Report for the Organic Farming Research Foundation

Title: Managing indigenous seed-inhabiting microbes for biological control against *Fusarium* pathogens in corn

Principle investigator: Lucas Nebert, M.S. Soil Quality, PhD Candidate, University of Oregon Environmental Studies, Science and Policy Program

Research Advisers

Brendan Bohannan, tenured professor University of Oregon Institute of Ecology and Evolution

Cindy Ocamb, associate professor and extension specialist, Oregon State University Department of Horticulture, Botany and Plant Pathology

Participating farmers

Andrew Still and Sarah Kleeger, Adaptive Seeds
Jeff Bramlett and Carri Heisler, Pitchfork and Crow

1. Summary

1.1 Project Overview

This project was initially approved from January 10th 2014 to January 10th 2015. Beginning spring of 2014, the project was first extended to May 10th 2015, and eventually to Dec. 31st 2015. While the field research was successfully conducted in 2014, we needed nearly the entire year of 2015 to troubleshoot DNA-based methods and to process the seed and plant samples for DNA-based pathogen detection and next-generation DNA sequencing.

In addition to optimizing DNA methods, by the end of the 2014 field season, the lab had established a high-throughput culture-based workflow to screen seedborne bacteria for their biological control ability against pathogenic *Fusarium* (section 3.1). Using this workflow, we found promising biocontrol candidates that could effectively reduce *Fusarium* infection by being applied directly to the seed. While it was too late to use one of our lab cultures in the 2014 field season, we took advantage of the extra 2015 field season to test two promising biocontrol candidates in an additional, small field experiment at Oregon State University.

This project will become a chapter of my PhD dissertation and part of it will likely become a publication in a peer-reviewed scientific journal. Given the wealth of DNA sequencing data generated with the grant, it is probable that many important findings have yet to be uncovered. Furthermore, I will continue to screen seedborne microbes for biocontrol against *Fusarium*. OFRF should expect to receive follow-up reports as I continue to dedicate more of my time to exploring the rich DNA sequencing dataset and continue culturing of beneficial microbes.

While the bulk of analysis of the DNA sequencing dataset is still ongoing, the DNA-based methods that have come out of this project provide important contributions to the rapidly-growing field of plant microbiome research. It is the first project, to my knowledge, that utilizes next-generation DNA sequencing to study microbial endophytes in corn. Moreover, it is the first of its kind to study the impact of seed treatments on the microbiome of the plant.

1.2 Research purpose

The purpose of this research project is to determine the ability of seedborne microbes to control pathogenic *Fusarium* in open-pollinated, organic flint corn. Futhermore, it is to investigate how seed disinfection treatments may affect the efficacy of microbial inoculants and any disease caused by *Fusarium*. *Fusarium* is nearly ubiquitous in corn, as the fungus is well-adapted to the biochemistry of corn (e.g. 2-Benzoxazolinone, BOA; see Saunders & Kohn, 2009), and is efficiently inherited in its seeds. Furthermore, *Fusarium* can remain in the soil ecosystem for long periods of time, particularly if it is rich in organic matter (Cindy Ocamb, personal comm.). Thus, pathogenic strains will build up in corn crops over the years, and can be transmitted to new farms via the seed. In addition to threatening crop production, seedborne *Fusarium* presents a risk to human and animal health, as it can produce carcinogenic mycotoxins that may go undetected into animal feed and into our food supply (Bacon, Glenn, & Yates, 2008)**.**

When not in its pathogenic state, *Fusarium* is considered an endophyte. Endophytes are bacteria or fungi that reside inside the plant, often systemically, without causing apparent disease. Like *Fusarium*, many endophytes can utilize seeds to transmit across plant generations. However, the vast majority of

seedborne bacterial and fungal species are not pathogenic. In treating seeds against pathogens like *Fusarium*, seed companies may inadvertently be removing beneficial seedborne endophytes. To find strains of bacteria that are antagonistic to seedborne Fusarium, it makes sense to screen bacterial endophytes, particularly those found in the seed, where the bacteria would be intimately competing with Fusarium for this specialized ecological niche. Inoculation of seeds with beneficial microbes, rather than disinfecting the seed, may prove to be a more judicious approach to controlling pathogenic seedborne *Fusarium*.

With the advent of better DNA methods such as next-generation DNA sequencing, we are able to study microbial ecology for more depth and clarity than ever before. In agriculture, we can use this technology to understand how practices affect the composition and integrity of microbial communities in the soil and inside our crops. We sought to utilize this technology to understand the effects of antimicrobial seed treatments and biocontrol microbial inoculants in relation to *Fusarium* crown rot in organic systems.

1.3 Project Objectives

Below are the proposed project objectives, each followed by a summary of the extent they were accomplished.

Objective 1: Measure the efficacy of seed inoculation with indigenous seed-inhabiting microbes for biological control against *Fusarium* pathogens.

Objective 1 results summary. We proposed to search for corn seed-inhabiting microbes that were promising candidates for field-testing against *Fusarium*. By the time the 2014 field season had begun, we had yet to find an adequate biocontrol bacterium from seeds, so we went with the proposed contingency plan, and used a commercially available biocontrol inoculum. We opted for a blend of bacteria that had been field-tested by OSU collaborator Cindy Ocamb, and proven effective at reducing *Fusarium* crown rot and increasing yield in conventional sweet corn F1 hybrids. Overall, we found that effectiveness of this inoculum depended on the farm on which it was used. By spring of 2015, we had discovered two promising seedborne bacterial endophytes after further developing our methodology for screening bacteria against pathogenic *Fusarium* in growth media and on corn seedlings (section 3.1). We set up a small field trial at OSU in 2015 and found promising results from one of the isolates, which merits future testing in organic systems (section 3.2).

Objective 2. Test combinations of disinfecting seed treatments and biological seed treatments for persistent reduction of seed-borne *Fusarium* pathogens.

Objective 2 results summary. We developed a series of hypotheses (section 2.1) for how seed treatments would affect *Fusarium* crown rot in the field and the proportion of seedborne *Fusarium* found in harvested seeds. We planted at organic farms Adaptive Seeds and Pitchfork and Crow, in addition to Oregon State University Botany and Plant Pathology Farm, which is maintained to have a high *Fusarium* pathogen pressure. During the plant pollination stage on each farm, we measured the degree of crown rot across seed treatment plots. We also measured the treatment effects on the yield and amount of *Fusarium* inherited in the seeds. We observed different, and sometimes opposite, responses to seed treatments, depending on the farm and on the source of the seed (section 2.4)

Objective 3. Determine how disinfecting seed treatments and biological seed treatments impact the seed-associated microbial communities as a whole.

During the 2014 field season, we collected tissue samples from the crown and harvested seeds across all treatments, and extracted DNA to study entire microbial communities present in the samples. Due to the unexpectedly long time required to obtain an adequate high-throughput sequencing dataset from these samples, and the sheer size of the dataset (10 million+ microbial DNA sequences from 192 seed and plant samples), we have not yet determined how disinfection or inoculation treatments affected microbial communities as a whole. Data analysis for next-generation sequencing is still ongoing.

Objective 4. Detect novel microbes associated with the corn plant's resistance to *Fusarium*.

We have successfully isolated bacteria from corn seed that limited the growth of *Fusarium* in petri dishes and reduced *Fusarium* infection in lab-raised corn seedlings. A small field trial in 2015 proved that one strain of bacterial species, *Arthrobacter ilicis*, was associated with less *Fusarium* crown rot and a higher yield. However, these results were marginally significant, due to small sample size and high variation in the field, and further testing is needed. Through this initial study, we have developed a high-throughput workflow that screens bacterial isolates from seed for anti-*Fusarium* bacteria, allowing for the further discovery of biocontrol candidates that can be tested in the field. Furthermore, we have produced a dataset that contains microbial DNA sequences from crown tissue samples across a range of crown rot. Using this dataset, we can discover species of bacteria and fungi that are associated with plants that were resistant to *Fusarium* and tailor our microbial isolation methods to culture them.

Objective 5. Make research methodology and results transparent and transferrable for public education, discourse, and utilization.

We strongly believe that research methodology should be open and shared so research is transferrable to different farming systems. We have developed microbial DNA-sequencing-based and microbial-culture-based methods that can be utilized together to discover beneficial microbes across different corn varieties, and crop species, depending on the demands of the local community and geography. We have begun publishing our research methods and results online at <www.microbialinheritance.org>. I have worked with a computer programmer to create an interactive data visualization tool, where data produced with this grant can be uploaded online for web users to view and interact with. Currently, this feature displays simple histograms of different microbes across samples and corn varieties. However, we see this as a working model to promote further interest for expanding this capability. In addition to sharing research methods and results from field research, I have been promoting interest in this topic among the organic seed community by allowing seed growers to send me seed samples for identification of seedborne microbes.

2. Field Experiment 2014

The 2014 field study was the main project conducted under this grant, conducted across two organic farms and an experimental field at OSU. It was designed to address a series of hypotheses pertaining to the practice of disinfecting seeds and inoculating seeds with biological control bacteria, and the effects on pathogenic *Fusarium* in open-pollinated flint corn.

2.1. Hypotheses

Hypothesis 1: Disinfecting seeds against seed-assocated microbes will leave plants more susceptible to soilborne pathogenic Fusarium. *Rationale*: Seed disinfection may remove seedborne microbes that would otherwise protect the seed from soilborne *Fusarium*.

Hypothesis 2: Disinfecting seeds against seed-associated microbes will significantly reduce the fitness of a plant, as measured by seed harvest. *Rationale*: Seed disinfection will remove beneficial seedborne microbes that may generally increase plant yield on organic farms.

Hypothesis 3: The biocontrol inoculum will reduce *Fusarium* crown rot and increase yield. *Rationale*: The biocontrol inoculum has been proven effective at decreasing *Fusarium* crown rot and increasing yield in conventional sweet sweet corn systems.

Hypothesis 4: Disinfecting seeds against seed-associated microbes will significantly increase the effectiveness of a biocontrol inoculum for reducing *Fusarium* crown rot, and increasing yield. *Rationale:* Seed disinfection will reduce microbial competition for the biocontrol inoculum, allowing it dominate the seedling and be more effective against *Fusarium*.

Hypothesis 5: Seeds from different sources will respond differently to seed treatments, and *Fusarium* pathogen pressure. *Rationale*: Despite being genetically similar to each other, seeds are expected to respond differently to seed treatments, due to carry-over of different microbial communities from different farms.

2.2 Experimental Design

Seeds that had been saved from the 2013 season by Adaptive Seeds and Pitchfork and Crow were treated with four different seed treatments before planting on each respective organic farm (Table 1). Both seed sources were planted together on the OSU BPP Farm for a direct comparison. In all cases, seeds were planted according to a randomized block design, to account for variation across the fields. Planting dates for the organic farms depended on their own availability for soil preparation. In all cases, seeds were spaced at 12” intervals. Seeds were planted about a month later on the OSU BPP to increase heat stress in maize seedlings, which is known to increase *Fusarium* pathogen pressure. Although not part of the experimental design, the Pitchfork and Crow experimental plots developed significant weed pressure over the growing season, which should be considered a factor affecting research outcomes.

Seed sources

The seeds used in this experiment belong to a northern flint variety called Cascade Ruby-Gold, which was bred by author and plant breeder Carol Deppe, from New England varieties Roy Calais flint and Byron. Adaptive Seeds (AS) grew the variety in 2012, before giving some to Pitchfork and Crow (PC). Both farms independently grew out this selection in 2013. During the 2014 field experiment, both farms grew their own 2013 selections, and the 2013 selections from AS and PC were both planted in the OSU field, for direct comparison. The seeds only differed over a single year of mass selection, so it is unlikely that these diverse populations diverged significantly from each other in terms of underlying genetics. We presume that most differences are due to epigenetic factors such as carry-over of distinct microflora.

Table 1: Experimental Setup of 2014 Field Season

	Adaptive Seeds (AS) organic farm	**Pitchfork & Crow (PC)** organic farm	**OSU BPP (OSU)** *Fusarium* field
Amount planted	1200	1536	3456
Seed source planted	AS 2013	PC 2013	AS 2013 + PC 2013
Planting date	15 May	29 May	20 June
Treatments	4 total: Control Disinfection Inoculation Disinfection + Inoculation	4 total: Control Disinfection Inoculation Disinfection + Inoculation	8 total*: AS & PC: Control, Disinfection, Inoculation, Disinfection + Inoculation
# Replicate plots / treatment	15	24	18
# Plants / replicate plot	22	20	24
# Plants for disease scoring	30	24	36
Fertilization method	Perfect Blend 100 lbs/acre	Stutzman's 4:3:2 2,000 lbs/acre	12:29:10:8 450 lbs/acre
Notes	Ideal growing conditions	High weed pressure	High pathogen pressure

Seed disinfection treatment

For disinfection of the seed, we soaked seeds in 240ppm peracetic acid (PAA) for 4 hours at room temperature, in flasks placed in a rotary shaker at 120 rpm. The use of PAA on seeds is approved by OMRI, as an alternative to hydrogen peroxide. To disinfect deeper into the seed following PAA soak, the seeds were then submerged in a sterile water bath at 60°C (140°F) for 5 minutes before being transferred to ice water to cool them to ambient temperature. Control seeds were simply soaked in room-temperature sterile water for 4 hours. Seeds were allowed to air dry before applying the inoculum.

Biocontrol inoculum

Following disinfection, seeds were treated with a biocontrol inoculum, a cocktail of 8 bacterial strains isolated by Cindy Ocamb that together have been shown to be antagonistic to Fusarium in corn: *Methylobacterium mesophiliccum*, *Rhodococcus erythropolis*, *Kocuria varians*, *Pseudomonas diminuta*, *Streptomyces violacceusniger* subsp. violaceusniger, *Streptomyces roches* subsp. rochei, *Streptomyces lavendulae* and *Bacillius megaterium*. The strains were cultured, maintained, and a seed inoculum was produced by TerraMax under the label MicroAF (http://www.terramaxag.com). It is important to note that the bacteria in this blend were not originally isolated from maize, but from rhizosphere soil of pine seedlings. Therefore, they are likely to be generalists rather than specialists on maize. Nevertheless, we felt this blend would allow us to test our methods and, in line with the proposal, further our understanding of how seed inoculants might interact with organic seed disinfection methods in remediating pathogenic *Fusarium*. The inoculant was cleared by OMRI before being applied to the fields of Adaptive Seeds and Pitchfork and Crow organic farms. To apply the inoculum, seeds were coated with inoculum at ten times the recommended dose (1 ml per gram of seed) and allowed to dry overnight

before planting the following day. Seeds not receiving the inoculum received the equivalent volume of water.

Plant Sampling

During the pollination stage on each of the three farms (approximately 9 weeks after planting), a subsample of corn plants were pulled out of the ground to measure the degree of crown rot. Crown rot was measured by slicing the base of the cornstalk in half lengthwise to expose the crown tissue, and scoring its relative darkness as a proxy for the amount crown rot. To standardize across samples, we took pictures of the affected tissue with a camera phone, using the flash setting, through a 10cm-long paper roll. The interior of the makeshift picture chamber was painted black, except for a white section to normalize each picture for the amount of exposure (Figure 1). The relative darkness of the crown was quantified using ImageJ software package. A subset of crown tissue samples were taken for DNA extraction and microbial community analysis using qPCR and next-generation DNA sequencing.

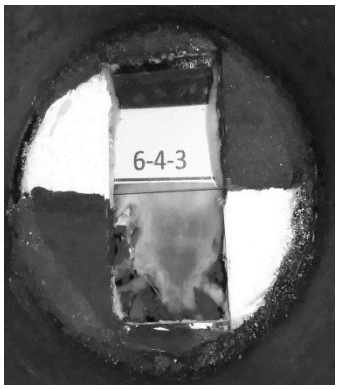

Figure 1*: Example of a sample photo for scoring crown rot. The crown tissue is the darker triangle in this picture, and is the interface between the roots and the shoot of the corn plant. The relative darkness is used as a measure of crown rot. The darkness of each crown is calibrated to the painted light and dark portions of the photo chamber.*

Corn plants were allowed to dry down in the field before harvesting cobs, approximately 14 weeks after planting. Cobs were dried by the respective farms before being shelled. Yield was measured by cob weight and seed weight per replicate plot. A subset of seeds were set aside for DNA extraction and microbial community analysis.

2.3 DNA methods

Seed samples were surface sterilized and aseptically ground-up in a sterilized ceramic burr coffee grinder. Crown tissue was removed with a sterile scalpel. To extract DNA from these samples, we used the MoBio PowerPlant DNA isolation kit. As the kit is intended for isolating plant DNA, we modified it to allow for more efficient extraction of microbial DNA from bacteria and fungi embedded around the plant tissue. To break apart fungal cell walls, we included two freeze-thaw cycles in liquid nitrogen, before bead-milling to further break apart cells for DNA extraction. Furthermore, we added 0.1mm glass beads to the kit's bead-beating vials, which are typically used for breaking apart microbial cell walls, in addition to the kit-standard 1mm steel beads.

Next, we amplified fungal and bacterial DNA from the corn samples (90 crown samples and 88 seed samples) using polymerase chain reaction (PCR). It proved difficult to amplify microbial DNA without inadvertently amplifying plant DNA, because the samples contained an overwhelming amount of plant DNA (approximately 98% of DNA in the sample), and the conventional bacterial and fungal marker genes that we targeted share significant similarity with DNA sequences of the plant genome. Indeed, the majority of laboratory time spent was dedicated to optimizing and troubleshooting PCR methods to obtain sufficient quantities of DNA from bacterial and fungal marker genes, so that microbial endophyte communities could be characterized. The optimized methodology will be published in a separate paper and will likely contribute to future research on corn-associated microbes.

Once amplified, the bacterial and fungal DNA were modified to include a DNA sequence identifier barcode that links it to the particular sample it originated from. All DNA was pooled together in a sequencing *library*, and added to the Illumina MiSeq sequencer, which can read up to 25 million DNA sequences. These DNA sequences are used to identify with microorganisms are present in each sample. Working with an Illumina MiSeq instrument at Oregon Health Sciences University (OHSU), we obtained 10 million sequences for the sequencing run, which averages out to several hundred thousand microbial DNA sequences per sample.

To quantify the amount of *Fusarium* in a particular sample, we utilized a method called quantitative PCR (qPCR), as next-generation sequencing is considerably less reliable for discerning relative abundance of particular microorganisms in a sample. With this method, we target particular species to amplify from the DNA sample, and the amount of amplification corresponds to the amount of that species that is present in the sample. We optimized amplification of several genes belonging to (i) all fungi, (ii) the genus *Fusaruim* and (iii) fumonisin mycotoxin-producing *Fusarium verticillioides*.

2.4. Results and Discussion from 2014 Field Trial

Statistical analysis

To identify the effects of seed treatments on crown rot and corn yield, I conducted a series of multiple-factor ANOVA tests. To find general treatment effects across all farms, an ANOVA test was conducted, with the factors *farm*, *disinfection*, and *inoculation*. Additionally, separate multiple-factor ANOVA tests were conducted for each farm. The main factors on individual farm ANOVA tests were *disinfection* and *inoculation*, and *seed source* was also a factor on the OSU farm, where both seed sources were planted. The ANOVA models also accounted for interaction effects between the main factors.

Overall differences between farms

The flint corn variety performed differently at Adaptive Seeds (AS), Pitchfork and Crow (PC) and OSU Botany and Plant Pathology farms, which differed significantly in management and biological selection pressures. Adaptive Seeds produced an exceptional harvest of corn, with minimal weed pressure. In contrast, the other farms had moderate (OSU) to severe (PC) weed pressure, and moreover lost very significant proportion of yield to moldy and damaged cobs, caused by lepidopteran ear worms (Table 2). Correcting for earworm damage by looking at the largest, unaffected cobs, AS cobs were still 20% larger than cobs at PC and OSU. As expected, plants grown at OSU presented the highest amount of crown rot. PC's intermediate amount of crown rot may be attributed either to soilborne *Fusarium* (not measured) or higher plant stress, which may have been caused by extensive weed pressure, or perhaps a later planting date (2 weeks later) that exposed the seedlings to more heat stress.

Seed sources demonstrate different susceptibilities to crown rot

Regardless of seed treatment, seeds originating from AS had significantly less crown rot than those from PC when grown under high pathogen pressure at OSU, suggesting an inherent resistance to pathogens in the AS seed lot. As the seeds are only distinguished by a single year of mass selection, and did not differ significantly by size, it is presumed that these differences are due to the carry-over of seedborne microflora.

Table 2: Overview of Crown Rot and Seed Harvest results of 2014 field season

	Disease	**Harvest per plot**	
	Crown rot index (0-1)	Seed harvest (g)	Avg. cob weight (g)
Whole farm averages			
Adaptive Seeds	0.59 a	102 a	104 a
Pitchfork and Crow	0.62 b	69 b	78 b
OSU BPP	0.70 c	64 c	75 b
Multivariate analyses	P-values less than 0.20 (direction of interaction, where applicable)[A]		
All farms			
Farm	<0.001***	<0.001***	<0.001***
Disinfection			
Inoculation		0.193 (+4%)	
Farm x Disinfection		0.163	
Farm x Inoculation		0.030*	0.012*
Disinfection x Inoculation	0.070* (–)		
OSU BPP			
Seed source	0.020* (1%)		
Disinfection			
Inoculation	0.150 (–1%)		0.078* (-4%)
Seed source x Disinfection		0.063* (–)	0.150 (–)
Seed source x Inoculation			0.084* (–)
Disinfection x Inoculation			
Block	0.006**	0.002**	<0.001***
Adaptive Seeds			
Disinfection			
Inoculation		0.120 (+12%)	0.023* (+5%)
Disinfection x Inoculation		0.152 (–)	
Block	0.043*		<0.001***
Pitchfork and Crow			
Disinfection		0.039* (–13%)	0.141 (-4%)
Inoculation			
Disinfection x Inoculation	0.064* (–)		0.145 (–)
Block	0.152	<0.001***	<0.001***

[A]The values in parentheses represent either (a) the degree and direction of a factor's effect on the mean of the dependent variable, or (b) for interaction effects between factors, a negative (–) denotes that the factors have an antagonistic interaction, and a positive (+) denotes they have synergistic interaction.
* = p<0.10; **=p<0.01; ***p<0.001

Seed disinfection increases the effectiveness of the biocontrol inoculum for reducing crown rot

The biocontrol inoculum only marginally reduced crown rot on OSU, where pathogen pressure was high, but did not affect the incidence of crown rot on organic farms. The main effect of the biocontrol inoculum may be confounded with its interaction with other factors. Analyzing all farms together, we see that effectiveness of the biocontrol inoculum for reducing crown rot appeared to be dependent on whether or not the seeds had been disinfected prior to inoculation, as evidenced by a significant interaction effect between the two factors in the model (p=0.070, Table 2, Figure 2A). As predicted in **hypothesis 4**, it appears that seeds that were disinfected prior to inoculation had significantly less crown rot, than those that had not. This same general trend is observed in each individual farm, but it is only statistically significant at Pitchfork and Crow.

Seed disinfection affects yields depending on farm, seed source

We hypothesized (**hypothesis 2**) that disinfection would generally reduce the fitness of the plant, as measured by yield. However, this trend was not evident when comparing metrics of plant yield across all farms (Table 2). Examined on a per-farm basis, disinfection significantly decreased yield at PC by 13% (p=0.039), but tended to increase yield at AS (not significant). Seed source might play a major role in these observations. At OSU, where both seed sources were grown, the effect of disinfection depended significantly on the source of the seed (p=0.063); plants originating from PC yielded less in response to the seed disinfection treatment, while plants from AS yielded more, recapitulating the trends observed on the individual organic farms (Figure 2B). One interpretation of this observation is that seeds originating from PC contained a yield-enhancing microbial community that may have been lost with disinfection, while seeds originating from AS yielded better after their inherited microflora was removed, possibly due to the removal of seedborne pathogens such as *Fusarium*. The differences in microbes between the seed lots is pending further DNA analysis of the microbial communities.

The biocontrol inoculum affects yield depending on farm, seed source

The effectiveness of the biological inoculum on increasing yield depended strongly on the farm on which it was applied, as evidenced by a significant *farm* x *inoculation* effect on seed harvest and cob size (p=0.030 and p=0.012 respectively). Inoculation treatments yielded significantly higher at AS, but no effects were observed at PC. Contrary to **hypothesis 3**, inoculation decreased yield at OSU (p=0.078),

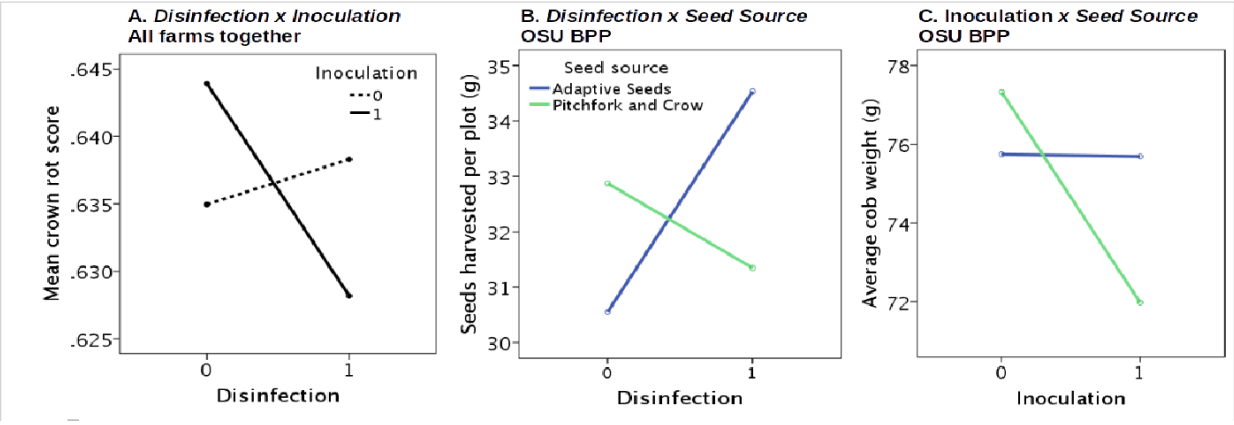

Figure 2: Significant interaction effects in the 2014 field season

despite also tending to decrease crown rot at OSU. Seed sources grown at OSU differed in their yield response to the inoculant (p=0.084). Seed from PC was negatively affected, apparently driving the overall trend, while AS was not affected (Figure 2C).

2.5 DNA based analysis of 2014 field season: quantitative PCR of *Fusarium*

With quantitative polymerase chain reaction (qPCR), we can estimate the quantity of a gene of interest within a DNA sample. By using DNA probes (primers) that target a particular type of organism, or group of organisms in a sample group we can quantify the abundance of that group in a particular sample. Here, we developed methods to quantified total fungi, the amount of *Fusarium* and the amount of fumonisin producing *Fusarium vertcilliodes*.

Crown tissue

Overall, PC had the highest average amount of *Fusarium* in its crown tissue, followed by AS and then OSU (Table 3). This was against what was expected, as OSU was considered to have the highest amount of *Fusarium* in the soil, and indeed had the highest amount of crown rot between the farms. Soil testing for *Fusarium* across all farms is ongoing. The interpretation of these results are confounded by the fact that *Fusarium* species present are not necessarily pathogenic.

As expected, crown tissues from plants that had been treated with the biocontrol inoculum were generally more likely to have reduced counts of *Fusarium*, compared with those receiving no inoculum, by a factor of 46% (p=0.053). However, this trend seemed to be driven by observations on the organic farms, AS and PC, as it was interestingly not evident at OSU. There were few trends when analyzing each farm separately. However, as hypothesized, disinfection increased the incidence of *Fusarium* in crown tissue at OSU, by over twofold (0.052). This trend was not evident at the organic farms, which have less soilborne pathogen pressure.

Table 3: Summary of results from a qPCR based approach to quantifying* Fusarium*, myxotoxigenic* Fusarium verticillioides*, and total fungi in crown and seed samples.

		Crowns		**Seed Harvest**					
		Fusarium		Total fungi		*Fusarium*		Fumonisin-producing *Fusarium*	
Whole farm Averages		# of gene copies per ng of extracted plant DNA							
	Adaptive Seeds	194	ab	25709	a	13	b	6.5	b
	Pitchfork and Crow	3600	b	25882	a	14	b	5.9	b
	OSU BPP	110	a	31189	b	3	a	1.0	a
Multivariate Analysis		P-values less than 0.10 (direction and degree of effect, where applicable)							
All Farms									
	Farm			0.009	**	0.003	**	0.000	***
	Disinfection							0.045	*(+72%)
	Inoculation	0.053	*(-46%)						
	Farm x Disinfection	0.011	*						
	Farm x Inoculation	0.040	*						
	Disinfection x Inoculation	0.020	*						

Seed harvests

Similar to trends observed in crown tissue, and also against expectations, seeds harvested from OSU had fewer counts of *Fusarium*, and even had less mycotoxigenic *Fusarium verticillioides* than seeds from the organic farms. Interestingly, seeds from had significantly higher counts of fungi than the organic farms. We observed few effects of the seed treatments on the incidence of *Fusarium* in the seed harvest. However, it appears that plants from seeds that were disinfected produced seeds with significantly higher fumonisin-producing *Fusarium* (+72%, p=0.045). This last observation fits with **hypothesis 1**, which states that disinfection will increase susceptibility of seeds to *Fusarium*.

3. Culture based microbial methods

3.1 High-throughput screening of biocontrol bacteria against Fusarium

During the granting period, we optimized a high-throughput screening method for discovering strains of bacteria that can control pathogenic *Fusarium* in corn. Research assistant Wes Horton successfully defended his undergraduate thesis on this sub-project in spring of 2015. We are optimistic that these methods can be adapted as needed to different corn varieties and pathogens, and are excited to share them with the community.

First, bacteria must be isolated from the seed. To improve the viability and culturability of seedborne microbes, the seed must be either fresh (i.e. not yet dried down) or pre-germinated for 24-48 hours. As we are interested in symbiotic microbes, we surface sterilize seeds to prevent the isolation of incidental contaminating microbes on the seed surface. Seeds are surface-sterilized by shaking seeds in detergent, 3% bleach and 95% ethanol solutions for 10 minutes each, before rinsing in sterile water. Seeds are either placed directly on the agar-based growth medium, cut in pieces first with a sterile razor, or ground-up in a sterilized ceramic burr coffee grinder and spread into growth media in a slurry. Intact, cut, or ground seeds are either added to potato dextrose agar (PDA), a general growth medium or PDA containing 0.5 mg L^{-1} of 2-benzoxazolinone (BOA), a method that was adapted from Glenn et al. (2001). BOA is an antimicrobial secondary metabolite produced by corn. Microbes that can grow in BOA are considered to be well-adapted to the germinating corn seedling environment, when BOA concentrations in the plant are usually the highest. While this method was suggested for the isolation of *Fusarium* pathogens(Glenn et al., 2001), our lab has proven it can be an effective way to isolate beneficial microbes as well.

Bacterial isolates are then inoculated into a liquid PDA + BOA medium to quantify their resistance to BOA. The best-performing bacteria are trialed against pathogenic *Fusarium*, usually an aggressive strain of *Fusarium culmorum*. The bacteria are inoculated at the edges of a petri dish containing either PDA or PDA+BOA, in which the middle is placed a 4mm culture plug of *Fusarium*. As the *Fusarium* grows radially outward through the growth medium, its growth may be inhibited by the bacterium (Figure 2). Those microbes that significantly reduce Fusarium growth are then trialed in seedlings.

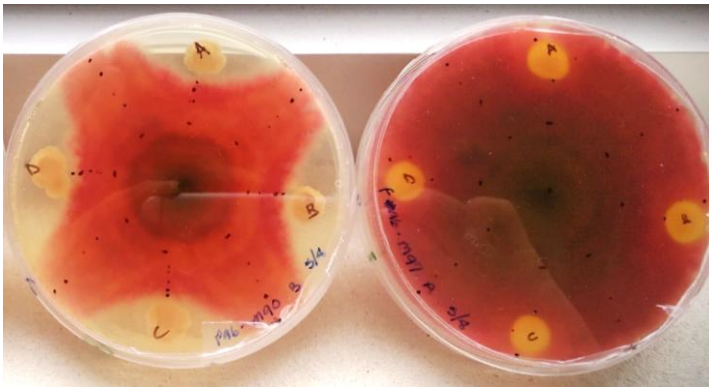

Figure 3: Example of seedborne bacterial inhibition of Fusarium, left, versus no inhibition, right.

Seedling trials

Bacteria that reduce *Fusarium* growth in petri dishes are then trialed in corn seedlings themselves. Seeds are soaked in a suspension of 100,000 bacterial cells per ml for 4 hours, rinsed in water and then placed on water agar for germination inside of a petri dish. After two days, the emerging radicle is inoculated with a spore suspension (about 500 spores contained in a 50 ul water droplet) of pathogenic *Fusarium*. Seedlings are grown in the petri dish for a week, after which they are scored on a scale of 0-8 for visual necrosis in the radicle and leaf parts. Those bacteria that significantly reduce pathogen damage on seedlings were are then candidates for greenhouse or field studies.

3.2 2015 Field Trial of seedborne biocontrol bacteria

During 2014, we found two seedborne bacterial strains that were effective at reducing *Fusarium* pathogen damage in flint corn seedlings grown in a petri dish, of the species *Bacillus subtilis* and *Arthrobacter ilicis*. To see if their success in the lab translated to success in the field, we prepared a smaller field trial in 2015 at the OSU BPP farm. We had not yet optimized the seed inoculation practice, nor trialed them in a greenhouse, so this field trial should be considered preliminary, and further testing is needed.

Experimental Setup

The experiment consisted of four treatments of two factors:

1. *Control*
2. *Bacillus subtilis*
3. *Arthrobacter ilicis*
4. *Bacillus subtilis* + *Arthrobacter ilicis*

Seeds were soaked for 2 hours in PBS with $1X10^7$ bacterial cells ml^{-1} and allowed to air dry overnight before planting the next day. Control plants received only sterile PBS buffer during the soaking period. Seeds were planted at OSU on June 19th 2015, at 1' spacing in a randomized block design pattern. During the pollination stage, a subset of the plants were measured for crown rot. After 15 weeks, plants were harvested, and their yield was measured, in terms of cob weight and lbs of seeds produced.

Results of 2015 field season

A heat wave in early July following seedling emergence left the corn plants stunted and bushy. Furthermore, in some plots, most of the seedlings had been removed due to bird activity. Despite these suboptimal field research conditions, we were still able to see some patterns caused by the microbial inoculant. There was an interaction effect between *Bacillus subtilis* and *Arthrobacter ilicis* such that *B. subtilis* negatively affected possible biological control properties of *A. ilicis*. In fact, this strain of *B. subtilis* tended to be detrimental to the plant both in terms of crown rot and harvest, though none of these observations were statistically significant. Upon removing *B. subtilis* from analysis, *A. ilicis* appeared to reduce the amount of crown rot in the field by a moderate 9.5% (p=0.080).

Low plant survivorship across plots due to bird predation and uneven watering during the heat wave introduced variation in the harvest measures, possibly obscuring some results. Nevertheless, plants inoculated with the seed-associated strain of *A. ilicis* produced cobs that were about 19% heavier on average than the control (t-test, p=0.070), although yield on a per-plant basis was not significantly different than control.

4. Education and Outreach

While seedborne microbes have been studied for several decades, publicly funded research, seed treatments and farming practices have been overwhelmingly focused on seedborne pathogens. To date, the beneficial properties of seedborne microbes are poorly understood and characterized, although there have been a few ground-breaking studies outlining their importance (i.e., Johnston-Monje & Raizada, 2011). We would like this research project to increase public awareness and understanding of seedborne endophytes, an addition to contributing to general scientific knowledge of plant-microbe symbiosis.

As our understanding of seedborne microbes is still in its infancy, we feel there is yet little practical knowledge to impart to the public. However, we know that by generating public interest, much more knowledge can be produced by the community. For this reason, we launched the Community Research Network, in which OP corn seed growers can send in their seeds for microbial community analysis (www.microbialinheritance.org/network). To date, we have received 70 seed samples from over a dozen corn varieties grown by over 20 individuals. During the 2014 and 2014 field seasons, we have been processing these samples in parallel with seed and crown samples from the OFRF funded experiment. With this growing community-generated data set, we will be able to ask broader questions across cultivars, geographies, and farming methods.

We plan for this data to be shared freely online, in the form of a data repository and interactive data visualizations. With the help of computer programmer Robin Nelson, a computer programmer, we set up a working model of what this interactive data visualization might look like. We utilized Shiny Web App, which interfaces with R, a commonly-used statistical analysis package. Shiny will allow us to maintain a web page where users can interact with a continually updated dataset. Currently, we have produced a histogram of a subset of the data, to as a proof of concept (Figure 4). However, we will soon include many more samples from the Community Research Network, and also come up with more ways to visualize the data.

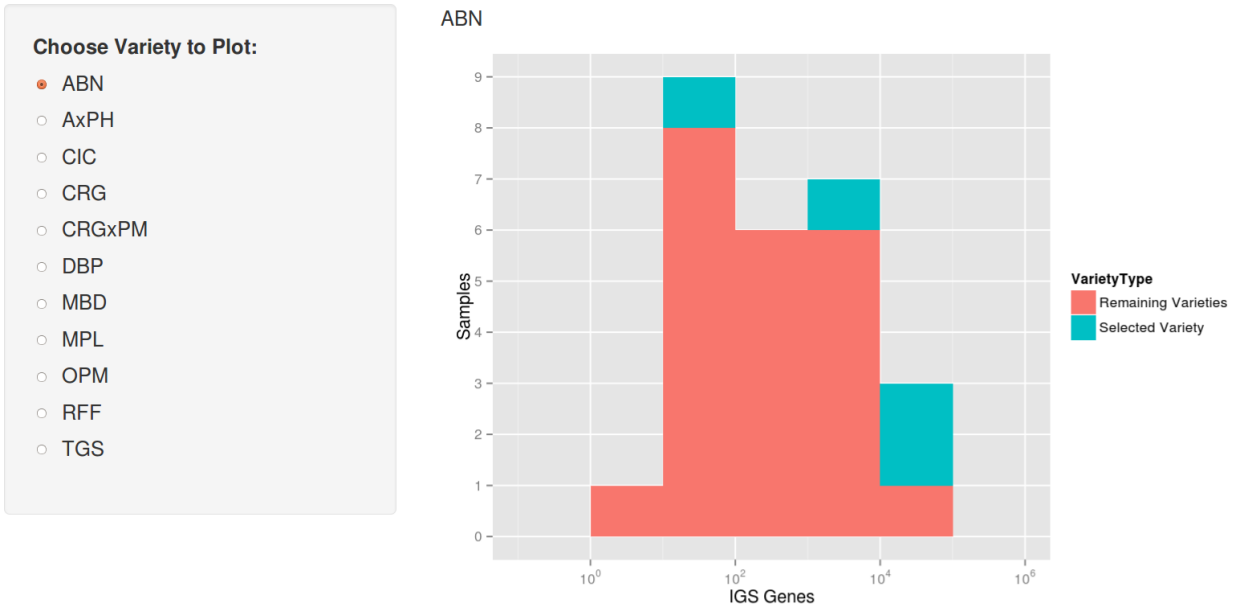

Figure 4: Screenshot from interactive data visualization of a small dataset produced by the Community Research Network. In this histogram of all seed samples tested, "IGS genes" stands for the abundance of Fusarium in the sample. One can select a corn variety to see where it falls on the histogram. In this case ABN, (Abenaki flint corn) samples, shown in blue, were disproportionately on the right side of the histogram indicating they had a higher incidence of Fusarium.

Expanding the community research network

For the 2015-2016 academic year, University of Oregon has granted the principle investigator and PhD candidate, Lucas Nebert, a full-year fellowship to expand the Community Research Network and develop a business or non-profit oriented towards testing of seedborne microbes. We will continue to take the methods and insights we learned from this research project and build capacity for larger-scale seed testing of microbes. We plan to improve the website (www.microbialinheritance.org) and increase visibility with social networking.

5. Conclusions

We have successfully accomplished the proposed research project, pending analysis of the next-generation DNA sequencing dataset that was produced during the final weeks of 2015, after a series of delays caused by methodological hurdles. The OFRF should expect updates as we uncover more information from the dataset, which includes over 10 million DNA sequences. With the current data on hand, we can still make several general conclusions about the practices of seed disinfection and inoculation.

Conclusion 1. Disinfection of seeds can increase a plant's susceptibility to pathogens

We observed cases in which seed disinfection was detrimental to a plant's success, reducing yield (see Pitchfork and Crow, Table 2), and actually increasing the transmission of mycotoxigenic *Fusarium* passed on in the next generation of seed (Table 3). The practice of seed disinfection should be used with discretion, and alternative methods should be considered.

Conclusion 2. Seeds are a viable source of biological control bacteria

We were able to find a promising anti-*Fusarium* biological control bacterium that we isolated from the seeds of an organic, open-pollinated flint corn variety local to the Pacific Northwest. Disinfection of seeds should be weighed against the fact that seeds can contain beneficial microbes. To our knowledge, this is the first instance where seedborne bacteria have been used as biological control for controlling disease in the plants from which they originated. We plan to redouble our efforts and screen more seedborne bacteria for their biological control abilities of *Fusarium*.

Conclusion 3. Disinfection of seeds can improve the effectiveness of microbial inoculants

We observed a consistent trend to suggest that seed disinfection increases the ability of biological control inoculants to reduce disease and increase yield (Table 2, Figure 2A). As a practice, microbial seed or soil inoculants could be preceded with OMRI-approved seed disinfection methods for improved efficacy in the field.

Conclusion 4. Epigenetic factors play an important role in the response of plants to pathogens and seed treatments

One of the most important factors affecting Fusarium crown rot was the source of the seeds. As these seed lots only differed based on a single growing season on separate farms, and were selected in masse, it is unlikely that observed differences had to do with genetic divergence. Rather, we presume seedborne microbes play a significant role in differences between seed lots. We hope to better understand the role of microbial ecology after we have more time to interpret the results from the next-generation sequencing dataset.

Acknowledgements

Thank you to the OFRF for funding and granting an extension to this project. Special thanks to Sarah Kleeger and Andrew Still of Adaptive Seeds and Carri and Jeff Bramlett and Carri Heisler of Pitchfork and Crow for their interest and participation in this research, and their ongoing promotion of this work with seedborne microbes. Furthermore, the project would have impossible without the help of undergraduate students Wes Horton, Anna Lundberg, Adrian Robins, Madi Raines, and Eric David.

Works Cited

Bacon, C. W., Glenn, a. E., & Yates, I. E. (2008). Fusarium Verticillioides : Managing the Endophytic Association With Maize for Reduced Fumonisins Accumulation. *Toxin Reviews*, *27*(3-4), 411–446.

Glenn, A. E., Hinton, D. M., Yates, I. E., & Bacon, C. W. (2001). Detoxification of Corn Antimicrobial Compounds as the Basis for Isolating Fusarium verticillioides and Some Other Fusarium Species from Corn.

Johnston-Monje, D., & Raizada, M. N. (2011). Conservation and diversity of seed associated endophytes in Zea across boundaries of evolution, ethnography and ecology. *PloS One*, *6*(6), e20396.

Saunders, M., & Kohn, L. M. (2009). Evidence for alteration of fungal endophyte community assembly by host defense compounds. *The New Phytologist*, *182*(1), 229–38.

Financial Accounting of Expenditures

We have grouped the expenditures below according to those in the proposal for direct comparison. See Table 4 on the next page for a more comprehensive financial accounting.

Budget expense	**Proposed**	**Actual**
Field work expenses	1000	613
Farmer Compensation	2000	2000
DNA Sequencing	5000	4738
Laboratory Costs	3176	3000
Website data visualization	2000	2070
Total	13000	12597

Table 4: Budget Summary

	Price	Recipient	Item	Date
Field Work				
	900	Pitchfork and Crow	90% farmer compensation	19-Aug-14
	900	Adaptive Seeds	90% farmer compensation	2-Sep-14
	100	Adaptive Seeds	10% farmer compensation	10-Nov-14
	100	Pitchfork and Crow	10% farmer compensation	10-Nov-14
Subtotal	**2000**			
Microbial culturing and general labware				
	63	Sigma Aldrich	Peracetic acid	20-May-14
	101	FisherSci	Potato dextrose broth	21-May-14
	71	Sigma Aldrich	BOA	12-Aug-14
	109	FisherSci	Sterilization Filters	28-Jan-15
	173	Neogen	Fumonisin testing kit	24-Jun-15
	84	UO Science Stores	General lab expenses	1-Nov-15
Subtotal	**601**			
DNA Extraction and Purification				
	208	Zymo	DNA clean and Concentrate	10-Jun-14
	411	MoBio	Plant DNA extraction	5-Sep-14
	470	MoBio	Plant DNA extraction	14-Jan-15
	301	MoBio	Microbial culture DNA extraction	29-Jan-15
	536	MoBio	Plant DNA extraction	10-Mar-15
	649	Beckman Coulter	DNA purification beads	24-Jun-15
Subtotal	**2575**			
PCR and DNA Sequencing				
	83	New England Biolabs	NdeI restriction enzyme	10-Jun-14
	128	FisherSci	Exosap	13-Jun-14
	514	PNA Bio	PCR clamp	10-Sep-14
	60	FisherSci	PCR Primers	10-Mar-15
	26	FisherSci	PCR primers	19-Mar-15
	460	Kapa Biosystems	qPCR reagents	8-Apr-15
	90	FisherSci	PCR Microplates	14-Apr-15
	460	Kapa Biosystems	qPCR reagents	4-May-15
	460	Kapa Biosystems	qPCR reagents	11-May-15
	151	New England Biolabs	NdeI restriction enzyme	8-Jun-15
	79	FisherSci	PCR Primers	9-Jun-15
	13	FisherSci	PCR primers	19-Jun-15
	510	Kapa Biosystems	qPCR reagents	25-Jun-15
	1704	OHSU	MiSeq sequencing run	
Subtotal	**4738**			
Budget summary				
Travel expenses	613	Lucas Nebert	Driving expenses	
Farmer compensation	2000			
Laboratory Costs	3176			
DNA Sequencing	4738			
Website work	1900	Robin Nelson	Wages, online data visualization	
	170	Fringe benefits		
Total Expenses	**12597**			
Left over in budget	403			