RNAi as a biocontrol agent against invasive plant species in wildfire-affected areas of Waterton National Park

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The spread of invasive plant species has been a growing concern in Southern Alberta, especially as species such as *Centaurea stoebe* (Spotted Knapweed) and others deplete nutrients in the local environments. This issue is further exacerbated by wildfires, as seen after the 2017 Kenow wildfire which devastated large swaths of land in Waterton Lakes National Park. Since the fire, many invasive species have been posing a higher threat in the park than in pre-wildfire years. Previous efforts to mitigate this problem have utilized manual volunteer labor and insects as biological controls to clear away invasive species, both of which have yielded slow success rates. A potential solution to this issue utilizes RNA interference (RNAi) as a novel herbicide that targets and eliminates invasive species. RNAi is a gene silencing method utilizing a eukaryotic cell's immune response. Engineering an RNAi system that targets specific essential genes in an invasive species can be an effective solution to eliminating said species. In doing so, the environment near the system's deployment can be improved with greater efficacy and lower labor costs. A benefit of using RNAi technology is that it can be designed to prevent non-target species from being impacted by the herbicide. Since the gene-silencing technology is designed to only target a 21-25 nucleotide sequence, by choosing a sequence that is specific to the species of interest, the potential for damage to other plants is reduced. However, a concern that may arise from the implementation of this solution is the potential for the novel herbicide to disrupt the established relationship between the invasive species and any previously introduced insect controls. To establish an effective RNAi herbicide and avoid these issues, further research is required.

Keywords: Invasive, plants, *Centura stoebe,* spotted knapweed, RNAi, dsRNA, siRNa

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Background

The population levels of invasive plant species within Waterton Lakes National Park in Alberta, Canada have risen after the 2017 Kenow wildfire which scorched 19,308 hectares of land (Figure 1) (Parks Canada, 2019a, 2019b). In the aftermath of a wildfire, resources that are vital for plant survival are often imbalanced (Zouhar et al., 2008). This setting is advantageous for Waterton's invasive species to easily dominate a post-wildfire environment. In a susceptible habitat, invasive species monopolize the available resources (Zouhar et al., 2008) resulting in insufficient resources for the area's native species to utilize for survival. During a post-wildfire period, invasive plant species have an advantage through sheer competitive numbers and can overtake post-wildfire sites (Coogan et al., 2019; *Zouhar et al.*, 2008).

Invasive plant species are species that are not native to the area they are found in and often cause harm to the environment in which they reside (Kumar Rai & Singh, 2020). Invasive species are a potent threat to their environments because they make it harder for native species to survive, reducing biodiversity. An immense growth rate, vast overpopulation, and overuse of the surrounding resources create an imbalanced environmental dynamic, making invasive species problematic. (Weber, 2017). Invasive species, such as *Centaurea stoebe* (spotted knapweed) have created this disadvantaged scenario in Waterton.

C. stoebe is one of 5 different strains of invasive knapweed found in Canada (Nature Conservancy of Canada, n.d.). It was originally brought to North America by European ships in the late 1800s (Blair & Hufbauer, 2009; Bourchier & Hezewijk, 2013). Spotted knapweed is difficult to contain, as it can produce more than 1,000 seeds that may lay dormant in the soil for at least 5 years (Pokorny et al., 2010). These circumstances cause knapweed infestations years after the plant has already been supposedly eradicated. The effects of spotted knapweed on surrounding species are substantial as the roots of a mature knapweed plant may emit allelopathic chemicals (Blair & Hufbauer, 2009; Pokorny et al., 2010) and inhibit the germination of surrounding species. Knapweed's ability to change nitrogen cycles can alter soil microbial communities, increase sediment yield and erosion, while also corrupting plant diversity. This makes spotted knapweed highly disruptive to surrounding ecosystems (Knochel & Seastedt, 2009).

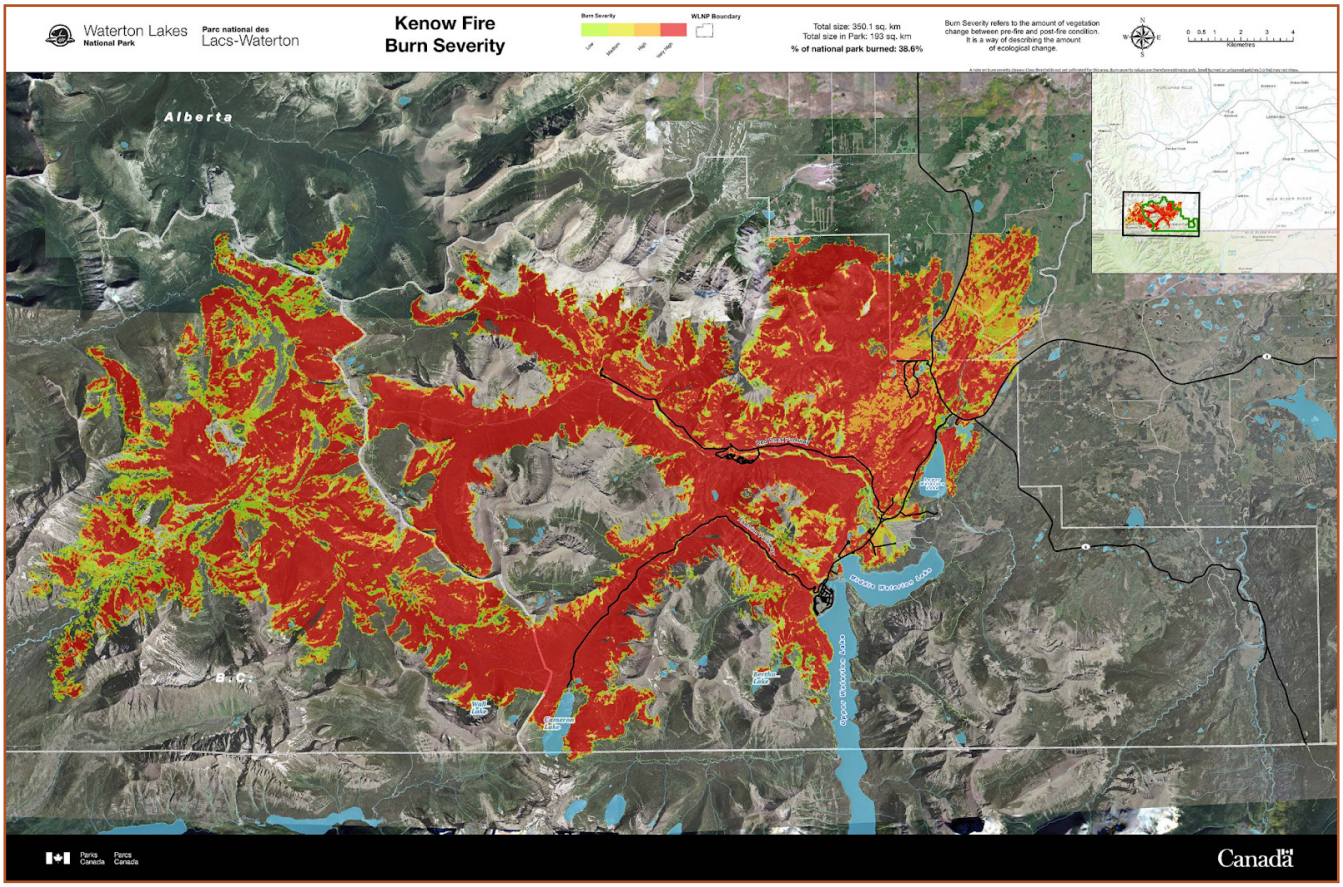

Figure 1. *Kenow fire burn severity map. In total, 38.6% of Waterton Lakes National Park was burned. The burn severity ranges from mild (lime green), to very high (red). Severity corresponds to the change in vegetation (Parks Canada, 2021).*

Spotted knapweed's ability to grow in a large range of soil types and habitats (Corn et al., 2006) has caused the plant to be spread across 8 Canadian provinces (BC, Alberta, Saskatchewan, Manitoba, Ontario, Quebec, New Brunswick, and Nova Scotia) with the largest concentration in southwestern Canada (Nature Conservancy of Canada, n.d.).

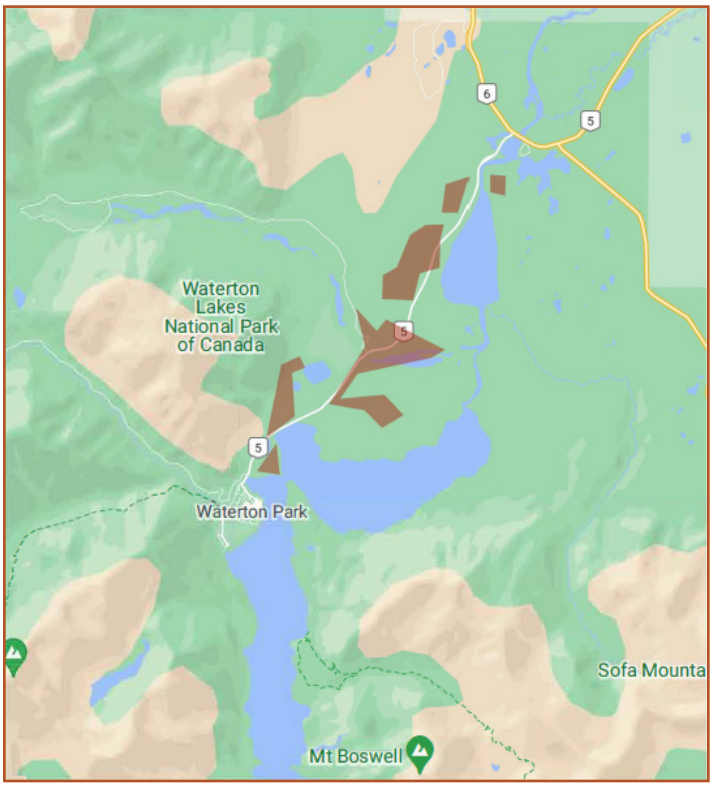

Figure 2. *Area in Waterton Lakes National Park treated for knapweed (2014-2017) (Kapoor, 2019).*

Currently, invasive plants are being combated with herbicides. Although herbicides are created to target plants, they can also be toxic to humans and wildlife (Nicolopoulou-Stamati et al., 2016). Considering the WHO goal of Global Health Security, chemical herbicides are not the best treatment against invasive plant species due to the biosafety and bioethics concerns (Albanese, 2019). As biosafety deals with protection from harmful incidents, chemical herbicides pose health concerns for both field workers and consumers lacking the means of properly washing herbicide-treated produce. These herbicides do not meet WHO's ethical objective. Furthermore, the extensive use of chemical herbicides may lead to increased resistance and pollution. Through our project, we want to research the parts of knapweed that make it thrive

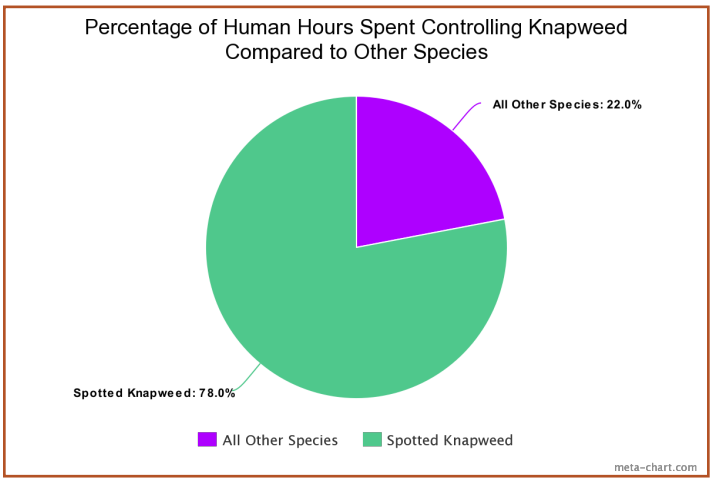

Figure 3. *Comparison between the number of hours spent and herbicide used on spotted knapweed and other invasive species in Waterton Lakes National Park (Kapoor, 2019).*

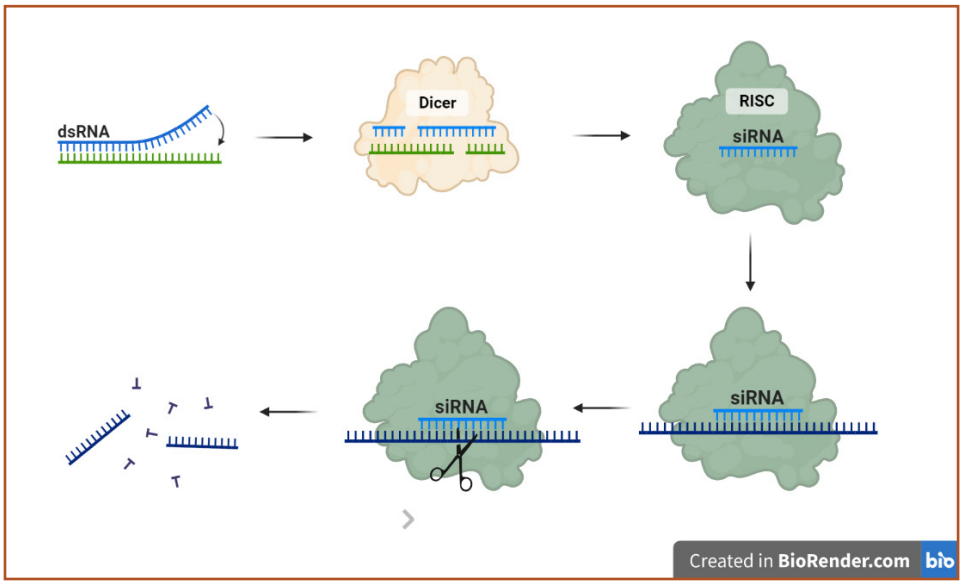

Figure 4. *RNA interference is triggered by double-stranded RNA processing by Dicer, followed by the recruitment of RISC, complementary base-pairing between the siRNA and target mRNA sequences, and subsequent degradation of the target mRNA.*

in its environments, and using our proposed RNAi-based herbicide, combat only genes specific to this species. With the herbicide exterminating knapweed and knapweed only, other plants and animals in the environment will not be negatively affected.

RNA interference (RNAi) is a cellular mechanism that silences gene expression and is a vital part of an organism's immune response (Agrawal et al., 2003). Gene silencing occurs naturally in a wide variety of eukaryotic cells through the processing of double-stranded RNA (dsRNA). In general, upon viral infection, the viral dsRNA is cut into small interfering RNAs (siRNAs) which are approximately 21-25 nucleotides long (Rogers, 2017) by an RNase II enzyme named Dicer. The siRNAs then bind to the RNA-induced silencing complex (RISC). The siRNA is unwound into single-stranded RNA which hybridizes with a messenger RNA (mRNA) target, where a protein such as Argonaute (Slicer) cleaves the mRNA/RNA strand pair (University of Massachusetts Medical School, 2018), rendering the target gene "silenced" (Figure 4) (Rogers, 2017).

The utilization of RNAi ensures long-term silencing and gene knockdown (Schmitt, 2018), which makes it highly efficient. The ability to specify a gene sequence is vital to our project; the gene sequence that we use must be unique only to knapweed so that other organisms are not harmed unintentionally. In addition, pre-designed RNAi reagents are accessible for our team at the University of Lethbridge, as opposed to similar, more expensive methods such as CRISPR-Cas9 or TALEN. RNAi necessitates the synthesis of a specific gene sequence combined with the simple transfection into target cells; meanwhile the other two processes require more complicated cloning methods (Michael & Boettcher, 2015). With our RNAi herbicide, we hope to replace the need for volunteers to laboriously weed Waterton by hand.

As mentioned, since the 2017 Kenow wildfire, the *C. stoebe* threat to the Waterton Lakes National Park region has grown. Our project proposes to devise a safe and efficient method of terminating *C. stoebe* within the Waterton Lakes National Park region to limit competition with native organisms for restricted resources, halt biodiversity decline, and prevent the altering of this specific habitat. We believe that our goal is attainable through designing an RNAi-based herbicide specific to the *C. stoebe* plant species. Our solution addresses the UN sustainability goal #13 (United Nations, n.d.-a) which is taking urgent action to combat climate change and its impacts.

Forest fires contribute to greenhouse gas emissions (Climate Atlas of Canada, n.d.) so, by limiting the invasive species rapidly reproducing in the Waterton region, we will assist in the battle against the continuous struggle of climate change. Our solution also addresses the UN sustainability goal #15 (United Nations, n.d.-b) which is to protect terrestrial ecosystems and halt biodiversity loss. As previously mentioned, C. *stoebe* endangers the environment and it's elimination will decelerate biodiversity loss. Our project's goals will help prevent *C. stoebe* from overtaking the ecosystem within Waterton. We intend to execute this goal by recognizing and respecting biosafety, biosecurity, and bioethical considerations, leaving surrounding flora and fauna within the region unharmed.

Systems Level

The solution we propose consists of an RNAi-based herbicide which will be sprayed onto patches of invasive plant species to stop their growth while also keeping surrounding organisms unharmed. The herbicide will be absorbed through the plants via the stomata and will be the primary means of entry into the plant. Fundamentally, our RNAi-based herbicide involves the termination of a protein which is essential for the invasive plant's survival or reproduction. Double stranded RNA (dsRNA), the key trigger molecule for RNAi (Fletcher et al.,, 2020), is first designed to complement the mature messenger RNA sense strand we wish to silence. We will be producing our custom dsRNA via *in vitro* transcription, which is an RNA synthesis method that utilizes a purified linear DNA template and T7 phage RNA polymerase (Thermo Fisher Scientific, n.d.). This method can generate up to 200 µg of RNA (Thermo Fisher Scientific, n.d.) and is the primary method in which we will create dsRNA. Our custom dsRNA will then be applied as a foliar spray (Tenllado & Díaz-Ruíz, 2001), allowing the plant to uptake the dsRNA through the stomata in the leaves (Dubrovina & Kiselev, 2019).

The absorbed dsRNA is then cleaved into small interfering RNA (siRNA) by the RNase-III enzyme termed DICER (Bennett et al., 2020). The cleaved siRNA, measuring approximately 21 nucleotides in length (Rogers, 2017), is then separated into the sense and antisense strands by the RNA-induced silencing complex (RISC). Sense strands are then discarded and disintegrate in the cell's cytoplasm. The antisense strand of the dsRNA remains bound to the RISC complex, then in turn binding to the sense strand of the mature messenger RNA (mRNA) that we wish to silence. The specific base pairing allows for precise and accurate cleavage of the target mRNA, ultimately resulting in the silencing of the gene (Heigwer et al., 2018).

As shown in figure 4, a simplified diagram of the important steps in RNAi, DICER cleaves dsRNA into siRNAs. RISC also cleaves the siRNAs sense and antisense strand. Then, RISC binds the antisense and sense strands together to direct cleaving of the mature RNA resulting in translation inhibition (Limera et al., 2017). Henceforth, the plant will no longer be capable of producing the protein encoded by the silenced gene (Rogers, 2017).

Through laboratory testing, the amount of herbicide required to reliably kill the target plant will be determined, as well as the time it takes for the RNAi to take full-effect. Although the seeds of knapweed have the ability to stay dormant in the soil for 8 to 10 years (Pokorny et al., 2010), we will also test the effectiveness of our herbicide on the seeds. If the dsRNA cannot penetrate the seeds, we will either target a gene essential to seed production or extend the duration of treatment to be performed annually for a minimum of 8 years. The herbicide itself will be approximately the viscosity of water, making it suitable for spraying through most commonly available spray bottles. For larger applications, there is the possibility of using a mobile herbicide sprayer that can identify and spray plants autonomously.

Our herbicide will only be effective on the plant species we desire to terminate due to the specificity of our siRNA sequence. By choosing the dsRNA antisense strand that is fully unique to the sense strand of the mature messenger RNA we wish to silence, we can rule out the concern that our herbicide would terminate any surrounding organisms. Since the dsRNA we choose will only be specific to the plant species we want to terminate (if said dsRNA were to be absorbed into an organism that is not our targeted species), RISC would not be able to bind the two sense strands together. Therefore, no silencing of the gene would occur. This process is advantageous because it eliminates the concern of disrupting the ecosystem our herbicide is in. Furthermore, this process allows for easier spraying and eliminates the potential human and wildlife health risks (Nicolopoulou-Stamati et al., 2016).

Device Level

Gene Selection Through Bioinformatics

To determine which gene(s) we will target in *C. stoebe*, we will conduct a BLAST (Basic Local Alignment Search Tool) search to determine which segment of the homolog is unique and whether or not it is a lethal phenotype. A lethal phenotype in plants causes the plant to stop growth at various stages of development, which causes the organism to die or halt seed production. The only genome for spotted knapweed in the National Center for Biotechnology Information was for its chloroplast, which made finding a unique gene more difficult. A Clp protease gene which codes for Clp protease proteolytic subunit in the chloroplast was found to be unique and have a lethal phenotype. The ClpP gene was chosen as the target gene that our siRNA construct will bind to, triggering RNA interference in the plant. Our other potential target is Adt2, an arogenate dehydratase that is essential for seed development.

Saccharomyces cerevisiae as a model organism

A method to test the siRNA construct will be through the use of *Saccharomyces cerevisiae* or baker's yeast. *S. cerevisiae* does not normally contain RNA interference machinery, so the strain that will be used for this project will be engineered to possess both RISC and Dicer pathways in order to participate in RNA interference. This specific strain was obtained from the Bartel lab at MIT ((Drinnenberg et al., 2009). A red fluorescent protein (RFP) reporter gene will be used within the yeast cell, which we will bind with our target gene from *C. stoebe*. These pathways and genes are essential because when the siRNA interacts with the target gene, it forms double-stranded RNA (dsRNA), triggering Dicer and RISC. When this takes place, Dicer will cleave the dsRNA, cutting the RFP gene in the process. If the RNAi treatment is successful, the yeast will repress translation for the red protein, ensuring the cells no longer possess red fluorescence. This will indicate that the siRNA construct was successful in both binding and cleaving the target gene. Furthermore, the decrease in fluorescence before and after RNAi treatment can be quantified as a function of time to give us an idea of what we might see in the plant testing phase.

Parts Level

Our parts will be rather simple for this project, as we only need to produce the siRNA.

The constructs will include the siRNA genes in plasmids flanked by an IPTG inducible T7 promoter (BBa_I712074) upstream and a double terminator (BBa_B0014) downstream. This will allow for efficient transcription of our siRNA *in vivo* or *in vitro*. We anticipate producing a few different plasmids with different siRNA sequences to test which sequence is best for silencing gene expression in our model system and the knapweed itself.

Safety

Conventional herbicides possess the ability to stimulate inadvertent environmentally unfriendly repercussions to the surrounding desirable plant species. The majority of plant injuries occur from post-emergent herbicides (Al-Khatib, n.d.; U.S. Fish and Wildlife Service, n.d.) When sprayed in high volumes, conventional herbicides hold the potential to travel through the air and unintentionally but negatively influence desirable flora in the area or areas nearby. Post-emergent herbicides may provoke injury symptoms such as yellow spotting, purpling of the leaves, necrosis, general and interveinal chlorosis and mottled chlorosis (Al-Khatib, n.d.). Traditional herbicides are non-selective, and they are toxic to extensive varieties of plant species and are not exclusive to the weeds. The incorporation of RNAi into our herbicide will safely eliminate the chances of this occurring. We will ensure our RNAi-based herbicide will solely be active on the plant species that we specifically assign it to eliminate.

Traditional herbicides affect multiple plant types, including some non-invasive species (U.S. Fish and Wildlife Service, n.d.). When traditional herbicides are sprayed in large quantities, they may travel through the air and affect desirable plants in other locations, even if there are no susceptible non-invasive plants in the very close vicinity. They can also affect humans and animals, both due to some toxicity or loss of vegetation. It is also possible for herbicides to contaminate key sources of water (U.S. Fish and Wildlife Service, n.d.). In contrast, the RNAi technology we will be using can prevent invasive species such as *C. stoebe* from reproducing, without significantly impacting the ecosystem by killing the plants (Mezzetti et al., 2020). RNAi can also be tailored to a particular plant species, preventing it from affecting desirable plants in the area (Lundgren & Duan, 2013). Spotted knapweed is consumed by animals such as sheep (Webmaster et al., 2021), and studies have shown that spraying plants with RNAi herbicides does not increase their toxicity to animals or humans (Rodrigues & Petrick, 2020).

We will initially only be testing our project in a lab setting to ensure that it is effective and without side effects using appropriate controls. We will also test it on other similar plants to ensure they are not negatively affected.

Discussions

To test our RNAi herbicide, a yeast model will be utilized. We will be using the model organism *S. cerevisiae*. Testing our RNAi herbicide requires the enzyme dicer and RNA-induced silencing complex, or RISC. Our yeast strain is engineered to have both of these so it will be able to participate in RNAi and silence genes. The reporter gene in our yeast model will be RPF, causing the organism to be red. We will acquire an essential gene(s) from knapweed as our target gene and connect it to the RPF gene so we can engineer it to express the RNAi's effect on knapweed. Then, a piece of plasmid which codes for siRNA will be given to the yeast so it can bind to the target gene. Introducing siRNA to the coherent target gene and RPF will produce dsRNA which triggers dicer and RISC. Assuming our RNAi herbicide is successful, with the dsRNA attaching the siRNA to the target gene, the entire cell cleaves and therefore is silenced. We will be able to tell whether our siRNA and target gene are complementary by the color of the yeast; if it is no longer red, it means the RPF, which is connected to the target gene, has been successfully silenced as well (Duina et al., 2014).

Our herbicide will be tested primarily on spotted knapweed seeds along with other types of knapweed. Furthermore, we will conduct experiments on other plant species, specifically those surrounding spotted knapweed in Waterton to make sure it does not negatively affect other wildlife like existing chemical herbicides, and closely related plants, such as cornflower (*Centaurea cyanus*) (Cho et al., 2012). Finally, we will conduct direct testing on the insect control agents previously mentioned. These experiments will be set in a controlled environment with appropriate heat and moisture settings to ensure we acquire the most probable results.

From the yeast model experiment, we hope to silence a targeted fluorescent gene in the yeast organism with RNAi. The result of successful silencing would result in a decrease in fluorescence. This will be similar to how we want to eliminate the specific vital gene of an invasive plant species, resulting in its removal from the affected land. But, the application of the herbicide should not kill other unrelated species in the area, and will only affect the invasive species. There are concerns that herbicide usage will disrupt the relationship between invasive species and pre introduced biological insect controls (Zavaleta et al., 2001). A possibility emerges where depleting the biological control's food source (the invasive species), will cause them to die off, leaving behind invasive seeds that have yet to germinate and further disrupt local ecosystems (Zavaleta et al., 2001). For this reason, we propose the solution of targeting a gene specific to plant reproduction. The ideal outcome is that Waterton's native species are able to access resources that were once under a monopoly by the invasive plant (Baiser et al., 2011). In the future, we hope to expand our technology to address other invasive species as well. For those with no biological controls in place, it would be pertinent to target genes essential

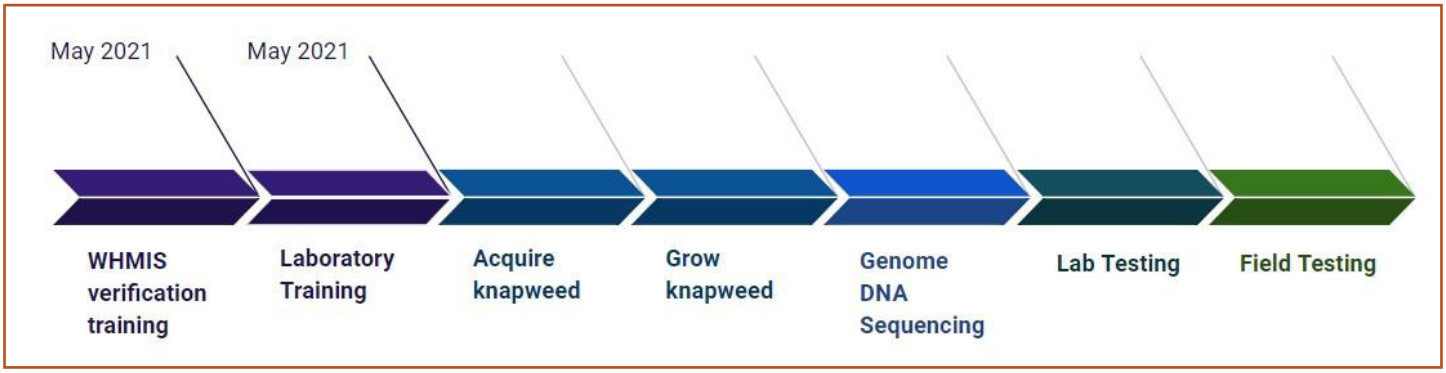

Figure 5. *A timeline for the development of our RNAi treatment for spotted knapweed.*

to that plant's life, resulting in a faster removal of that species, which can not only be implemented in Waterton, but throughout global areas dealing with similar invasive conflicts.

Next Steps

The first major step that we must take towards our project is to make sure that all our team members are well-informed of procedures in the wet lab and are confident in the safety precautions. We will do this through a WHMIS training course to certify the ability to work in a level 1 lab, and go through lab skills training to ensure team members are prepared to conduct experiments. After this, we plan to acquire samples of knapweed to better understand the logistics of our product deployment and tailor our project accordingly. One of our goals for the future is to sequence the genome of the knapweed to find critical genes specific to the species that we can use as a target for our RNAi herbicide system. Beyond this, we will do more laboratory testing to validate the efficacy and safety of our project. As demonstrated in figure 5's timeline, after we have conducted thorough and comprehensive experiments that affirm these things, we can start to consider the opportunity to test this project in the field.

Author Contributions

Every author contributed to gathering information and research. A.D., M.F., and L.H. wrote the abstract. Additionally, A.D., A.N., A.Q., R.S., and S.Y authored the background section. M.F., and S.H. wrote the systems level section. S.B. composed the device level section. L.K-W. wrote the parts level. H.M., A.N., and M.W. authored the safety section. A.D., X.L., and R.S. wrote the discussions section. M.B., G.G., and L.H. wrote the next steps section. M.K. developed the video.

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