Project Title: Improving the production, distribution and post-release monitoring of *Puccinia punctiformis*, a naturalized rust fungus for the biological control of Canada thistle, in the West.

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Amount Requested: Year 1 (2017): $15,120; Year 2 (2018): $57,549; Year 3 (2019): $26,793
Total: $99,462
Total Match: $102,403
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Project Goals and Supporting Objectives
Project goals: The primary goal is to implement the biocontrol of a major invasive weed, Canada thistle (*Cirsium arvense*) in Washington, Idaho, Utah, Montana and Wyoming. Implementation will be accomplished through the transfer of newly developed protocols from the Colorado Department of Agriculture, where the rust *Puccinia punctiformis* is currently being used as a biocontrol agent, to five states that lack this biocontrol option. Furthermore we will enhance our production capacity enabling us to supply teliospores (the infective stage of the rust) to our cooperators. We will also supply cooperators with a monitoring protocol and we will coordinate the multistate network of cooperators and make certain information concerning establishment and efficacy of the agent is shared between cooperators. Supporting objectives: Our objectives are to develop and train a network of cooperators in the five states such that each state has the knowledge and capacity to utilize the rust for Canada thistle control and has a monitoring program in place. We will also enhance our ability to produce the infective spore stage and to make supplies available to our cooperators.

Project Justification/Urgency
The Colorado Department of Agriculture (CDA) initiated work on *P. punctiformis*, a naturalized rust fungus for the biological control of Canada thistle, in 2013. The CDA was the first to implement and track the biocontrol potential of *P. punctiformis* on a large scale basis extending state wide. At this point the CDA is prepared to expand the *P. punctiformis* biological control project to other western states, making it a regional project and enabling end users in other states to have access to this Canada thistle biological control agent. The CDA will model the sequence of steps for regional implementation after the development of our program in Colorado. Our goal is to bring weed managers in five additional states to the point where they can sustain an efficacious Canada thistle biocontrol program. This project addressed the three priority project identified for the Biological Control of Invasive Plant program.

Canada thistle is a serious invasive weed
Canada thistle is one of the most serious and widespread invasive weeds worldwide and in North America, it thrives in a number of different ecological settings including farmland, ranchland, meadows and riparian areas (Tiley 2010). Canada thistle is a long lived deep rooted perennial with an extensive horizontal root system that spreads primarily through rhizomatous growth, giving rise to one of its common names, creeping thistle. Plants may also spread through seed although this is less common. Plants form patches (colonies) which are very difficult to eradicate using conventional methods, in fact tiling can result in a dramatic spread of a patch owing to the fact that pieces of rhizome may act as propagules. In Colorado alone estimates of the extent of the Canada thistle infestation range from well over 100,000 acres up to 400,000 acres.

Do we have biocontrol options?
Biological control of Canada thistle with the two most commonly used agents, the gall fly Urophora cardui and the stem weevil Ceutorhynchus littura, has been largely unsuccessful (Price and Schwarzlaender in prep.). This

is in spite of the fact that both agents are widespread, especially the gall fly, and there is substantial demand for biological control of this weed, for instance the Colorado Department of Agriculture received over 150 requests per season for Canada thistle biocontrol agents prior to discontinuing the program in 2012. With high demand and a lack of agents driving urgency, we explored the possibility of using the naturalized rust fungus, *Puccinia punctiformis*, for Canada thistle control.

The rust *P. punctiformis* has a complex life cycle and is fatal to infected plants

P. punctiformis has a complex life cycle adapted to the biology and life history of Canada thistle (Berner et al 2013) which is the single host for the pathogen and on which the pathogen is specific (Guske et al, 2004). The fungus has long been known to be widespread in North America (Olive 1913) and over 100 years ago it was suggested as a biological control agent for Canada thistle (Wilson, 1969). Use of the pathogen against CT has been hampered by the inability to achieve systemic infections, which are infections that enter the root system and eventually prove fatal. Recent work by Berner et al (2013) has provided additional information on the infective spore stage as well as the infection court (stage and location of the plant through which infection occurs). Based on new observations and the observed fungal life cycle (Fig 1) Berner et al (2013) hypothesized that teliospores produced in mid to late summer were the infective stage and the new rosettes of late summer and early fall provided the infection court. They reasoned that as plants senesced and infected leaves dried and broke apart the teliospores would be carried around on leaf fragments and these fragments would fall onto newly developing rosettes which appear in the autumn. Once spores contacted new rosettes under the mild and relatively moist conditions of autumn they germinate, producing basidiospores which send overwintering hyphae into the plants through the rosettes, and eventually into the root system. After the fungus has entered the root system the plant (genet) will eventually die from the infection even though it might continue to send up shoots (ramets) that do not appear diseased but may be stunted (Berner et al 2015a). Eventually the genet will send up systemically infected shoots which are tall and spindly but most striking is that they contain pycniospores (spermagonia) and receptive hyphae which fuse to produce aeciospores, but these structures are self-incompatible and crossing must occur between infected plants. After outcrossing, possibly aided by insects attracted to the unusual flowery fragrance of the infected shoots, the pycniospores merge with hyphae to form dark reddish brown aeciospores. The infected shoot dies and the root system supporting it also dies while the aeciospores spread to neighboring plants where they infect leaves, producing uredinia which eventually transform to telia and produce the infective teliospores.

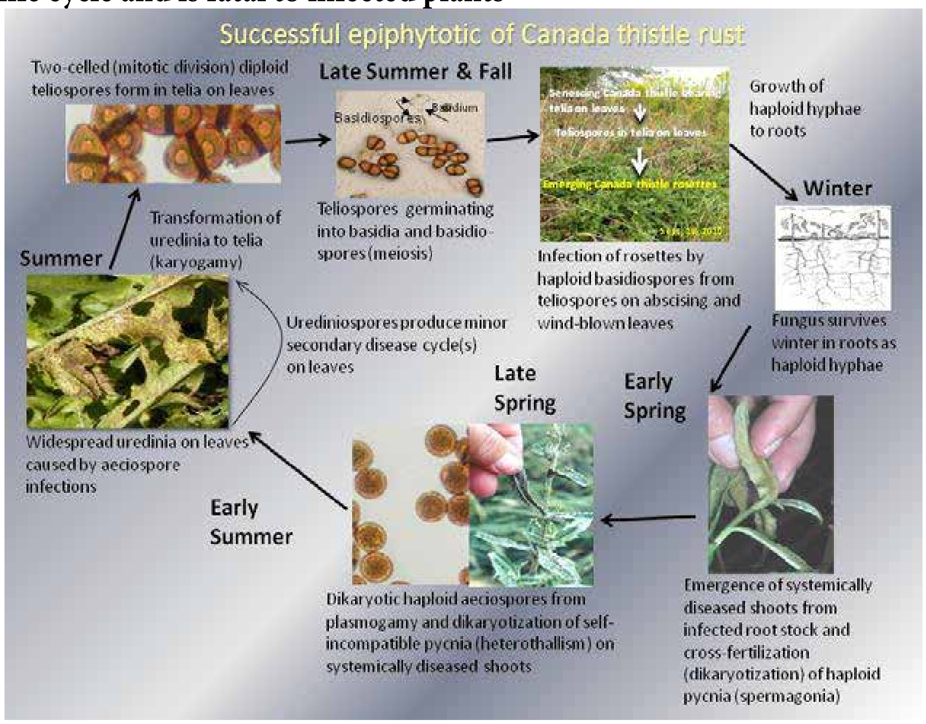

Figure 1. Life cycle of the rust fungus showing seasonal timing of the spore stages in relation to Canada thistle life cycle (from Berner et al 2013)

The unusual life cycle of *P. punctiformis* made it difficult to work with until now

P. punctiformis has an unusual and complex life cycle that makes it difficult to work with. It was identified as a possible control for Canada thistle as early as 1893 (Wilson 1969) but a lack of understanding of the life cycle and general difficulty in establishment of systemic infections has hampered development of the rust for routine use

Spring and early summer aeciospores form from the fusion of pycniospores (on one infected plant) and receptive hyphae (on another infected plant). Aeciospores are formed in dark brown aecia and they are unable to initiate a systemic infection, but can initiate an above-ground infection cycle that eventually leads to teliospore production

Aeciospores form uredinia in which form urediniospores which may propagate, or may form teliospores. At this stage the CT leaves turn yellow and are spotted with uredinia or telia. The primary way to distinguish them is by looking for 2 celled structures (teliospores) instead of single cells (urediniospores)

Telia (upper panel) produce teliospores which are 2 celled structures (lower panel) that begin to appear in the field in mid to late summer replacing urediniospores (the round spore in the lower panel). Teliospores germinate to produce haploid basidiospores which can infect a Canada thistle rosette. Neither urediniospores nor aeciospores can initiate a systemic infection.

Figure 2. Spore stages of *P. punctiformis* and their developmental timing and function in relation to Canada thistle biocontrol

against Canada thistle. The rust has been considered on multiple occasions as a potential biocontrol agent but the promise of the rust remained unfulfilled probably for two reasons. First, the rust life cycle is complex and easy to misinterpret. If the wrong spore stage is used or if the wrong infection court is targeted the infection rate will be low or nonexistent. Second, even if infection is achieved the rust acts slowly, and if biocontrol practitioners look for results in a 1-2 year time frame they won't be satisfied. The work of Berner and colleagues has helped in the formulation of protocols for infection. First, the life cycle has been more clearly described. Use of the most obvious spores (dark brown aeciospores, Fig. 2) would result in failure or at best a low infection rate as the aeciospores would have to initiate an aboveground infection and cycle through uredinia and telia in order to infect the root stock. A systemic infection cannot be initiated if urediniospores are used instead of teliospores and this could be confusing since they look the same to the naked eye. Care must be taken to distinguish teliospores so they must be viewed under a microscope or hand lens to verify that they are two celled (Fig 2). The next consideration is discovery of the infection court. Berner and colleagues had the best success infecting rosettes that appear in the fall and timing the infection to coincide with a high probability of evening dew (Berner et al 2013). Finally, another confounding element of *P. punctiformis* biology is that the fungus may exist for some time as a root parasite, hidden from view and causing no overt symptoms in a genet, in fact healthy looking ramets may appear in spite of a root infection (Berner et al 2015).

Figure 3. Infected leaves are harvested and dried after most spores present are teliospores (as opposed to urediniospores). Dried teliospore-bearing leaves are ground in a blender into a coarse mixture of leaves and spores which is applied to rosettes in the fall.

The CDA has a long term Canada thistle biocontrol program in place

The CDA initiated work on *P. punctiformis* in 2013 with the cooperation and assistance of Dr. Dana Berner from the USDA Agricultural Research Service Foreign Disease-Weed Science Research Unit in Ft. Detrick, MD. Berner and colleagues had documented significant declines in CT densities following systemic *P. punctiformis* infections within CT patches. His work on *P. punctiformis* included field trials in Maryland, Russia, Greece and New Zealand where once systemic disease was established stem densities declined significantly (average 63% decline in2.5 years) and in some cases the Canada thistle disappeared entirely (Berner et al 2013). We are the first to implement and track the biocontrol potential of *P. punctiformis* on a large scale basis extending state wide.

Our Canada thistle biocontrol program includes the identification of teliospores in the field, harvest and processing of teliospores (Fig 3) and storage for use later in the season (teliospores are usually harvested in August while rosettes are infected in September) or even saving them for subsequent seasons in a large chest freezer for inoculum storage at -80°C. Patches of Canada thistle are inoculated in the field and permanent transects have been set up to monitor the patches for the appearance of systemically infected shoots as well as to measure the density of Canada thistle shoots in the years after inoculation . We have deliberately targeted Canada thistle patches in diverse settings including the higher meadows of the Rockies down to the lower drier areas of Colorado's western slope and out on the eastern plains in native grasslands and agricultural areas . At this point we are prepared to expand the project to other western states, making it a regional project and enabling end users in other states to have access to this Canada thistle biological control agent. We will model the sequence of steps for implementation after our own implementation steps in Colorado during which we received the expert advice of Berner and his colleges, including field visits and follow up visits by Dr. Berner. After we had become able to locate and process sufficient teliospores we opened the program to landowners who are now able to request our services and assistance in establishing the rust fungus. Our goal is to bring weed managers in five additional states to the point where they can sustain an efficacious Canada thistle biocontrol program.

Approach

1. Conduct workshops in five western states to instruct and assist weed and resource managers in the development of *P. punctiformis* for CT biocontrol. CDA personnel will use protocols and experiences derived from the *P. punctiformis* project in Colorado to develop Canada thistle biocontrol programs in five western states; Utah, Wyoming, Washington, Idaho and Montana. The CDA will assist weed and resource managers in each state to identify systemically infected plants in the field, identify telia (and teliospores) in the field, collect telia bearing leaves, process and store telia, inoculate rosettes in the field, and monitor progress of the rust fungus in the field. These steps occur during the seasonal course of infection and it will require three trips to each state by CDA personnel. The goal of the first trip, which will occur in the spring of 2018, will be to identify systemic infections in the field. The goal of the second trip, which will occur in midsummer, will be to identify teliospores on the leaves of plants adjacent to the systemically infected shoots (which will be dead and probably difficult to identify by mid-summer). The goal of the third trip will be to assist in the inoculation of uninfected patches in the late summer or fall of 2018. In the late fall of 2018 or early spring of 2019 a summary workshop will be held in each state to instruct resource managers and landowners in the use of Canada thistle rust to treat Canada thistle patches.

2. Develop improved procedures to produce and harvest infective teliospores and provide teliospores, as well as procedures for their production, to our cooperators. We will also improve procedures to inoculate susceptible stage Canada thistle patches to initiate systemic *P. punctiformis* infections. The Palisade Insectary currently has two 15X15 meter plots (450 m2) of irrigated land planted in Canada thistle and devoted to tracking the decline of Canada thistle following *P. punctiformis* infection, as well as monitoring *P. punctiformis* to better understand the phenology of rust development in western Colorado. The Palisade Insectary will plant an additional four 15x15 meter irrigated squares in Canada thistle and track teliospore development and yield after inoculating the squares with two different levels of aeciospores during the early season. Artificial aeciospore inoculations have not been used as a means of elevating teliospore levels although it would seem to have an excellent chance of working (Berner et al 2015b). The goal will be to maximize teliospore yield from Canada thistle patches, store teliospores stocks in our -80°C freezer and provide teliospores to our cooperators when needed. We will also incorporate improvements in teliospores production into our standard protocols.

3. Continue to monitor previously inoculated Canada thistle patches across Colorado (140 monitoring sites) using permanently marked transects to measure Canada thistle density and occurrence of infected shoots with a rapid protocol similar to the SIMP protocol developed by researchers at the University of Idaho. These data will be shared with end users in the other five states to help guide their programs.

Expected Products and Outcomes

We will start Canada thistle biocontrol implementation programs in Idaho, Utah, Wyoming, Washington and Montana using locally collected rust fungus *Puccinia punctiformis*. We will develop a training protocol and train natural resource managers and landowners to recognize *P. punctiformis* infections and use naturally occurring infections as a source for inoculum in statewide programs, much like Colorado already has (Priority 1). We will develop and provide detailed collection and processing protocols for infective spores (Priority 1). We expect that development of systemic infections within Canada thistle patches will result in a significant decline in CT stem densities. We will provide our monitoring protocol to users in other states, enabling them to perform a rapid quantitative assessment of biological control impacts (Priority 2) and Canada thistle decline. We will also enhance our ability to collect infective teliospores by developing a protocol to infect and harvest teliospores from cultivated Canada thistle (Priority 1). Finally we will continue to monitor 140 sites infested with rust fungus in what has become a large scale monitoring project to track Canada thistle biocontrol in Colorado (Priority 2 and 3). We will share all information with cooperators in the other five states to enable full and rapid development of the rust fungus as a biological control agent for Canada thistle.

The most substantial product and outcome will be to further the development of an effective biocontrol option for Canada thistle and to make it available within a multi-state region.

Timetable:

Year 1 (2017): Till and plant four irrigated squares (225 m2 each) in Canada thistle and inoculated with teliospores in the fall of 2017. These patches will be used for teliospore harvest as well as for testing methods to increase teliospore harvest. Since finding infected CT patches is best done in the spring we will plan to begin the multistate development phase in the spring of 2018 but planning will begin during the summer and fall of 2017. Contact resource managers in Idaho, Montana, Wyoming, Washington and Utah and develop a schedule for training during the 2018 field season. Continue monitoring 140 Canada thistle sites across Colorado. The costs of monitoring in 2017 will be defrayed using other funds.

Year 2 (2018): CDA personnel will travel to each of the five states four times, three during field season and one after. The first scheduled trips will be in the spring to locate infected CT patches. The second trip will be in mid-summer to locate and assist in gathering teliospores while the third trip will be in the fall to assist in inoculation of CT patches. The last trip will be in mid to late fall to hold a workshop for resource managers and landowners summarizing the project with a step by step methodology for implementation of CT biocontrol. Teliospores will be harvested and stored at the Palisade facility and teliospores stocks will be made available to cooperators. Methods for increased teliospores production will be evaluated. We will continue monitoring 140 Canada thistle sites across Colorado.

Year 3 (2019): CDA staff will contact end users in the five states to see if further assistance is required for implementation of Canada thistle rust as a biocontrol agent. CDA staff will also compile and distribute information gained in 2018 on the efficacy and use of rust as a Canada thistle biocontrol agent. The 140 Colorado field sites will be monitored and the teliospore production gardens will be continued. Information gained from the four state program, the 140 field sites in Colorado as well as the teliospore production garden will be compiled and sent out to end users in the four states.

Literature Cited

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Budget Justification
A portion of the requested funds will go toward the partial support of a high level technician with extensive experience working with *P. punctiformis* as well as working with resource managers and land owners in Colorado. She will coordinate the multi-state program. During Years 2 and 3 we will hire seasonal staff members to assist in monitoring release sites and in maintaining the Canada thistle garden. Seasonal staff are usually selected from top level students at Colorado Mesa University and such positions are often used as stepping stone into natural resources management careers. Travel funds will go entirely toward travel necessary for training cooperators through field demonstrations and workshops.

Salaries ($68,544)
Canada thistle technician, $4,032/month, 0.25 FTE in years 1&3, 0.5 FTE year 2, total salary $48,384 Seasonal staff member, $15.50/ hr. year 2 and $16/ hr. year 3, 4 months per season, total salary $9920 year 2 and $10,240 year 3.

Fringe benefits ($14,918)
25% salary for full time staff ($12,096), 14% salary for seasonal staff ($2,822)

Supplies
Supplies will be purchased by the CDA and will be part of Matching Costs

Travel ($16,000)
This includes mileage, per diem and hotel for 15 field trips (3 trips to each of 5 states @ an average of $700/trip) and 5 workshops at the end of 2018 (average of $1,100/ workshop for staff time and facilities costs).

Total Direct Costs ($99,462)

Matching Costs ($102,403)
Match includes 1 month of PI's salary per year ($6,400 x 3) plus 25% fringe per year ($1,600 x 3): $24,000. Match also includes one month per season of Joel Price's salary plus fringes ($4,100 + $1,025 X 3= $15,375) and one month per season of John Kaltenbach's salary plus fringes ($5,600 + $1,400 X 3=$21,000). Joel is co-lead for the Canada thistle project for the CDA and John is in charge of eastern Colorado and will work with cooperators in Wyoming. It also includes use of the Palisade Insectary greenhouses and head house for growing Canada thistle as well as space for processing and storage of spores, including a -80°C freezer ($150/month for 36 months= $5,400) and use of 900 m2 of irrigated land for growing CT to produce teliospores. The cost of the land plus irrigation plus tilling and routine maintenance (weeding, fertilizer, irrigation, weed barrier) is estimated at $450/ month for 7 months per year for three years ($9,450). Other supplies include bags, gloves, shears, blenders and storage containers for frozen teliospores, seed spreaders, U-posts, monitoring frames, garden tools, shipping and shipping containers with a total cost of approximately $2,000/year ($6,000). A leased vehicle from state fleet will be used for travel within Colorado to visit monitoring sites. Fleet vehicle cost plus mileage costs are approximately $2,500 per season ($7,500). Unrecovered indirect
costs on the federal request, which are calculated at the CD!'s federally negotiated rate of
16.28% of salaries and fringe benefits. Salaries and fringe benefits total $84,326 x .1628 = $13,728 unrecovered indirect.