Bio-based antibacterial seed treatments to improve soil and plant health

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III. Nontechnical Summary

The overall objective of this project was to perform a prove-of-concept study to develop bacteriophage-based seed treatments. In detail, the objectives were broken down as follows: 1) incorporate active bacteriophages into liquid polymeric seed coating formulations; 2) coat active bacteriophage in polymer solutions onto seeds; 3) test the protection of bacteriophage from the environment; 4) demonstrate that the seed treatments did not affect seed and seedling behavior; and 5) determine the efficacy of the treatment against soil-born and seed-borne bacterial diseases. This project was a new area of research for both investigators and required the hire of a new graduate student to focus their time on achieving the objectives. This student started in August 2016 which marked the start of the concentrated effort to achieve the project objectives.

Our efforts focused on incorporating bacteriophages into polymer solutions, coating them onto seeds, storing the seeds at different environmental conditions, and testing the coatings effects on seed germination and vigor (objectives 1, 2, 3 and 4). We used three polymers, that are typically used in commercial seed treatment formulation: Polyvinyl alcohol (PVOH), Polyvinylpyrrolidone (PVP) and Poly(methyl vinyl ether) (PMVE). CN8 bacteriophages were integrated into these polymers and bacteriophage activity was tested (Objective 1). When compatible combinations were found, we coated these treatment mixtures on maize seeds and placed the coated seed onto a plate containing *CMN* to determine if the bacteriophages stayed active. After incubation, the active bacteriophages lysed the bacteria around seeds indicating that the treatment retained active bacteriophage on a maize seed (Figure 1). After this success, we

initiated a detailed study analyzing both, the survival of CN8 bacteriophages in liquid polymer solution and when coated on maize seeds. During drying on corn seed bacteriophage concentration decreased with all polymers indicating that a stabilizer would be essential in the coating formulation (Objective 2). Sucrose, Whey Protein Isolate, Maltodextrin, D-Mannitol, and Skim Milk were tested in coating formulations for stabilization properties. Whey Protein Isolate (WPI) demonstrated the greatest stabilization properties in which it did not lose any active bacteriophage during the drying process.

Seeds treated with successful polymer-stabilizer-bacteriophage formulations were then stored at 4 ˚C, 10 ˚C, and 26 ˚C, to allow for observations to be made on a wide range of temperatures (Objective 3). Treatments containing WPI as a stabilizer, allowed bacteriophage to remain active after four months of storage at 26 ˚C and seven months of storage at 4 ˚C and 10 ˚C. Stabilization and storability of treated seeds shows that these polymer-WPI-bacteriophage coatings have the ability to be treated at a seed conditioning plant then shipped to the farmer and stored for a few months prior to planting. This is crucial in the chain of events, and allows for this treatment to directly fit in with the current practices in the seed treatment market.

Treatment formulations were then tested for their effect on seed germination and seedling vigor (Objective 4). Treatments were broken down into individual ingredients and treated on corn seed. Treated seeds were then germinated and tested for seedling vigor using dry seedling weight. Statistical analysis was conducted and no significant difference was seen among any of the treatments for germination or vigor. Indicating that the developed treatments do not inhibit germination or vigor.

The final objective of testing the efficacy of the treatments against Goss's Bacterial Wilt in corn was not reach as of December 31st, 2017. Time constraints of the long bacterial growth of CMN played a large role in the pace of this research project. CMN bacteria takes a minimum of 3-4 days to grow and because of this, some of the research goals had to be pushed back on the timeline. In addition this funding timeline was cut short due to the shutdown of the Leopold Center which did not allow us to reach the last objective. Studies evaluating the effectiveness of the treatments will be done at a later time.

In the end, the central question asked in our study was to determine if bacteriophage can be stabilized on seed and used to control bacterial plant diseases. Currently, the only treatments for bacterial diseases are copper compounds and antibiotics, which both have resistance issues. In this study we demonstrated the proof of concept that bacteriophages can be incorporated into seed treatment polymers, coated on seed and stored for long periods of time without losing activity. We demonstrated that these developed treatments had no inhibitory effect on germination or vigor. Future studies will determine their effectiveness against bacterial diseases, but the stabilization of the treatment on seed was crucial before evaluating their efficacy. Similar bacteriophage treatments are now possible against other plant pathogens providing a new biological bactericide with less environmental impact.

IV. Detailed Report

A. Introduction

As plant pathogens, seedborne and seed-transmitted bacteria and fungi cause losses in agronomic and horticultural crops in the hundreds of millions of dollars per year worldwide. For

example bacterial disease in corn caused by *Clavibacter michiganense subsp. nebraskense* (Goss's wilt) can occur at any stage of plant development and lead to yield losses of 20% to 60% (Ruhl et al. 2009, Jackson et al. 2007, Treat et al. 1990). In 2011 a Goss's wilt outbreak in Iowa caused severe losses (Robertson 2011).

Standard procedures to prevent seedborne and seed-transmitted diseases in plants are the application of seed treatments. Bacterial diseases are often controlled with copper compounds. These can lead to copper-resistant bacteria strains, especially if weather conditions favor the development of the bacterial disease (Abbasi et al. 2002). For the control of bacterial speck both copper-based bactericide and streptomycin have been used with varying success and development of resistance in the bacteria (Wilson et al. 2002). A lot of the chemical treatments are non-specific meaning they not only attack the pathogens, but also beneficial organisms on the seeds and after planting in the soil. These treatments can significantly impact the environment and decrease the soil health (Koller et al. 1999, Wang et al. 2009), potentially requiring the addition of further chemicals to increase the yield. The use of alternatives means for pest control can reduce the threat of pest resistance development and promote a sustainable farming environment.

Biocontrol agents, like beneficial bacteria, fungi or biological derived molecules (Harman 1991, Shoda 2000) may not negatively affect the soil, sometimes even improve the soil health (vanDiepeningen et al. 2006), and may be used in organic farming. For example, a combination of foliar and seed application of beneficial bacterial strains led to a reduction in bacterial-speck-disease severity up to 32% in field trials (Ji et al. 2006). There are commercially available biocontrol agents against nematodes (VOTiVO, Bayer Crop Science) and against fungi (Serenade and Sonata, Bayer CropScience) based on non-pathogenic bacteria (Whipps 2009).

Bacteriophages, a naturally-occurring virus against bacteria, are a possible biocontrol agent against bacteria. Bacteriophages have been used as biocontrol agents in greenhouses and field trials with varying success (Frampton et al. 2012). Challenges included interactions with other microbes, contaminated phage-preparations and loss of phage viability in the presence of chemicals. As with chemicals bacteria can develop resistance to bacteriophages requiring a careful selection of phages for use as biocontrol agents. Often combining multiple phages binding to different receptors for the same host in a 'phage cocktail' can prevent resistance due to receptor mutations (Tanji et al. 2004, 2005). Choosing phage that are unaffected by the 'clustered regularly interspaced short palindromic repeat (CRISPER) array' (Deveau et al. 2008), can also prevent bacteria resistance, since about 40% of all sequenced bacteria contain this feature potentially able to prevent phage replication (Al-Attar et al. 2011).

Another challenge is the influence of environmental conditions on phage stability. Phage applied in the phyllosphere suffer degradation under UV light, which requires protective formulations and specific timing for foliar applications of phage (Balogh et al. 2010, Balogh et al. 2003, Iriarte et al. 2007, Jones et al. 2007). Nonetheless Omnilytics developed AgriPhage products against tomato and pepper spot, which are commercially available and recognized by the Organic Materials Review Institute in 2006 to be compatible with organic food production (Monk et al. 2010). In soil bacteriophage can survive at least one month (Assadian et al. 2005) depending on

environmental factors such as pH, and moisture. In a greenhouse trial bacteriophage survived 4 month and were able to protect tomato seedlings against *Ralstonia solanacearum* (Fujiwara et al. 2011) when applied to the soil or to cuts in the roots of the seedlings. Bacteriophages have also been directly applied to seeds. For example, Adachi et al. (2012) successfully coated rice seeds with bacteriophages by immersing them into a suspension of phage against *Burkholderia spp.* reducing the number of diseased seedlings. Basit et al. (1992) adsorbed phage against *Bradyrhizobium japonicum* to soybean seeds with phage reducing nodulation without the additions of seed coating formulations.

Differently from the studies above, we applied bacteriophages in combination with polymeric seed coating formulation to the seeds before planting. Mixing seed coating formulations with active ingredients before coating phages onto seeds will increase their adherence to the seeds and reduce dust-off, while improving the seed flow, plantability and appearance (Taylor 2003, Taylor et al. 1990). Seed coatings can also reduce seed imbibitional injury (Baxter et al. 1986, Willenborg et al. 2004) or retard seed germination by reducing polymer permeability (Johnson et al. 1999, Ni 2001, Pecinovsky 2005). However, these polymers can negatively affect seedling emergence when environmental stress is not severe enough to break the polymer (Willenborg et al. 2004); when the active ingredient associated with the polymer becomes phytotoxic to the seed in the laboratory and in storage (Goggi 2009); or when the polymer interferes with seed germination pathways. Not only the polymer, but also the seed treatments themselves can affect seed germination, vigor, and storability (Goggi et al. 2009, Krueger et al. 2012, Mbofung et al. 2013). Even organic seed treatments based on plant essential oils may have unintended effects on seed quality (Christian et al. 2008). It is essential that all new seed treatments are evaluated for their efficacy against the intended target pathogen, but also evaluated for their effect on unintended targets such as soil health, seed germination, vigor and storability.

In the case of bacteriophages, seed coating formulations can address a number of challenges encountered in their use as biocontrol agents: 1) phage viability can be increased, since the phage are isolated from the environment both during storage (temperature, moisture and light fluctuations) and during or after planting (mechanical stress, temperature and moisture fluctuations, presence of soil microbes); 2) mixtures of phages, which usually show better efficiency with less bacterial resistance (Tanji et al. 2004, 2005) than single phage treatments, can be applied to the seeds at the appropriate ratios, independent on their absorptivity to the seeds; and 3) phage can potentially be combined with other seed treatments, since a multilayer coating can separate phage from potentially harmful chemicals.

The overall objective of this proposal was to perform a prove of concept study to develop bacteriophage-based seed treatments. We combined the advantages of polymeric seed coating formulation and phage-based biocontrol to achieve a treated and storable seed in which the phage are protected from the environment. In this prove of concept study we will used a single phage against *Clavibacter michiganense* subsp. *nebraskense*—the bacteria causing Goss's wilt in maize in combination with different seed coating polymers.

To achieve our overarching objective we completed the following specific objectives:

(1) *Incorporated active bacteriophages into liquid polymeric seed coating formulation.* We tested the survival of the phages in commercially available seed coating formulations (faster product development) and polymeric solutions/suspensions with defined molecular weight and charge (better scientific understanding of the interactions of phages with materials).

(2) *Coated active bacteriophage in polymer solutions onto maize seeds.* We coated the seeds by dipping them into polymer bacteriophage mixtures and air drying them for 24 hours.

(3) *Tested protection of phage from the environment.* We stored the coated seeds at temperatures between 4 °C and 26 °C for up to seven month testing for phage activity every couple of weeks.

(4) *Demonstrated that the seed treatment do not affect seed and seedling behavior***.** We performed standard germination and vigor testing as stated in the AOSA handbook..

B. Project Design, Methods, and Materials

1. Bacteria

Bacteria inoculum for *Clavibacter michiganense subsp. Nebraskense (CMN)* was prepared from a 96 hours bacteria culture that was grown on Nutrient Broth Yeast Extract (NBY) medium (8 g nutrient broth, 2 g yeast extract, 2 g K_2HPO_4 (anhydrous), 0.5 g KH_2PO_4, 974 mL distilled water, 15 g agar, 25 mL 20% glucose, and 1 mL 5% $MgSO_4$) at 26 ºC. One colony from this bacteria culture was selected and used to inoculate a flask of NBY media (did not contain agar). The flask was put onto a mini bench top shaker (Fisher Scientific incubating mini-shaker) at 350 rpm and 26 ºC for 72 hrs. This liquid bacteria culture was then stored at 4 ºC.

CMN cultures were enumerated on NBY medium agar (1.5%agar) and concentrations were reported as colony forming units (CFU) per milliliter.

2. Bacteriophages

CN8 bacteriophages were propagated on CMN overlays. 50 µl from the -80ºC glycerol stock (25% glycerol, 25% distilled water and 50% CN8 bacteriophages) was spread on overlays consisting of 10 mL NBY bottom agar (1.5% agar) and 10 mL NBY top agar (0.5% agar). The top agar contained 100 µl of CMN liquid bacteria culture. This plate was then incubated for 72 hrs at 26 °C. After incubation 5 mL of sterile lambda buffer was added to the plate and the top agar was scraped off using a sterile smear stick and placed into a sterile 50 mL centrifuge tube. The mixture was vortexed until homogenous. Mixture was separated into 15 mL conical tubes and centrifuged at 6500 rpm for 5 min. The aqueous was decanted and passed through a 0.22 µm filter. The filtrate that passed through the filter now contains the CN8 bacteriophages. The concentration was determined using serial dilutions and plated onto an overlay. The concentration is reported as plaque forming units (PFU) per milliliter. CN8 bacteriophages solutions were stored at 4°C.

3. Polymer and coating solutions

Three polymers with a vinyl-backbone and different functional groups were used to coat bacteriophages on maize seeds: Polyvinylpyrrolidone (PVP, 58,000 g/mol), polyvinylalcohol (PVOH, low molecular weight), and poly(methyl vinyl ether) (PMVE, 30% solution in water). Polymer were dissolved in deionized water (10% w/v) and stored at room temperature before

use. PVOH required heating at 80 ºC for 20 min to dissolve. Seed treatments contained 3.5 w/v% polymer, 2 w/v% stabilizers and $5.0x10^7$ to $5.3x10^8$ PFU/mL CN8 bacteriophage in water. Stabilizers included Whey Protein Isolate (WPI), Skim Milk, Sucrose, Maltodextrin, and D-Mannitol. Solutions were prepared fresh before use.

4. Coating maize seeds

We used both the vortex and the soaking method to coat maize seeds with coating solutions. The vortex method consists of putting maize seeds into a 50 mL conical tube and adding 1 ml of the polymer-bacteriophages coating solution to the tube (30 maize seeds). The tube containing the seeds and the coating solution was then vortexed for 2 min. After the 2 min the seeds were taken from the tube and placed onto a sterile petri dish inside a biological-safety cabinet and left to dry for 24 hours.

The soaking method involves completely submerging the maize seeds in the coating solution. Maize seeds were added to a 50 mL conical tube and the coating solution was poured over the top of the seeds until the seeds were completely submerged in 10 mL of the coating solution. Completely submerged maize seeds were left in the solution for 15 min. After submersion the seed were placed onto a sterile petri dish inside of a biological-safety cabinet and left to dry for 24 hours.

5. Maize seed sterilization and Accelerated Aging Test

Maize seed samples were surface sterilized in 8% sodium hypochlorite for 10 min and rinsed 3 times in sterile distilled water. The seeds were transferred to sterile aluminum foil and allowed to air-dry overnight in a biological safety cabinet at ambient temperature.

The Accelerated Aging test (AA) consists of placing the maize seed on an elevated screen in a single smooth layer. The screen containing the seeds is placed into an acrylic box that contains 40 ml of water. The box is covered with a tight-fitting lid and placed into the AA chamber, in which the seeds are exposed to 43°C and 95-99% relative humidity. The maize seeds were left in the chamber for 96 hours. The seeds are removed from the chamber and promptly re-sterilized using the method above.

6. Germination and Vigor

Germination tests were conducted using the rolled brown paper towel method (AOSA, 2017). In short, 50 seeds were placed down the middle of two damp paper towels and one damp paper towel was placed on top of the seeds. Paper towels were rolled up and a rubber band was placed around the paper towels to hold the seeds in place. Two replicates of 50 seeds were used for each treatment, placed in their own bucket, and covered with a plastic bag that was secured with a rubber band. Buckets were randomly placed into modified food service carts (Lincoln Foodservice Products). Germination was performed at 25 °C for 7 days in the dark, and rating using a standard scale (AOSA, 2017) occurred on day 7.

Seedling vigor was determined by separating germinated seeds from the roots and shoots, and drying the roots and shoots at 75 ºC for 16-20 hrs. Dry seedlings were weight and the average weight per seedling calculated (AOSA, 2009). Germination and vigor experiments were replicated twice which replications planted 7 days apart.

C. Data and Discussion

On Seed Drying with and without stabilizer

All CN8 bacteriophage solutions dried on seed showed infective bacteriophages after drying (Figure 2). Seeds placed directly on an overlay showed visual lysis of *CMN* bacteria which decreased after drying, and storage (Figure 1). While quantitative analysis of the lysis area is related to the number of infective bacteriophages for two dimensional materials, e.g., paper (Meyer et al., 2017), for three dimensional seeds inconsistent contact between the seeds and the overlay made this analysis too inconsistent. Visual lysis was used only to confirm that bacteriophages in the biocontrol coatings can infect bacteria in a moist environment, e.g., seeds planted in soil.

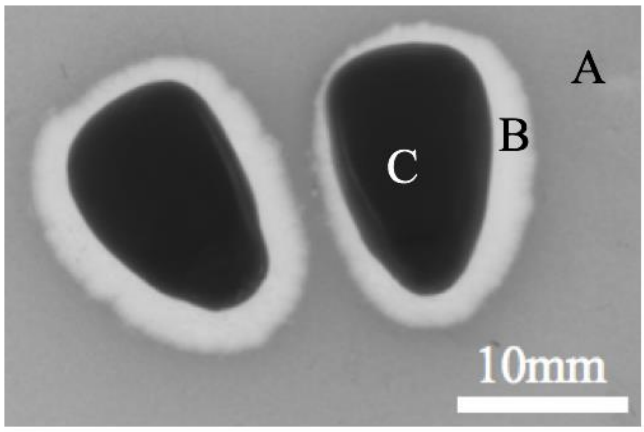

Figure 1. Maize seeds (C) coated with CN8 bacteriophages (5.0x10^8 PFU/mL) in 10% PVOH on an CMN lawn (A) after 72-hour incubation. The lighter area around the seeds (B) indicates lysis of CMN by infective CN8 bacteriophages.

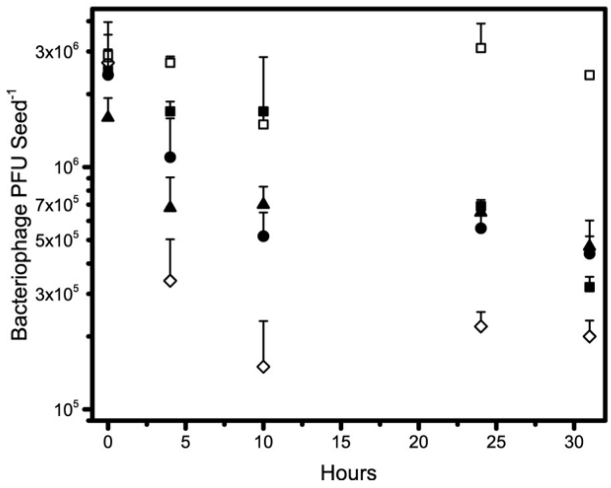

Figure 2. Average CN8 bacteriophage concentration on maize seed during drying. Treatments consisted of: open diamond – no polymer, closed square – PVOH, closed circle – PMVE, closed triangle – PVP, open square – PVOH + WPI. Error bars indicate two times started error and only negative error

bars shown for visual purposes.

For qualitative analysis we dissolved the coating in buffer and determined the number of bacteriophages in that solution. The number of infective bacteriophage in the solution and with that on the seeds depended on the time of drying and the polymer and stabilizers used in the biocontrol coatings (Figure 2). The number of infective bacteriophages decreased significantly during the first 10 hours of drying and more slowly after that. For most coating formulations the number of infective bacteriophage leveled off after 24 hours. For bacteriophage applied from buffer only 8% survived at 24 hours, while with polymers 23% to 41% survived. Adding WPI to PVOH led to dry biocontrol coatings without any loss of bacteriophage infectivity.

We tested maltodextrin, D-Mannitol, sucrose, lactose, whey protein isolate, and skim milk which have been shown to stabilize proteins or bacteriophages during drying (Clark et al., 1962; Merabishvili et al., 2013; Vonasek et al., 2014). When drying CN8 bacteriophage in PVOH (3w/v%) with the presence of the sugars or proteins the amount of infective bacteriophages was 0.86 to 3.39 times better than in PVOH alone. Sugar-based stabilizers were less effective (0.86 to 2.68 times) than protein-based stabilizers (2.86 to 3.39 times), skim milk and whey protein isolate retained the greatest number of infective bacteriophages.

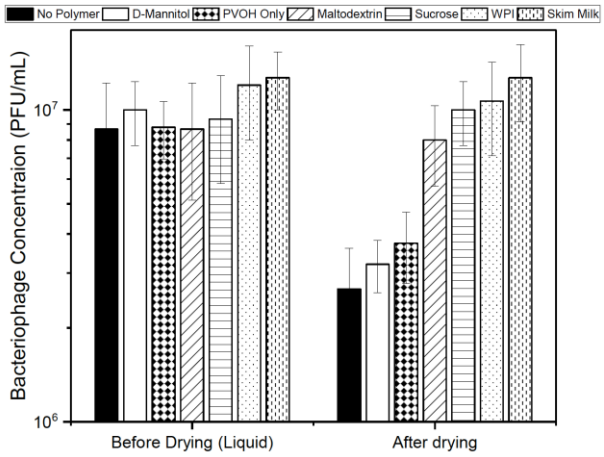

Figure 3. Stability of CN8 bacteriophages in PVOH polymer film before and after drying. Mixtures contained 3.5 % polymer, 2 % stabilizer and 94.5 % bacteirohpages. All polymers follow the same trend, PVOH data shown. Error bars indicate two times standard error. All polymer follow same trend, only PVOH data shown.

Storage depending on temperature

A lower storage temperature led to longer storage stabilities which was further improved by adding WPI. At 4°C all treatments even those without polymer showed activity for two months, with polymer and WPI the storage time could be extended to four months (Figure 4). After that time the number of infective bacteriophage decreased from PVOH and PVP with WPI, over PMVE with WPI, to PVOH and PVP without WPI, while the other treatments showed no infective bacteriophage.

After three month at 10°C showed infective bacteriophage (Figure 4). One month later the treatments without polymer and with PMVE had no infective bacteriophage. Two months later at five months seeds coated with PVP had no infective bacteriophage.

Storage stability further decreased when seeds were stored at 26°C with only seeds coated with PVOH and WPI showing any infective bacteriophages after two months. After one month PVOH coatings and polymer coatings with WPI were the only ones showing infective bacteriophages.

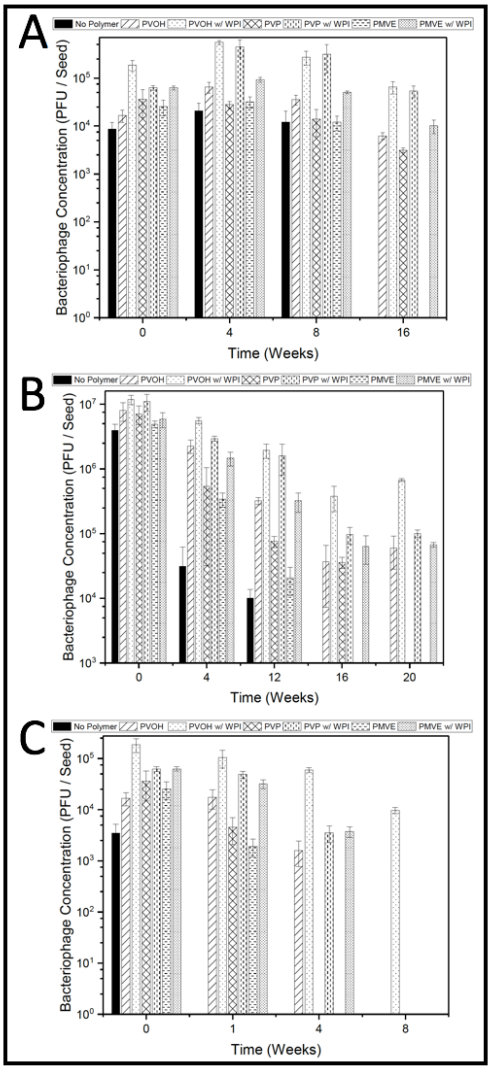

Figure 4. Stability of CN8 bacteriophages on treated maize seed as a function of time. Seeds were stored at 4 ºC (A), 10 ºC (B) and 26 ºC (C). Treatments were as follows: control (no polymer or stabilizer), PVOH, PVP, and PMVE each with and without the addition of WPI. Error bars indicate two times standard error.

Germination and Dry Seedling Weight

All treatments containing bacteriophages, stabilizer, or polymer did not have an effect on the germination rate ($P > 0.05$) (Figure 5) Germination rates ranged from 97.5 % to 99.5 %. Seedling vigor was determined by the dry weight test (AOSA, 2009). The means of seedling dry mass

ranged from 42.1 mg to 49.2 mg. Means were not significantly different (P > 0.05) (Figure 5). Results indicated that all polymers could be used for the treatment of corn while not affecting germination rate and seedling vigor.

Treatment	Avg. Germination (%)	Avg. Dry mass / Seedling (mg)	Avg. PFU / seed
Controls			
No Treatment	98	42.1	
Sterile deionized water	99	47.1	
Polymer, Stabilizer and Bacteriophage			
WPI Only	98	49.2	
PVP Only	99.5	48.6	
PVOH Only	98.5	44.1	
PMVE Only	98.5	47.6	
PVP + WPI	97.5	48.6	
PVOH + WPI	98.5	45.7	
PMVE + WPI	99.5	47.7	
Phage Only	99	49.2	9.05E+03
PVP + WPI + Bacteriophages	97.5	44.8	1.51E+06
PVOH + WPI + Bacteriophages	98.5	46.4	2.08E+06
PMVE + WPI + Bacteriophages	99	48.6	1.08E+06
F Test	ns	ns	

ns = not significantly different

Abreviations: PVP - polyvinylpyrrolidone, PVOH - polyvinylalcohol, PMVE - Poly(methyl vinyl ether), WPI - Whey protein isolate

Figure 5. Germination rate and seedling weight for treated corn seed. Treatments were broken down into their individual components. ns indicating that samples were not significantly different from each other.

D. Conclusions

Our results indicate that bacteriophages can be incorporated into seed treatment polymers, coated on seed and stored for long periods of time without losing activity. We demonstrated that these developed treatments had no inhibitory effect on germination or vigor. With these results, disease control studies will now be possible. Future studies will determine their effectiveness against bacterial diseases. Stabilization of the treatment on seed was crucial to evaluate before evaluating their efficacy, because newly developed treatments must fit into the industry handling procedures for treatment and storage. This will allow these new bacteriophage treatments to get to market faster without requiring seed conditioning plants to use new storage and handling conditions. Similar bacteriophage treatments are now possible against other plant pathogens providing a new biological bactericide with less environmental impact.

E. Impact of the Results

The objectives of the project were achieved with time permitting. Due to the Leopold Center shutting down, our project was cut slightly short and we did not get to the disease control studies. The results that we did gather will pave way for the use of bacteriophage based products in the seed treatment industry. Bacteriophage seed treatments have shown prior disease control success (Adachi et al., 2012; Basit et al. 1992), but have not been stabilized on seed. Stabilization on seed allows for the efficacy to remain for longer periods of time. This provides the ability for seed processing plants to treat the seed, store the seed, and ship the seed to the farmer while not losing any efficacy of the treatment. In addition, bacteriophage based treatments provide farmers with an option to combat soil-borne and seed-borne bacterial diseases that they did not have

before. With the issues surrounding the use of copper compounds and antibiotics there are not any options to control bacterial diseases. It will also provide this same benefit to organic farmers as bacteriophage are a natural organism and can be used in organic farming.

F. Outreach and Information Transfer

Publications

A publication presenting all of the information from this project will be submitted to the Journal Biotechnology and Bioengineering.

Education and Outreach

Results were presented as an oral presentation at the Iowa State University Seed Treatment workshop in 2017. The presentation was in Ames, IA and the audience consisted of industry and academia personnel. Results were also presented in a poster at the 2017 Leroy & Barbara Everson Seed Symposium in Ames, IA. The audience again consisted of industry and academia personnel.

Cooperative Efforts and Student Support

This project supported one Master's graduate student in the Agronomy department. Four undergraduate research students, and one high school research student helped the graduate student with data collection.

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H. Leveraged Funds

There were no other funds leveraged for this project.

I. Evaluation

There was no formal evaluation of the project.

V. Budget Report

A. Total request was $72,219. Expenditures year one - $18,674; year two - $50,560. Expenditures in year two were much higher than year one, since project work only really started halfway through the first year.

B. The primary expenditures for the grant were salaries and laboratory supplies, including seeds. There were no major differences in expenditures during years one and two.

C. No other funds were leveraged for the project.