatments. Plots remained in fallow for the rest of the year, with one additional tillage treatment in October, 2021. Seeding in 2022 was delayed until late May, due to heavy rains that limited access to experimental plots (Supplementary Figure 1). Prior to seeding in the spring of 2022, the plots were prepared with assigned tillage treatments. Crops were terminated in late August 2022 with respective tillage treatments (Supplementary Figure 3).

Data Collection

All data were collected within one week prior to crop termination during each study year, which was approximately timed with the observable development of *P. punctiformis* teliospores on infected *C. arvense* stems. *Cirsium arvense* patch boundaries were mapped as polygons with an Emlid Reach RS2+ GNSS receiver. Mapping was completed on the WGS84 geographic coordinate system (Brunner, 1998), and was post-processed in ArcGIS Pro using corrections

from the Montana State University GPS base station. GPS polygon data from 2020 was used to relocate discrete patches throughout the study.

To determine the effects of tillage on *P. punctiformis* infected stem density and the growth rate of infected stems through time, a total count of *C. arvense* stems with signs of *P. punctiformis* infection were collected from within each discrete patch, during each year of the study. To evaluate the interaction between *C. arvense* and *P. punctiformis* infection within tillage treatments, stem counts and cover estimates were collected by randomly hand throwing 1-m^2 quadrats within each discrete patch. The number of quadrats per patch was scaled based on patch size, to account for spatial variability of discrete *C. arvense* patches within plots. Sample sizes ranged from three quadrats in patches with a small spatial area to nine quadrats in patches with a large spatial area. Within each quadrat, stem counts were taken from asymptomatic and symptomatic *C. arvense*, along with visual estimates of ground cover that included: *C. arvense* cover, crop cover, and the combination of non-target weed and organic litter cover.

Data Analysis

To assess the effects of tillage on *P. punctiformis* infected stem density over three years, the total count of *C. arvense* stems with signs of *P. punctiformis* infection per patch was modelled using a negative binomial mixed-effects model ("glmer.nb" function in the R-Package "MASS"; Venables et al., 2023; Stoklosa et al., 2022). The fixed effects were tillage treatment, year, and the interaction between tillage and year. To account for repeated observations within the same *C. arvense* patch and replication, a unique patch identity was included as a random effect. The estimated marginal means and standard errors were calculated for the interaction between tillage treatment and year using the "emmeans" function in R-Package "emmeans"

(Lenth et al., 2023). Model selection was completed by comparing all potential models with a χ^2 drop in deviance test. Assumptions of homoscedasticity, normality, and influential observations were visually assessed (Ramsey and Schafer, 2012).

The relative growth rate (RGR) *P. punctiformis* infected stem density over three years was evaluated between tillage treatments using a linear model ("lm" function in R; Ramsey and Schafer, 2012), using the total count of infected stems per patch. The relative growth rate was calculated by subtracting the natural log of infected stem density per patch in 2022 with the natural log of infected stem density per patch in 2020, and dividing by the difference in time between 2022 and 2020 (Hoffman and Porter, 2002; Gurevitch et al., 2016). All assumptions were assessed using the methods previously described.

Ground cover data from the 1-m^2 quadrats was aggregated into mean cover estimates for each discrete patch for each year of the study ("aggregate" function in RStudio). Mean estimates of *C. arvense* ground cover (% cover/ m^2) were compared with tillage treatments and estimates of *P. punctiformis* infected stem ground cover (% cover/ m^2). *Cirsium arvense* cover was modeled with a linear mixed effects model ("lmer" function in the R-Package "lmerTest"; Kutznetsova et al., 2017) using fixed effects of year, *P. punctiformis* infected stem cover, crop cover, and a combination of non-target weed and litter cover. The random effect was patch ID. The best fit model was selected using the Akaike Information Criterion (AIC; Gurevitch et al., 2016) and assumptions were checked using methods previously described.

Additionally, *C. arvense* stem density data from the 1-m^2 quadrats was separated between quadrats where *P. punctiformis* infection was present vs. absent, then aggregated into mean *C. arvense* density estimates for each discrete patch for each year of the study. The change in *C.*

arvense stem density (stems/m^2) over time was evaluated, using a negative binomial mixed-effects model, between tillage treatments and between observations where *P. punctiformis* infection was present vs. absent. The fixed effects were tillage treatments, presence or absence of *P. punctiformis* infection, year, and the interaction between *P. punctiformis* infection and year. Pairwise contrasts between quadrats where *P. punctiformis* infected stems were present or absent, tillage treatments, and year were calculated using estimated marginal means. The random effect was patch ID. The model selection and assumptions were checked using methods previously described.

Results

Puccinia punctiformis Infected Stem Density and Growth Rate

There was no evidence for a difference in *P. punctiformis* infected stem density (stems/patch) between tillage treatments alone ($p = 0.661$), however infected stem density was different between study years ($p = 0.001$), with an interaction between tillage treatments and years (ANOVA $\chi^2 = 10.14, p = 0.006$). Infected stem density did not change through time in the standard tillage treatment ($z\text{-ratio} = -0.475, p = 0.883$); but it increased over three years in the reduced tillage treatment ($z\text{- ratio} = -4.408, p\text{- value} < 0.001$). During 2020 and 2021, there was no difference in infected stem density between tillage treatments (2020: $z\text{-ratio} = 0.393, p\text{-value} = 0.694$; 2021: $z\text{-ratio} = 0.745, p\text{-value} = 0.457$). The greatest change occurred in 2022 ($z\text{-ratio} = -2.181, p\text{-value} = 0.029$), when the estimated means for infected stem density decreased to (±SE) 2.8 ± 1.62 infected stems/patch in standard tillage treatment and increased to (±SE) 14.5 ± 7.13 infected stems/patch in the reduced tillage treatment (Figure 1; Table 1).

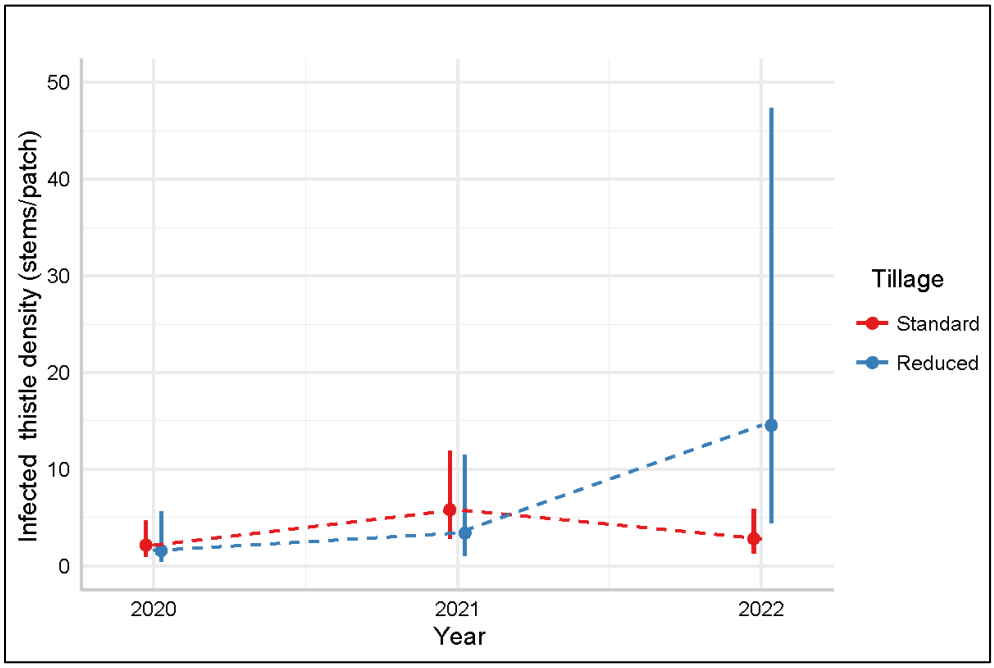

Figure 1: Predicted density (stems/patch) of *Cirsium arvense* (thistle) stems with signs of *Puccinia punctiformis* (thistle rust) infection between standard tillage and reduced tillage treatments between 2020 and 2022. The results come from a generalized linear mixed effect, negative binomial model.

The mean relative growth rates of *P. punctiformis* infected stems differed between tillage treatments, where the growth rate was highest in the reduced tillage treatment ($F = 5.65$, $p = 0.026$). The estimated mean relative growth rates of *P. punctiformis* infected stems was (±SE) 0.04 ± 0.36 infected stems/year in the standard tillage treatment and (±SE) 1.20 ± 0.33 infected stems/year in the reduced tillage treatment. The relative growth in the standard tillage treatment did not differ from zero ($p = 0.920$). The relative growth rate in the reduced tillage treatment was greater than zero ($p = 0.001$), indicating a positive growth rate of *P. punctiformis* infected stems.

Impact of *Puccinia punctiformis* on *Cirsium arvense* Stem Density and Cover

There was no difference in *C. arvense* ground cover between the standard and reduced tillage treatments over all three study years (ANOVA $\chi^2 = 0.117, p = 0.732$) where the estimated mean cover was (±SE) 37.3 ± 1.35% in the standard tillage treatment and (±SE) 35.4 ± 1.24% in the reduced tillage treatment over the three study years. The estimated mean *C. arvense* ground cover was (±SE) 13.9 ± 1.55% in 2020, (±SE) 45.5 ± 1.23% in 2021, and (±SE) 49.6 ± 1.52% in 2022, with the lowest cover observed in 2020 (*p-value* < *0.001*). After accounting for crop cover and the combination of non-target weeds and litter cover in the model, *C. arvense* cover was not impacted as the cover of *P. punctiformis* infected stems increased (ANOVA $\chi^2 = 0.112, p = 0.7376$). *Cirsium arvense* cover decreased by 0.7% on average, for every 1.0% increase in *P. punctiformis* infected stem cover (*t* = *-0.335*; Figure 2; Table 1).

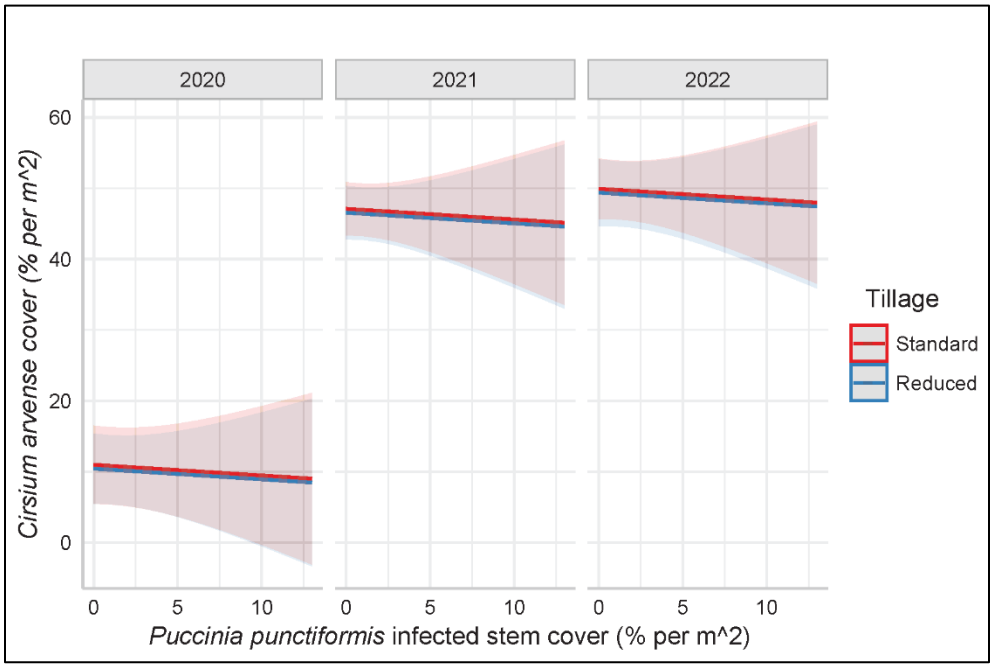

Figure 2: Predicted ground cover of *Cirsium arvense* in response to the ground cover of *Puccinia punctiformis* infected stems and tillage treatments from 2020 to 2022 at the Ft. Ellis Research Farm in Bozeman, Montana.

Table 1: ANOVA results for the *Cirsium arvense* ground cover (% cover/m^2) response to tillage treatments (standard, reduced), years (2020-2022), *Puccinia punctiformis* infected ground cover (% cover/m^2), crop ground cover (% cover/m^e), and non-target weeds + litter ground cover (% cover/m^2).

	df	**χ^2**	**p-value**
Tillage Treatment	1	0.117	0.7320
Year	2	119.031	<0.001
***P. punctiformis* Infected Cover**	1	0.112	0.7376
Crop Cover	1	48.404	<0.001
Non-Target Weeds + Litter Cover	1	178.309	<0.001

Cirsium arvense stem density was lowest in 1-m^2 quadrats that had *P. punctiformis* infected stems present, compared to quadrats where all *C. arvense* stems were asymptomatic (ANOVA $\chi^2 = 2.546$, $p = 0.1106$). However, there was no impact to *C. arvense* stem density from either tillage treatment (ANOVA $\chi^2 = 0.463$, $p = 0.4961$). There was an interaction

between the two categories of *P. punctiformis* infection and years (ANOVA $\chi^2 = 12.879$, $p = 0.0016$), where *C. arvense* density decreased from 2020 to 2022 when infected *C. arvense* stems were present (*z-ratio = 2.834, p-value = 0.0128*) and increased from 2020 to 2022 when all *C. arvense* stems were asymptomatic (*z-ratio = -2.128, p-value = 0.0842*; Figure 3; Table 2).

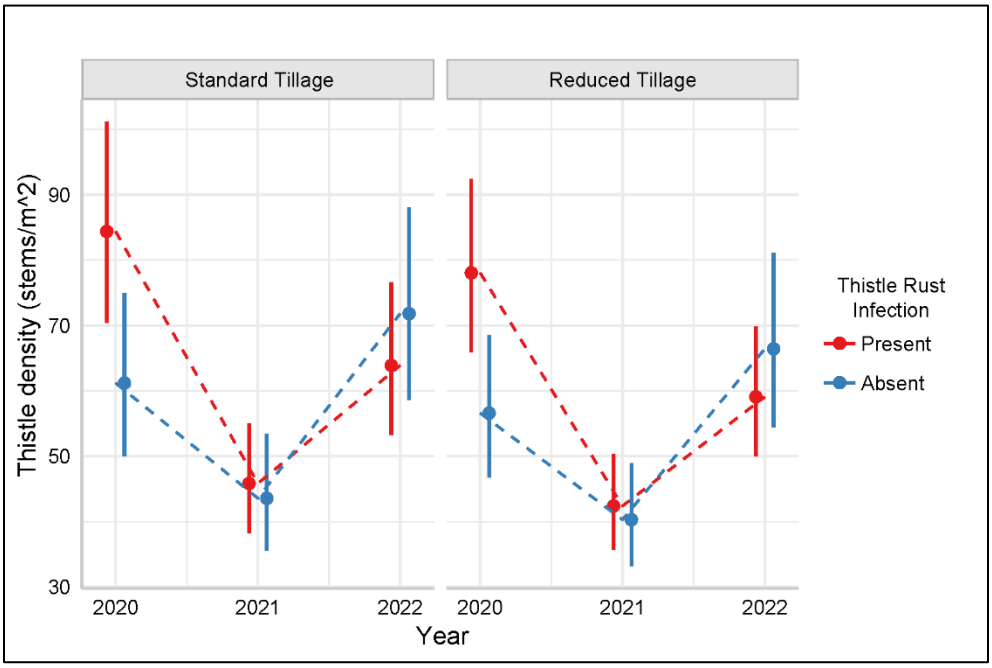

Figure 3: Predicted asymptomatic *Cirsium arvense* (thistle) stem density (stems/m^2) from 1-m^2 observations where *Puccinia punctiformis* (thistle rust) infected stems were either present or absent, between standard tillage (disc cultivation) and reduced tillage (mowing) treatments over three years.

Table 2: Pairwise contrasts of the estimated marginal means for *Cirsium arvense* stem density between observations where *Puccinia punctiformis* infected stems were either present or absent, the first and final years of the study, and tillage treatments. *Cirsium arvense* density was back transformed from the log scale, but all tests were performed on the log scale.

Tillage Treatment	***P. punctiformis* (Present/Absent)**	**2020 Mean *C. arvense* stem density/m^2**	**2022 Mean *C. arvense* stem density/m^2**	**Pairwise Contrast: *C. arvense* density by year**	**Ratio**	**SE**	**z-ratio**	**p-value**
Standard	Absent	61.2	71.8	2020 / 2022	0.852	0.0641	-2.128	0.0842
Standard	Present	84.4	63.9	2020 / 2022	1.321	0.1297	2.843	0.0128
Reduced	Absent	56.6	66.4	2020 / 2022	0.852	0.0641	-2.128	0.0842
Reduced	Present	78.0	59.1	2020 / 2022	1.321	0.1297	2.843	0.0128

Discussion

The development of integrated weed management toolsets is necessary for sustainable management of *C. arvense* (Davis et al., 2018; Orloff et al., 2018). Successful integration of *P. punctiformis* into organic systems has the potential to minimize *C. arvense's* impact on crop yield. In this study, we found that reduced tillage was associated with a higher density and a more rapid development of *P. punctiformis* infection compared to standard tillage. Also, lower *C. arvense* densities were associated with observations where the biocontrol was present. However, variable climate conditions that impacted tillage timing had a strong influenced the integration of *P. punctiformis* with tillage treatments.

The abundance of *P. punctiformis* infected stems per patch was not influenced by tillage during the first two years of this study, when tillage treatments occurred in the fall. However, there was a significant divergence between the tillage treatments after tillage occurred in the spring of the third year, where high spring moisture delayed seeding until late May. The surge in *P. punctiformis* infection in the third-year also influenced the growth rate of *P. punctiformis* infected stems, where incidence of infection increased at a higher rate in the reduced tillage

treatment compared to the standard tillage treatment. Reduced tillage systems, where mowing is used to manage *C. arvense,* may favor transmission of teliospores through mechanical dispersal, thus increasing the potential for *P. punctiformis* to infect new hosts (Bockus and Shroyer 1998; Peters et al., 2003; Demers et al. 2006). Our findings were consistent with previous studies, where reduced tillage with mowing increased densities of infected *C. arvense* (Demers et al. 2006), however, only when reduced tillage occurred in the spring. In contrast, standard tillage practices that invert soil A-horizons are generally utilized to manage soil- and residue-borne crop pathogens by displacing transmissible spores (Bockus and Shroyer, 1998; Peters et al., 2003). Additionally, root cutting from standard tillage practices could impact the pathogen's access to resources and the ability to move within the rhizomes (Berner et al., 2015), potentially causing premature death. Our findings align with general pathogen management, where standard tillage appeared to have no effects on *P. punctiformis* incidence or growth rate, with potential for a negative effect on *P. punctiformis* incidence when spring tillage was performed.

After three years, we observed no difference in *C. arvense* stem density or cover between reduced tillage and standard tillage treatments. However, over time, there was an interaction between quadrats where *P. punctiformis* infected stems were present and quadrats where *P. punctiformis* infection was absent. In the first year, quadrats where *P. punctiformis* infection was present had a higher *C. arvense* density than quadrats where *P. punctiformis* infection was absent. In general, areas of high host plant densities can be vulnerable to host-selective pathogens, where an abundance of healthy hosts can create optimal conditions for pathogen establishment and transmission (Burdon and Chilvers 1982). Yet over time, persistent infection can lead to a reduction in host vigor. For example, Berner et al. (2015) observed that *P.*

punctiformis infection caused a slow decline in *C. arvense* density over four years. Our *C. arvense* density results showed declining density in quadrats where *P. punctiformis* infection was present, while there was an increase in *C. arvense* density in quadrats where *P. punctiformis* was absent. However, the time allotted to this study was likely too short to capture the progressive impacts of *P. punctiformis*, assuming that long-term infection in *C. arvense* would cause greater reductions in host vigor. While the effects of standard and reduced tillage conditions on *C. arvense* density and cover were generally inconclusive, it was evident that pathogen pressure from *P. punctiformis* impacted the vigor of *C. arvense.*

The heavy precipitation in the spring of 2022 forced a management decision to till all plots late in the spring for weed management and seedbed preparation (Supplementary Figure 2). While un-replicated, the unexpected change in tillage timing, from fall tillage to spring tillage, highlighted the responsiveness of *P. punctiformis* to mechanical management in the spring. *Puccinia punctiformis* typically emerges on *C. arvense* stems during the spring, marking the beginning of the pathogen's sexual life-cycle and subsequent development of transmissible spores (Kentjens et al., 2023). Our results suggest that standard tillage in the spring, where infected *C. arvense* rhizomes are disturbed by cultivation, can limit or prevent the spring emergence of infected *C. arvense* stems. In contrast, the absence of *C. arvense* rhizome disturbance through reduced tillage in the spring, appeared to promote the emergence of infected *C. arvense* stems. The influence of tillage timing on *P. punctiformis* incidence suggests that increased infected stem densities can be influenced by the intensity of early season mechanical management, but requires further investigation in a study designed to evaluate the effects of tillage timing.

Overall, our results suggest that *P. punctiformis* integrated with reduced tillage practices, leads to increased disease incidence through time. While reduced tillage and standard tillage treatments did not have different impacts on *C. arvense* density or cover, we do conclude that *P. punctiformis* presence and increasing infection cover are associated with declines in *C. arvense* density and cover, respectively. Therefore, the potential for reduced tillage practices to increase disease incidence may, over time, cause greater declines in *C. arvense* density because of increasing pathogen pressure. The integration of *P. punctiformis* into tillage practices can be dependent on variable climates and logistical challenges of agricultural management, but incorporation of *P. punctiformis* with integrated weed management strategies can reduce *C. arvense* over time.

CHAPTER FOUR

SUMMARY OF FINDINGS/FUTURE RESEARCH

Organic management of *C. arvense* requires a systematic integration of management tools, whose complementary qualities reduce the weed's ability to establish, gather resources, grow, and reproduce. The fungal pathogen, *P. punctiformis,* has been identified as an effective biocontrol for *C. arvense* in relatively undisturbed environments, yet the efficacy of the pathogen in agricultural systems has remained unclear. The main purpose of this study to was to evaluate the impact of *P. punctiformis's* on *C. arvense* in agricultural systems, and it's potential to be integrated with cultural and mechanical tools. When integrated with cultural management, the combination of *P. punctiformis* and competitive annual crops interacted to reduce *C. arvense* stem and root biomass, more than individual use of either tactic. Additionally, the tested competitive crop sequence had a greater relative competition intensity when *C. arvense* was inoculated with the biocontrol vs. the non-inoculated (control). When the biocontrol was integrated with mechanical management, reduced tillage resulted in a greater abundance of *P. punctiformis* infection over time, when compared to standard tillage practices. Although reduced and standard tillage practices did not have different impacts on *C. arvense* abundance, the presence of *P. punctiformis* was associated with lower *C. arvense* stem densities.

Our findings suggest that *P. punctiformis* can effectively impact *C. arvense* when integrated with cultural management, but may be most effective in reduced tillage systems where disturbance is minimal. There is potential to build upon this research by exploring the interaction of *P. punctiformis* with competitive crop sequences that include multiple years of a perennial forage. The use of perennial forages for *C. arvense* management is advantageous because they

can produce competitive root systems and dense canopies, they can aide soil fertility, and can be harvested multiple times per year with minimal soil disturbance, resulting in a reduction of *C. arvense* carbon stores and a suppression of seed production when mowed (Hodgson, 1958; Carr et al., 2012; Jarvis et al., 2017; Favreliere et al., 2020). Furthermore, *P. punctiformis* may be successful in a multi-year perennial forage system, as the multi-year forage would facilitate slow development of the pathogen, the low disturbance regime would promote spore accumulation on the soil surface, and mechanical spore dispersal from mowing could increase the distribution range of transmissible spores.

Although our results show promising potential in favor of integrating *P. punctiformis* as a *C. arvense* biocontrol in organic crop systems, there are many research steps that must be taken prior to a widespread adoption of the pathogen. For example, the obligate biology of *P. punctiformis* limits the ability to artificially culture transmissible spores (Kentjen et al., 2023), making it difficult to utilize the biocontrol agent in a scalable manner. Research efforts are constrained by the availability of *P. punctiformis* inoculum, which is currently sourced from wild populations. There is a need for new development of *P. punctiformis* production techniques that yield a reliable supply of inoculum with low economic costs. One possible way to address the shortage of inoculum is to intentionally cultivate *C. arvense* in a controlled environment, where *P. punctiformis* spores could be produced for harvest and processing. Another option would involve a development of culture mediums that encourage the fungal life-cycle by simulating specific pathogen-host interactions that drive teliospore development.

To improve the efficacy of *P. punctiformis* as a biocontrol agent, there is a need for future research that explores pathogen-host interactions and the genetic factors that determine pathogen

virulence. *Puccinia* pathogens are known to be genetically diverse (Kolmer et al., 2012). Molecular techniques have been used to identify virulence factors in species that impact crops (Wan and Chen, 2011), and more recently, identify genetic characteristics and diversity between *P. punctiformis* populations (Berner et al., 2015; Henderson et al., 2018). There is a need to build upon previous work with a concerted effort to identify and isolate virulent strains of *P. punctiformis* that are fit for field applications. If virulent mechanisms and *P. punctiformis* strains can be identified, then there is potential to incorporate breeding or engineering techniques that produce offspring with desirable characteristics, ultimately improving its efficacy in applied settings.

If *P. punctiformis* is to become a publicly available biocontrol product in the United States, it will be necessary to meet the requirements of the USDA Animal and Plant Health Inspection Service (APHIS). These requirements are meant to regulate the importation, interstate movement, and environmental release of plant pests or noxious weeds, and provide permitting for release of non-indigenous weed biocontrol agents. Additionally, the biocontrol would need to meet individual state requirements for labeling and use as an organically certified product. Completion of the federal regulatory processes would expand access to the biocontrol for future research and application opportunities, effectively increasing *P. punctiformis's* potential as a *C. arvense* management tool.

This study is a contribution to a broad effort towards sustainable and long-term management of *C. arvense*. Our findings represent a novel use of the biocontrol agent, *P. punctiformis*, as part of an integrated weed management toolset in organic cropping systems of the North American Great Plains. Our results confirm that integration of *P. punctiformis* with a

cultural management tactic can be an effective way to reduce *C. arvense* vigor in a greenhouse setting, and that reduced tillage practices can facilitate an increase in *P. punctiformis* abundance in a field setting. With future research and development, it is our hope that *P. punctiformis* can play an active management role as a biocontrol agent, and that integrated weed management tactics can be adopted to sustainably manage *C. arvense* in organic cropping systems.

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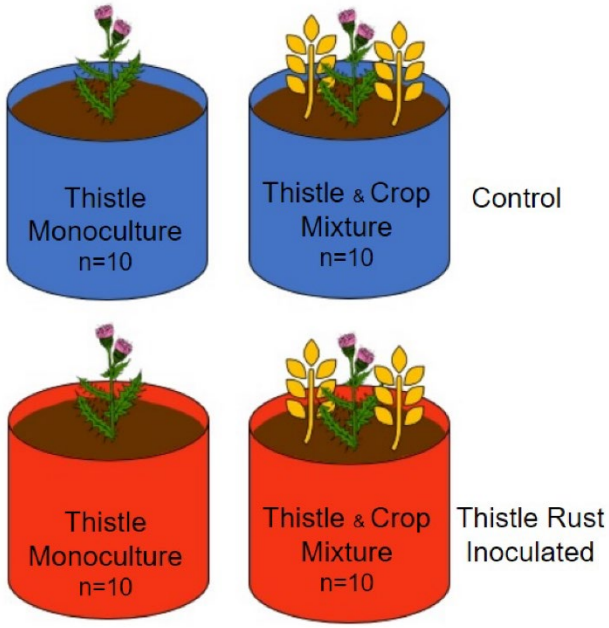
APPENDICES

APPENDIX A

IMPACT OF *PUCCINIA PUNCTIFORMIS* ON *CIRSIUM ARVENSE* PERFORMANCE IN A SIMULATED CROP SEQUENCE



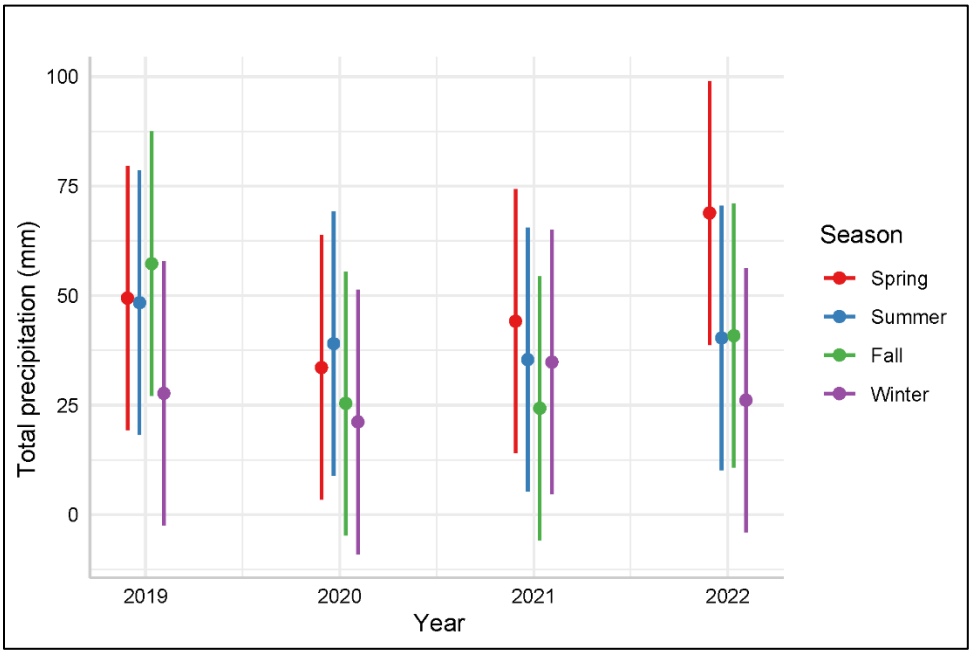
Supplementary Figure 1: Canada thistle growth was assessed within three levels of a competition treatment (crop monoculture, thistle monoculture, thistle & crop polyculture) that were nested into two levels of an inoculation treatment (control & thistle rust inoculated). Canada thistle was grown for 16 months in greenhouse pots, and evaluated for density and biomass within a 4-phase diversified crop rotation.

Supplementary Table 1: Duration *C. arvense* growth for each crop phase over the three trials (days)

	Fallow	**Wheat**	**Peas**	**Safflower**	**Total**
Trial 1	131	81	75	108	395
Trial 2	149	92	70	75	386
Trial 3	134	95	93	61	383
Mean Duration	138	89	79	105	388

APPENDIX B

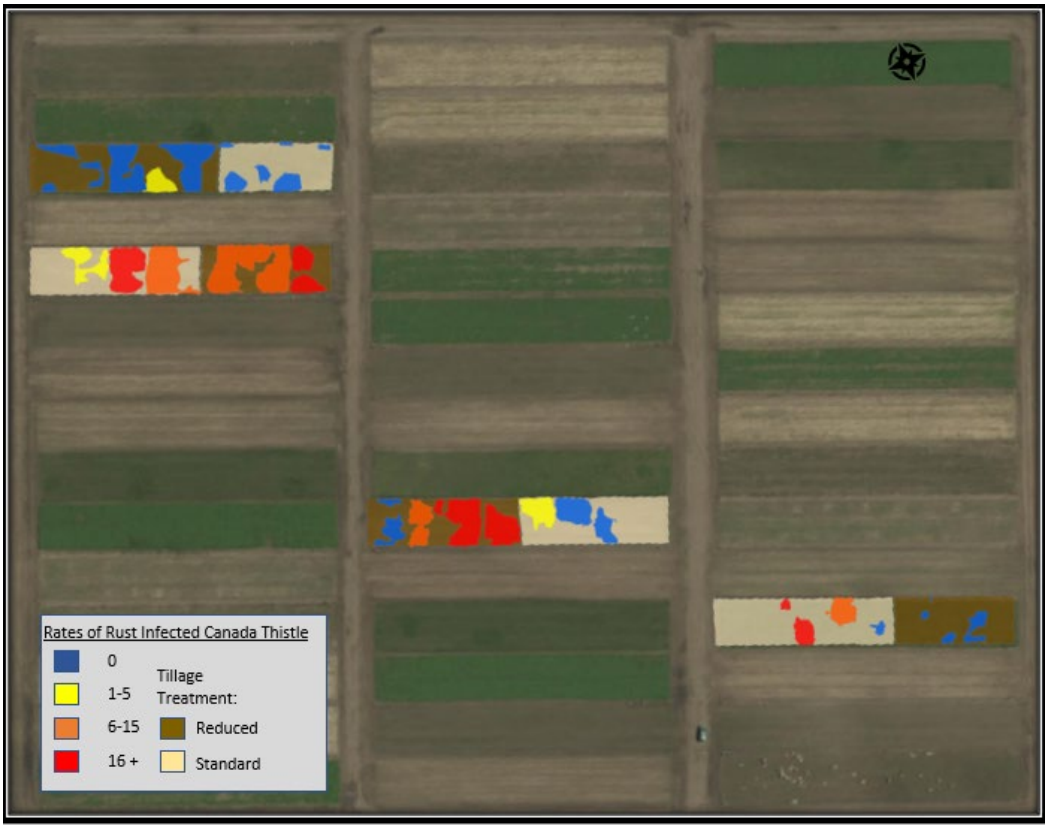
INTEGRATION OF *PUCCINIA PUNCTIFORMIS* INTO MECHANICAL MANAGEMENT FOR *CIRSIUM ARVENSE*



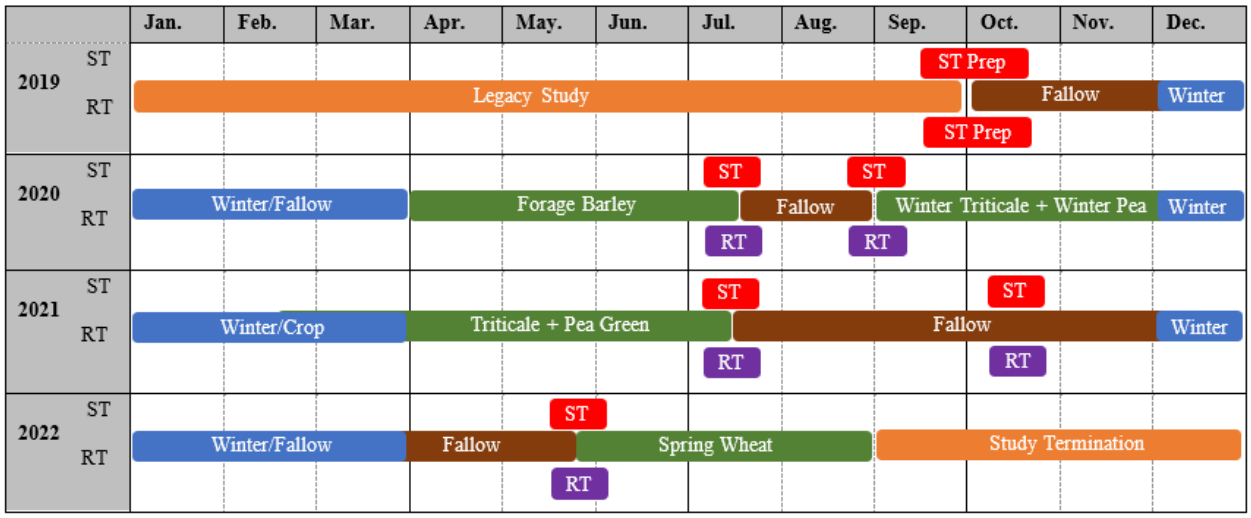
Supplementary Figure 2: Quarterly precipitation by year in Bozeman, Montana between 2019 and 2022.

Supplementary Table 2: Estimated seasonal and annual means for precipitation in Bozeman, Montana between 2019 and 2022.

	2019	**2020**	**2021**	**2022**
Mean spring precipitation (mm)	49.4	33.6	44.2	68.9
Mean summer precipitation (mm)	48.4	39.0	35.4	40.3
Mean fall precipitation (mm)	57.3	25.4	24.3	40.9
Mean winter precipitation (mm)	27.7	21.2	34.8	26.1
Mean annual precipitation (mm)	45.7	29.8	34.7	44.1



Supplementary Figure 3: Experimental plots at the Ft. Ellis Research Farm in Bozeman, Montana. Discrete *Cirsium arvense* patch boundaries were initially mapped 2020 and color categorized by the density of *Puccinia punctiformis* infected stems per patch. Plots were randomly assigned with standard tillage and reduced tillage treatments



Supplementary Figure 4: Experimental timeline from the integration of biological and mechanical management at the Ft. Ellis Research Farm.