Application of Muropeptides for Sustainable Management of Invasive Plant Species

Grant Proposal

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Abstract

Invasive organisms threaten the health of fragile ecosystems and endangered animals. The removal of such invasive species is of great importance to maintain high biodiversity in ecosystems. Invasive plant removal is often achieved through several methods: chemical removal, biological removal, and mechanical removal; however, each method has its own limitations and shortcomings. The pitfalls include damage to water systems, harm to native plants and animals, and the disruption of the soil microbial communities. A potential alternative for the herbicides frequently used in removal processes is the application of muropeptides. Muropeptides, fragments of bacterial peptidoglycan, can induce immune responses in plant species that have pattern recognition receptors recognizing peptidoglycan. After gathering a plant sample and isolating plant RNA, it will be tested for receptors then later submitted to application of muropeptides. Through the implementation of a killing assay, perception of peptidoglycan in plants with and without receptors will be observed. This research will contribute to a better understanding of the various pattern recognition receptors in plants and is expected to find muropeptide application to be a new possible biological removal method for invasives.

Application of Muropeptides for Sustainable Management of Invasive Plant Species

Invasive organisms reduce biodiversity across the globe, damaging both ecosystems and species. These exotic animals and plants often replace the native species through various methods such as predation, alteration of habitat, disease transmission, and out competition (Kumar Rai & Singh, 2020). Many invasive plants, like Kudzu, an invasive Chinese arrowroot, possess the ability to overshadow entire forests of native species, inhibiting their access to sunlight, and in turn, deactivating their natural photosynthesizing ability. Exotic species benefit from their evolutional abilities, that are in balance with their natural ecosystem, when relocated into previously uninhabited environments (Meyer et al., 2021). Lacking predators and parasites, these species can uncontrollably thrive, throwing off the biological system of checks and balances, and predators and prey. As climate change accelerates, the spread of invasive species is expected to intensify, further disrupting ecosystems, and threatening biodiversity, making it crucial to address this growing issue now to safeguard the health of our planet's natural systems. The strongest natural defense against climate change is biodiversity, only attained and upheld by the abatement of invasive species (Biodiversity - our strongest natural defense against climate change, n.d.). To limit the invasive plant presence in ecosystems, several removal techniques exist: mechanical, chemical, and biological. Mechanical removal involves cutting or pulling the plants out of the ground and is extremely labor intensive. This method limits environmental impact but is difficult and exhaustive to execute. Conversely, using chemicals to eliminate invasive species is effective and resource-efficient but has the potential to release toxins into the environment, posing risks to surrounding wildlife and water systems. Biological removal requires the use of natural enemies, such as plant diseases or insect predators, that will either outcompete the invasive species or directly target it. However, biological removal can have inadvertent consequences and requires high amounts of research on all possible outcomes as to not introduce control agents that can harm non-target species and disrupt the balance of the

ecosystem, potentially resulting in further loss of biodiversity (Pearson et al., 2021). Both chemical and biological removal have the potential to drive hundreds of species to extinction if not used properly and with thorough testing. Many of the herbicides used in killing invasive plant species damage surrounding native plants and have immense negative environmental impacts. There is no established method capable of effectively eliminating invasive plants while simultaneously preserving the health of surrounding species. This research seeks to investigate the escalating expansion of invasive, non-native plant species that have caused widespread degradation of the environment, water systems, agriculture, biodiversity, and essential ecosystem services in regions worldwide. Muropeptides are a main component of bacterial cell wall peptidoglycan, which is a crucial structural component that provides rigidity and strength in bacteria and helps them protect against osmotic pressure. Peptidoglycan receptors have been identified in some plant species, but very little research has been conducted on how they work (Andrea A. Gust, 2015). However, it has been established that different plant immune responses are linked to variations in the carbohydrate or peptide parts of the peptidoglycan (Erbs et al., 2008). Observing the effects of peptidoglycan on plants, this research has the potential to address the substantial environmental damage caused by invasive, exotic species across the globe. By gaining a deeper understanding of the mechanisms behind their spread and impact, this work could lead to more effective strategies for mitigating their disruptive effects on ecosystems. Invasive species are known to threaten biodiversity, alter habitats, and disrupt ecological balance, and thus, efforts to control and prevent their development could have profound benefits for the preservation of natural environments and the health of ecosystems worldwide.

Section II: Specific Aims

This proposal's objective is to not only reduce the environmental impacts of invasive plant species on ecosystems worldwide but also engineer a safe, eco-friendly removal

mechanism that prioritizes the health of native species before the removal of exotic ones. The long-term goals are to make ecosystems more stable, increase biodiversity, and build climate change resistant environments. The central hypothesis is that fragments of it peptidoglycan, known as muropeptides, kill invasive species via inflammation in a safe and sustainable way where other organisms in the ecosystem are not damaged. The rationale is that the application of peptidoglycan will only damage the plants that have pattern recognition receptors recognizing bacterial peptidoglycan. The work proposed here will hopefully lead to increased success in invasive species management and preservation of ecosystems with minimal environmental impacts.

Specific Aim #1: Test the hypothesis that peptidoglycan pattern recognition receptors recognizing bacterial peptidoglycan are conserved across plants.

Specific Aim #2: Test the hypothesis that peptidoglycan induces cell death in plant species.

Specific Aim #3: Test the hypothesis that there is improved delivery and better absorption when applied to the leaves of plants rather than the roots or stem.

The expected outcome of this work is to make ecosystems more stable and reduce the extremely dangerous impact that invasive plants have on ecosystems and native biodiversity.

Section III: Project Goals and Methodology

Relevance and Significance: Invasive species management is crucial to maintaining healthy environments for both people and wildlife. Controlling invasive species helps to prevent negative economic and environmental impacts such as the loss of biodiversity, the decline in human

health and safety, the acceleration of climate change, and loss the of resources such as the $1.288 trillion dollars that the United States has used in the last fifty years to combat invasive species (USDA, n.d.). Without efforts to control invasive species, many native plants and animals would be outcompeted for resources, as invasive species often have a unique ability to thrive and spread rapidly. Over time, native species may become endangered or even extinct. One invasive plant management removal technique is chemical removal; however, this often has dangerous environmental consequences. Chemical control methods typically involve the use of herbicides, which are easy to apply and have proven effective in controlling invasive plants in some areas. However, herbicides can lead to environmental pollution and pose risks to the health of humans and native animals and plants (Patten, O'Casey & Metzger, 2017; Qiao et al., 2019; Xie et al., 2019). Herbicides have the potential to impact non-target plants, contaminate soil and water systems, harm insects, and disrupt the food chain (*Use of Herbicides for Invasive Plant Control*, n.d.). This work is significant as it provides a possible alternative to these dangerous herbicides for the removal of invasive plant species.

Innovation: This project is unique as it employs muropeptides and peptidoglycan as a possible alternative to the dangerous and potentially harmful chemicals that have previously damaged ecosystems, agriculture, and water sources (Qiao et al., 2019; Xie et al., 2019). Current invasive plant removal methods include mechanical, chemical, and biological although each of the three have their own pros and cons. Mechanical removal requires human labor, chemical risks the health of the environment, and biological often has non-target effects. The usage of peptidoglycan is a form of biological removal that lacks the negative characteristics of other biological removal strategies. It can be effective and at the same time completely safe and sustainable for various ecosystems around the world.

Methodology: This study will begin by assessing pattern recognition of various plants, invasive and native. Arabidopsis thaliana will serve as my positive control, and human peripheral blood mononuclear cells (PMBCs). RNA from each plant species and control will be isolated and tested in a lab setting. To do this, a Qiagen RNeasy RNA isolation kit will be used. Data on the purity and amount of RNA will be collected and subsequently analyzed. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) will be performed to determine which pattern recognition receptors are exhibited by the sample plant species. The first step, reverse transcription, involves RNA extraction, conversion to cDNA, and priming. The next step will be Polymerase Chain Reaction (PCR) which requires amplification, thermal cycling, and lastly exponential amplification. Muropeptides will be purified then applied to new plant samples. The application will involve another test regarding whether the roots or leaves are better for maximizing the absorption. To do this, a well characterized reporter gene such as, β-glucuronidase will be used to express color change after the application of peptidoglycan.

Specific Aim #1: Test the hypothesis that peptidoglycan pattern recognition receptors recognizing bacterial peptidoglycan are conserved across plant species.

The objective for this specific aim is to determine whether or not peptidoglycan pattern recognition receptors are present in many of the invasive and native plant species. In doing so, these plants can be better understood and possibly become a target for removal via muropeptides.

Justification and Feasibility: The approach is to harvest RNA samples from various plants in addition to both positive and negative controls then analyze whether these receptors are present using an RNA isolation kit. After isolating the RNA, it will be applied to a nanodrop to determine both the purity and concentration of RNA in each sample. The rationale for this approach is that the RNA can be isolated, analyzed, and then examined. The methods for this

specific aim help determine if peptidoglycan pattern recognition receptors recognizing bacterial peptidoglycan are conserved across plants by determining whether they are conserved in several invasive and noninvasive samples as well as both a positive and negative control. It is known that treatment with peptidoglycan from *Staphylococcus aureus* triggers plant responses, including medium alkalinization, increased cytoplasmic calcium levels, nitric oxide production, camalexin synthesis, and the post-translational activation of MAPK pathways (Gust et al., 2007). In Arabidopsis, the perception of peptidoglycans involves two receptor-like proteins (RLPs), AtLYM1 and AtLYM3, which contain lysine motif (LysM)-based ectodomains that specifically bind peptidoglycans (Willmann et al., 2011). It is believed that peptidoglycan perception in plants involves a multimeric receptor system that includes peptidoglycan-binding LysM-RLPs and signaling-transducing LysM-RKs, like CERK1 (Zipfel, 2014). This hypothesis builds upon the fundamental information available on peptidoglycan perception and tests various plants for the pattern recognition receptors that recognize bacterial peptidoglycan.

Summary of Preliminary Data: The data below pertains to the amount of RNA and the purity of the RNA in each plant species and the positive control. A260/A280 and A260/A230 are measurements of a sample of RNAs purity.

Table 1: Amount and purity of RNA in plant samples. *This data was collected in a lab setting via an RNA isolation kit and demonstrates the purity and concentration of the RNA samples that will later be analyzed for peptidoglycan pattern recognition receptors recognizing bacterial peptidoglycan.*

Plant	ng/µL	A260/A280	A260/A230
Rhamnus cathartica L.	1498.3	1.2	0.49
Euonymus alatus	3862	1.16	0.52

Celastrus scandens	3191.5	1.32	0.61
Lathyrus oleraceus	6476.2	1.71	0.59
Hypericum calycinum	9265.5	0.67	0.42
Positive control	930.1	1.95	0.34

In addition to the RNA samples, the results of the qPCR test are as seen in Figure 1 below. The figure illustrates the expression of three target genes, along with a negative control (H2O), in the selected plant sample. As anticipated, Arabidopsis thaliana successfully expressed all three genes of interest. This confirms that the experiment was conducted properly as the entire genome of this plant has been sequenced and published upon. Because of the LYM1 gene expression in both the Bittersweet and pea plants, the data suggests that muropeptides could be a viable method for biological removal, opening up potential applications in this area. One significant consideration is that the experiment is an n of 1, meaning it was performed only once and for its results to carry more significance and lead to stronger conclusions, the experiment needs to be repeated. For qPCR tests to be conclusive, they must be conducted multiple times. A limitation in this experiment is

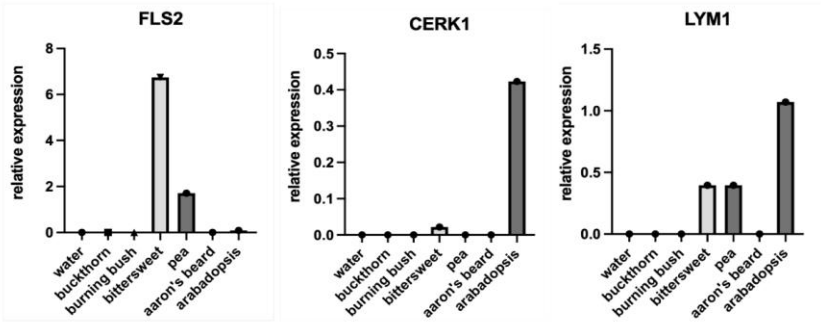


Figure 1: Expression of Genes FLS2, CERK1, and LYM1 in Plant Sample through qPCR. *This figure demonstrates the expression of three genes and a negative control, H2O, in the chosen plant sample. Because Arabidopsis Thaliana, as expected, expressed all three genes of interest the experiment can be considered successful. The fact that Bittersweet and the pea plant expressed the LYM1 gene opens up the door for the possible use of muropeptides as a biological removal technique.*

how the replicants varied from one another. This inconsistency could be due to impurities in the samples that could have potentially affected the accuracy of the results. It is crucial that the results are reproducible across multiple trials to draw definitive conclusions.

Expected Outcomes: This knowledge will be used for the treatment and eradication of invasive plant species. The expected result is that some of the plant species above will express the peptidoglycan pattern recognition receptors recognizing bacterial peptidoglycan.

Specific Aim #2: Test the hypothesis that peptidoglycan induces pyroptosis in plant species.

The objective for this specific aim is to determine the effects of peptidoglycan on plant species and test whether it possesses the ability to induce cell death in plants with the receptors and without them.

Justification and Feasibility: Our approach will be to apply peptidoglycan to various plant species in a killing assay to discover exactly what peptidoglycan does to each individual species with its own unique biological makeup. The rationale for this approach is that, by implementing peptidoglycan in a killing assay, the invasive management potential of peptidoglycan can be determined as well as its effectiveness in the eradication of plant species.

In previous research, pyroptosis, a form of programmed cell death, is typically characterized by both the rupture of the cell and the release of inflammatory molecules, such as interleukins (Saur et al., 2020). The hypothesis that peptidoglycan induces pyroptosis is based on research on NOD-like receptor-mediated plant immunity suggesting

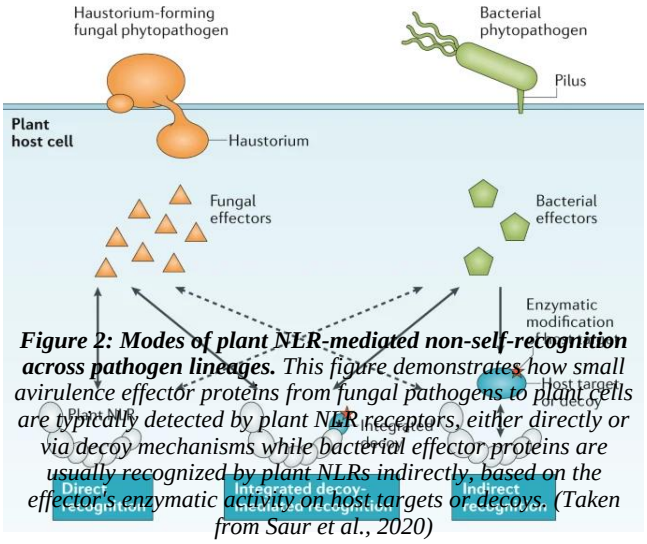


Figure 2: Modes of plant NLR-mediated non-self-recognition across pathogen lineages. *This figure demonstrates how small avirulence effector proteins from fungal pathogens to plant cells are typically detected by plant NLR receptors, either directly or via decoy mechanisms while bacterial effector proteins are usually recognized by plant NLRs indirectly, based on the effector's enzymatic activity on host targets or decoys. (Taken from Saur et al., 2020)*

cleaved gasdermins initiate pyroptosis by disrupting the plasma membrane, forming pores that increase its permeability (Lamkanfi M & Walle L, 2016).

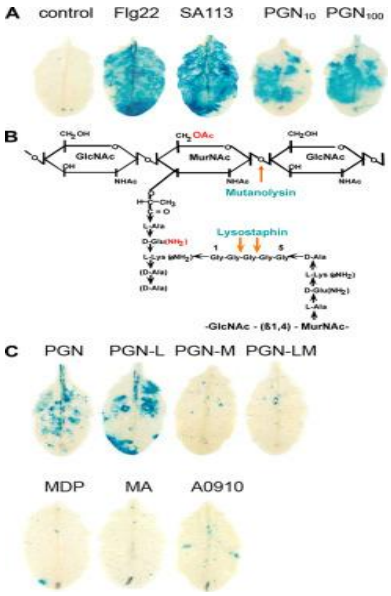
Expected Outcomes: The expected outcome of the killing assay is that some plants will undergo pyroptosis, demonstrating the potential use of peptidoglycan as an alternative for dangerous herbicides.

Potential Pitfalls and Alternative Strategies: If the application of peptidoglycan does not lead to pyroptosis, the various other possible effects of peptidoglycan will be observed and analyzed.

Specific Aim #3: Test the hypothesis that there is improved delivery and better absorption when applied to the leaves of plants rather than the roots or stem.

The goal of this specific aim is to evaluate if the leaves or roots are the most efficient part of the plant for absorption of peptidoglycan. This is important as it is believed that the amount of muropeptides absorbed by the plant will directly increases the severity of the immune response.

Justification and Feasibility: The approach is to infiltrate an invasive plant with an immune signal inducing synthetic peptide via both the leaves and the roots. The rationale for this approach is that we can test and analyze application to determine which part of the plant is the best administration location. To test this, a well characterized reporter gene such as, β-glucuronidase will be used to express color change after the application of peptidoglycan. This way, the absorption of the peptidoglycan can be quantified and comparable to that of the roots.



A previous study used a similar technique to characterize peptidoglycan recognition in Arabidopsis thaliana. The study investigated how peptidoglycan triggers early immune responses in plants, specifically examining medium alkalinization and the increase in extracellular pH. A concentration of 100 μg/ml PGN was found to be sufficient to trigger nearly maximal alkalinization and was used in later experimentation.

Figure 3: S. aureus peptidoglycan is perceived in A. thaliana plants*. Panel A shows the leaves from Arabidopsis PR1:GUS transgenic plants that were treated with various stimuli and the subsequent GUS activity. This indicates that peptidoglycan from S. aureus activates immune responses in Arabidopsis. Panel B illustrates the structure of S. aureus PGN and panel C presents a comparison of immune responses triggered by different forms of PGN.*

Expected Outcomes: The overall outcome of this aim is to determine the most effective administration method of peptidoglycan to invasive plants which is expected to be through the leaves of the plants.

Potential Pitfalls and Alternative Strategies: If there is no conclusive evidence for greater absorption between the parts of the plant then muropeptide application will not require targeting specific sections of plants.

Section IV: Resources and Equipment

This project requires the use of a lot of very specialized equipment and chemicals that are usually held in a lab setting such as a centrifuge, fume hood, chloroform, ethanol, trizol, RNase free water and spray, dry ice, RPMI medium, microscope, nanodrop, thermal cycler, RNA template, reverse transcriptase enzyme, DNA polymerase, muropeptides, forward and reverse primers, deoxynucleotide triphosphates, reaction buffer, and fluorescent dye.

Section V: Ethical Considerations

This project's goal is to harm and kill invasive plants in an environmentally beneficial way, although, there is a possibility that the finished product could also potentially kill native plants

which would damage ecosystems and reduce biodiversity. Although, this is a concern in the implementation of muropeptides to invasive species removal, not research. To keep myself and my colleagues safe throughout my experiment lab procedures such as fume hoods will be used as well as gloves, and more safety equipment when dealing with harmful chemicals.

Section VI: Timeline

My project will be complete by February 21st. Testing for my first specific aim will be done by January 20th. Testing for my second specific aim will be done by January 31st. Testing for my third specific aim will be complete by February 21st.

Section VII: Resources

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