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Utilization of polyanhydride nanoparticle encapsulated fungicide seed treatments

against seedborne and soilborne *Fusarium graminearum* on maize

by

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The student author and the program of study committee are solely responsible for the
content of this thesis. The Graduate College will ensure this thesis is globally accessible
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ABSTRACT

Fusarium graminearum is a soilborne and seedborne fungus that can cause significant losses on maize through a variety of different diseases, including seedling blight. Seedling blight is usually managed by utilizing fungicide seed treatments, but unfavorable germination environments and high levels of inoculum can still cause significant losses. Therefore, improved seed treatment efficacy is needed. Amphiphilic polyanhydride nanoparticles (PAN) have been used to enhance efficacy and provide sustained release of several active ingredients in animal systems and have potential for use in crop production.

To assess the potential benefits of PANs in seed treatment formulations, we performed two types of experiments with maize seeds. In the first experiment, seeds were inoculated with *F. graminearum* (seedborne inoculum) and then treated with PANs loaded with varying rates of fludioxonil (FLD) or thiabendazole (TBZ). In the second experiment, seeds were treated with uniform rates of PAN-encapsulated FLD or TBZ and exposed to unfavorable germination environments in the presence of soilborne inoculum. Controls included commercial rates of FLD and TBZ without PAN encapsulation, as well as untreated seeds and noninoculated treatments. Seedborne inoculum experiments with half rate of TBZ (0.025mg) loaded PANs protected root length similarly to full rate non-encapsulated TBZ, but PAN-encapsulated FLD did not perform as well as non-encapsulated FLD. In soilborne inoculum experiments, disease symptoms were severe and there were few differences among treatments. PAN-encapsulated TBZ performed similarly to non-encapsulated FLD and TBZ in shoot length when emergence was delayed from zero

to three weeks. However, PAN-encapsulated fungicides did not increase or prolong efficacy over non-encapsulated formulations.

PAN-formulated seed treatments have the potential to provide tailored release systems for fungicides to protect against soilborne and seedborne diseases under a variety of different environments and potentially using a range of active ingredients. Our results indicate that PAN-encapsulation may facilitate use of reduced rates for some active ingredients, but more work is needed to demonstrate their potential to prolong efficacy under delayed emergence conditions.

CHAPTER 1 LITERATURE REVIEW

Seedling Diseases of Maize

Over eighty percent of the world's food crops are grown from seed (CropLife, 2013), each of which are exposed to either pathogenic seedborne or soilborne microbes. These pathogens can cause damping off before or after emergence, resulting in uneven field stand and reduced yield. The main tactic to control seedling diseases are seed treatments. Seed treatments are usually comprised of a series of chemicals to protect against fungi, oomycetes, nematodes, and bacteria in the field, but are not always effective. Delays in germination due to cold wet weather prolong the exposure of seeds to pathogenic microbes, which can decrease the efficacy of these treatments (University of Minnesota, 2017).

In maize, seedling diseases have been a widespread and consistent concern wherever it is grown. Over the past few years, seedling blights alone have been responsible for losses from 48 to 150 million bushels of maize per year (Mueller, 2016a, 2016b, 2016c; Wise, 2012). There exists a wide variety of seedling diseases; a few common genera of causal agents include: *Fusarium, Pythium, Penicillium, and Rhizoctonia.* While *Penicillium species* infect plants at high temperatures and are often inhibitory to other fungi, *Pythium,* on the other hand, favors low temperatures, with some strains being able to adapt to higher temperatures and moist conditions (Munkvold and White, 2016). *Rhizoctonia and Fusarium* seedling diseases can occur under a wide range of temperatures and are therefore harder to predict and manage (Sumner and Bell, 1982).

Fusarium graminearum

Of the many maize seedling pathogens, *Fusarium graminearum* is responsible for root rot, seedling blight or damping off, stalk rot, ear rot, and head blight in a variety of different hosts, including: maize, soybean, wheat, etc. (Goswami and Kistler, 2004). Therefore, this well-rounded and multi-faceted fungus is an object of efforts to not only prevent infection of ear rot, but also the prevention of seed infection which will in turn prevent damping off and decreased yields. Furthermore, this pathogen is of great economic and health concern due to its ability to produce mycotoxins and their subsequent effects on livestock and humans (Escriva and Manyes, 2015; Hueza et al., 2014; Wild and Gong, 2010).

Fusarium graminearum is a soilborne and seedborne fungus. It primarily survives on maize or other host debris as mycelia, chlamydospores, or other survival structures and infects kernels after planting (Munkvold, 2003). However, the fungus can also survive in the soil without the presence of host debris and infect non-host crop and weed species (Parry et al., 1995).

Perithecia are produced on host residues over a wide range of temperatures, with the optimum being 28°C. After production of the perithecia occurs in the fall, ascospores are discharged into the air (optimal temperature: 16°C) in the spring after a diurnal drying and wetting period of the perithecia (Munkvold, 2003). Once *Fusarium graminearum* infects its host, it can kill off the seeds or seedlings, otherwise known as damping off, or persist in the host. If the fungus persists and does not cause damping off early in the season, it can cause stalk and crown rot later in the season (Munkvold, 2014).

Of great importance is the range in which *Fusarium graminearum* can impact agricultural production. Not only does it have a wide range of host species, but also the ability to infect and cause substantial yield and quality losses in each species via seedborne and soilborne inoculum. In maize and wheat alone, *Fusarium graminearum* can cause stalk rots, head scab, head blight, and seedling blight (Broders et al., 2007). This is especially important in states that use maize-soybean, maize-soybean-wheat, etc. rotations with reduced or no-till systems to prevent soil erosion and increase organic matter. Such agronomic practices increase the amount of host debris left behind and gives rise to a larger source of inoculum that have the ability infect the next year's crops. Furthermore, due to current trends of early planting the time needed for seed germination is extended due to exposure of seeds to low soil temperatures and high moisture, increasing an already prolonged window of susceptibility for seedling blight (Broders et al., 2007; Vandoren and Triplett, 1973).

Another topic of importance regarding *Fusarium graminearum* is its ability to produce mycotoxins. Mycotoxins produced by *Fusarium graminearum* include deoxynivalenol (DON) and zearalenone. Outside of their ability to cause harm to the consumers of their plant host, mycotoxins also play a role in infection. When mycotoxins are decreased, the severity of disease can also be lost and *Fusarium graminearum* may not be able to spread to other parts of the plant outside of its original infection court (Munkvold, 2003).

Seedborne infections are managed effectively by advances in seed conditioning that remove severely infected seeds from commercial seed lots. Despite this, *Fusarium*

graminearum inoculum in the soil and crop debris is a threat that must be managed (Munkvold, 2002).

The first cultural practice that is suggested is crop rotation. By rotating to non-host crops, the amount of debris that can be used for perithecia and chlamydospore production is reduced, which in turn decreases the amount of primary inoculum and decreases disease pressure (Mueller, 2010). Although this practice is suggested, it is usually unrealistic given that rotation crops used in corn producing states are also hosts for *Fusarium graminearum*. Tillage has the ability to reduce the amount of primary inoculum source available on top of the soil by burying crop debris, which will in turn reduce the amount of spores available to infect during anthesis and result in kernel infection on the ears (McGee, 1995).

Delayed planting dates are also suggested to decrease the likelihood of seedlings being subjected to potentially favorable environments for seedling infection and damping off. During periods of early planting, temperatures are usually unfavorable for seed germination and may cause stress that results in increased susceptibility in infection and that can result in loss of stand. The suggestion of later planting to avoid this situation, however, is rarely followed, due to its potential to result in decreased yields (McGee, 1995).

Lastly and most importantly, seed treatments are recommended to protect kernels from seedling blight and root rot early in the growing season. Seed treatments often come in cocktails to protect against multiple pathogens and can have various chemical activities for targeting pathogens and uptake into the plant (Broders et al., 2007; Rodriguez-Brljevich et al., 2009).

Most commercially produced seeds are treated with fungicides to protect from infection after planting or to deter seedborne fungal pathogens. These seed treatments usually result in improved emergence and yield in comparison to untreated seed (Munkvold, 2002). Activities of fungicides vary between contact, local systemic, and systemic activity. Contact fungicides are not taken up by plant tissues and must come into direct contact with its target to have an effect. Local systemic fungicides are taken up by the plant but not translocated throughout the plant. Lastly, systemic fungicides can be translocated throughout the plant (Munkvold, 2014). A combination of these fungicides can be favorable to target multiple pathogens and to reduce the risk of resistance development by varying the mode of action and control.

Seed treatments offer a series of advantages including placing the product directly on their respective target. Active ingredients are placed on the seed in order to provide protection to the seed and its roots during the "first critical stages of crop development" (Munkvold, 2014). By applying fungicides directly to the seed, the amount of active ingredient used can be reduced significantly. This practice also allows for more even treatment of the seed surface by use of precise treatment methods in comparison to sprays that may be unevenly dispersed based of off plant density or environmental conditions. By using seed treatments, the active ingredients also have the potential to remain accessible to the seeds longer by persisting in the spermosphere and rhizosphere for potentially weeks after planting (Munkvold, 2014).

Despite the fact that almost all maize seed is treated with some combination of fungicide, nematicide, or insecticide, there remains a need to improve their efficacy. As before mentioned, seedling blights have been responsible for the loss of over 300 million

bushels cumulatively in the past three years. In terms of root rot and seedling blights *Fusarium* seedling blight is responsible for the loss of 37.5 million bushels of maize per year. This was only superseded by Pythium damping off and nematodes in terms of root rot and seedling blights, which accounted for 93.6 and 81.5 million bushels of loss per year respectively (Mueller, 2016c; Wise, 2014). For these reasons, developing novel methods of seed treatment delivery is of great importance and interest in considering the future of disease management in agronomic crops.

Nanoparticles

One area for developing novel methods of seed treatment delivery is taking form in the field of nanoparticles. With decreased size of antimicrobial compounds, the active ingredients of interest present a larger surface area to encounter pathogens and their hosts (Ishida et al., 2014). Several kinds of nanoparticles have begun to be investigated for their potential use in agriculture, including a variety of metal based nanoparticles (silver (AgNPs), gold (AuNPs), iron, and a variety of others), as well as carbon nanotubes.

Carbon nanotubes have been investigated to determine their ability to penetrate seed coats and their potential impact on seed germination by Khodakovskaya et al. (2009). Their studies showed that carbon nanotubes have the ability to penetrate seed coats and roots, and can enhance water uptake in tomato seeds

Investigation of other metal nanoparticles in cocktail formations has also been explored in the management of *Pseudocercosporella herpotrichoides*, the causal agent of eyespot in wheat. Panyuta et al. (2016) found that utilizing a mixture of copper, zinc, iron, silver, and manganese nanoparticles protected wheat seeds more effectively than copper

nanoparticles alone when challenged with the fungus. This result was demonstrated in the decrease of stress products such as thiobarbituric acid reactive substances that are a result of lipid peroxidation.

Silver nanoparticles have been found to help manage *Bipolaris sorokiniana*, the causal agent of brown splotch disease in wheat, and *Phytophthora parasitica*, the causal agent of black shank in tobacco, and *P. capsici,* the causal agent of Phytophthora blight of cucurbits (Mishra and Singh, 2015; Mishra et al., 2014; Ali et al., 2015). In managing *Phytophtora* species, AgNPs caused encystment of spores and reduced mycelial growth in vitro (Ali et al., 2015).

A variety of mechanisms have been proposed to explain the antimicrobial activity of AgNPs. Among these is the interaction of silver ions with phosphorous found in DNA and sulfur-containing proteins. This mechanism would allow for AgNPs to inhibit DNA replication and enzyme and protein functions (Ali et al., 2015). The ability to inhibit universal biological processes presents a risk of nonspecific interactions and, pertaining to our work, phytotoxicity. Additionally, it is not yet understood which species (AgNPs, Ag+, or Ag complexes) is available when introduced into biological systems, making the determination of their effects and aggregations hard to pinpoint (Anjum et al., 2013).

Furthermore, studies on the uptake and phytotoxicity of AgNPs in different plant species have had variable results. Harajyoti and Ahmed (2011) demonstrated that AgNPs were deposited inside of root cells and caused damage to vacuoles and the cell wall of *Oryza sativa* (commonly known as Asian rice). Pokhrel and Dubey (2013) observed toxicity in *Zea mays*, in the form of inhibition of germination, but at rates no higher than 35%.

The variability of toxicity may be due to the size of the AgNPs used in the studies, with higher toxicity being credited to smaller particle size (Rico et al., 2011). Furthermore, as aforementioned, the AgNPs may also be tied up with different substances in the soil or organic matter, making the Ag ions unavailable for defense of the plant. Given the wide range of environmental factors contributing to availability of free Ag ions, as well as their potential to be phytotoxic, a less harmful antimicrobial nanoparticle is desired as a potential new disease management tool.

A possible alternative, and the topic of this thesis, may be found in the utilization of polyanhydride nanoparticles. These amphiphilic polyanhydride nanoparticles passively deliver payloads in a controlled manner that allows for a slower time release, and presents a method to decrease losses from leaching, volatilization, and degradation of fungicides (Ramos Campos et al., 2015). Current use of polyanhydride nanoparticles has been concentrated in the field of medicine and has displayed their ability to prevent degradation of antibodies, control release kinetics, and retain bioactivity of a range of therapeutics (Carrillo-Conde et al., 2014).

Materials used in these polyanhydrides include diacids of 1,6-bis(p-carboxyphenoxy) hexane (CPH) and 1,8-bis-(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) which are formed via melt polycondensation (Kipper et al., 2002). After the polymers are created they are synthesized into nanoparticles encapsulating their payloads via anti-solvent nanoencapsulation (Ulery et al., 2009). Given that these polymers are biodegradable and are able to be modified for desired degradation mechanisms, they are an intriguing candidate for managing disease.

Degradation characteristics of polyanhydrides nanoparticles was investigated by Torres et al. (2006) who noted that erosion is a combination of polymer degradation, water absorbance, as well as monomer diffusion. They observed that the hydrophobicity of CPH decreases water intake resulting in a characteristic surface erosion process in which only 8% of the mass of the polymer is degraded over four weeks. This characteristic makes CPH an ideal candidate for decreasing bulk erosion of other hydrophilic polymers, such as sebacic acid (SA) and CPTEG, and thus prolonging the release of a desired payload (Katti et al., 2002; Li et al., 2010).

Our proposed use of polyanhydrides utilizes the formulation of CPTEG:CPH nanoparticles to encapsulate thiabendazole, a systemic fungicide, and fludioxonil, a contact fungicide, both of which are known to be effective against *Fusarium* spp. and are used in Syngenta's Maxim Quattro corn seed treatments. To our knowledge, no other research has been conducted utilizing a tailored delivery system for application of fungicides to maize seeds challenged with a fungus, though there has been related research in which Ramos Campos et al. (2015) demonstrated that encapsulating fungicides using polymeric and solid lipid nanoparticles decreased fungicide cytotoxicity through sustained released of active ingredients and did not affect emergence of *Phaseolus vulgaris* (common bean).

Both of the chosen active ingredients are common fungicides included in seed treatment cocktails for the management of seedling diseases of maize. By instituting the use of polyanhydride nanoparticles bearing reduced payloads of fungicide, we hope to provide an alternative method to disease management that will assist in decreasing the rate, overall usage, and presence of fungicides in agricultural, aquatic, and other surrounding environments.

CHAPTER 2 MATERIALS AND METHODS

Rolled Towel Assay

Rolled-towel assays (Ellis et al., 2011) were implemented to assess efficacy of seed treatment fungicides in standard formulations compared to polyanhydride nanoparticle formulations on seed inoculated with *Fusarium graminearum*.

Fusarium graminearum isolate 27 *(isolated in Iowa, U.S.A)* was grown on potato dextrose agar (PDA) (BD, Franklin Lakes, New Jersey) for one week at ambient temperature (20-27°C). Mycelial plugs were then placed on Spezieller Nährstoffarmer Agar (SNA) (Leslie and Summerell 2008) to encourage sporulation. SNA plates were placed in the same conditions as the aforementioned PDA plates for ten days. Spores were gently agitated off SNA with sterile deionized water and filtered through sterile cheesecloth. The concentration of the resulting spore suspension was then quantified utilizing a hemocytometer and diluted to achieve a 1×10^5 spores/mL suspension.

Before seeds were placed into the spore suspension, they were prepared using a two-step process. Seeds were soaked in 10% bleach for 5 minutes and rinsed with sterile water, before soaking in deionized water for 5 hours to encourage imbibition of the spore suspension. Batches of 100 seeds each were then soaked in either 30 mL of 1×10^5 spore suspension or sterile water on a shaker set at 24°C at 92 rpm for approximately twelve hours.

After the inoculation soak, seeds were dried on paper towels in labeled letter trays within a biosafety cabinet for twenty-four hours before seed treatments were applied. Treatments included: water and planting polymer controls, thiabendazole (TBZ),

fludioxonil (FLD), Span 80 surfactant loaded nanoparticles (ENP), half rate 12% loaded thiabendzole nanoparticles (HTNP), full rate 12% loaded Thiabendazole nanoparticles (TNP), half rate 12% loaded fludioxonil nanoparticles (HFNP), and full rate 12% loaded fludioxonil nanoparticles (FNP) (Table 1).

Table 1. Codes and descriptions of seed treatments utilized for the rolled-towel assay.

Seed Treatment Abbreviations	Treatment Description
Control	Polymer and water
TBZ	Thiabendazole, water, and plantability polymer
FLD	Fludioxonil, water, and plantability polymer
HENP	Half rate of nanoparticle, surfactant, water
ENP	Full rate empty nanoparticle, surfactant, water
HTNP	Half rate of nanoparticle encapsulated thiabendazole, surfactant, water
TNP	Equivalent weight of commercial nanoparticle encapsulated thiabendazole, surfactant, water
HFNP	Half rate of commercial nanoparticle encapsulated fludioxonil, surfactant, water
FNP	Equivalent weight of commercial fludioxonil encapsulated in nanoparticle with surfactant, water

Each treatment was applied to inoculated seeds and non-inoculated controls for a total of eighteen treatments. Traditional seed treatments (fludioxonil and thiabendazole)

were vortexed with Becker Underwood 1197 plantability polymer and water, and the non-treated control utilized Becker Underwood 1197 plantability polymer and water alone. Nanoparticle treatments were placed in water (15uL of water per seed) before sonication. Seeds were sonicated using a VCX 130PB sonicator with a CV138 tip (Sonics and Materials, Newton, CT) at 30% amplitude to deter aggregation before application of nanoparticle suspension. Nanoparticle suspension was pipetted over the seeds in a plastic bag. After the suspension was applied, the seeds were rubbed together to assist in evenly applying of the suspension over each seed.

After the seed treatments were applied, seeds were dried in a biosafety hood overnight (~12 hours). Seeds were then placed onto labeled sheets of 12 x 22 #38 germination paper (Anchor Paper Co., St. Paul, MN). Fifteen seeds per treatment were placed atop two pieces of germination paper that were moistened with sterile water. After seed placement, a third piece of germination paper was placed on top of the seeds. The resulting compression was loosely rolled, as to not inhibit germination or growth, and placed in a sterile plastic lined 15-liter bucket that was filled with approximately 1 liter of water. Each roll was replicated three times, with each replicate placed into a separate bucket in the growth chamber at 24°C.

After one week, the rolled towels were removed from the chamber. Seedlings were analyzed for root length, shoot length, disease severity, and the total fresh weight of the plants per roll. Disease severity was measured on a one to five scale, with a five being most severe disease. Roots were then cut from the plant, patted dry and scanned using an Epson 1000X scanner in 24-bit color.

Root scans were analyzed using WinRhizo (Regent Instruments, Quebec, Canada) and assessed for the number of forks, surface area, overall length, and root tips. All data were then analyzed using SAS 9.4 for analysis of variance using PROC GLM. Mean separations were made using Tukey's honestly significant difference test at $P \leq 0.05$.

Delayed Emergence Assay

Efficacy traditional and nanoparticle-formulated fungicide seed treatments were assessed under delayed emergence conditions in growth chamber experiments with a *F. graminearum* – infested potting medium. Millet was rinsed with deionized water and strained (#500 sieve). After rinsing, two liters of millet were placed into aerated bags and autoclaved twice (90 minutes at 121°C) over a span of two days. The millet was then infested with ten-day old *Fusarium graminearum* PDA cultures. Colonized agar from five Petri dishes was placed into each sterilized bag containing 2 liters millet and then placed in growth chambers at 24°C for two weeks. Bags of infested millet were tossed once to evenly distribute the fungus. *Fusarium graminearum* grew for two weeks or until the millet mycelial mixture hardened. The inoculum was broken up and mixed into pasteurized field soil to create a 5% (v/v) soil infestation.

Infested and non-infested soul was placed into containers. Each conetainer represented one experimental unit and was labeled with a coded stake to differentiate treatments. Treatments were then randomized into 98-well trays and placed into the growth chamber at 13 or 24°C.

Seed treatments included: polyanhydride nanoparticles containing 12% loaded thiabendazole and Span80, 12% loaded fludioxonil and Span 80, Span80 loaded

nanoparticles, chemical grade thiabendazole, chemical grade fludioxonil, and a water and plantability polymer control. These treatments were planted both in infested and non-infested soils, for a total of twelve treatments with ten seeds planted for each treatment, totaling 160 seeds per delayed emergence environment.

Table 2. Codes and descriptions of seed treatments utilized for the delayed emergence assay.

Seed Treatment Abbreviations	Treatment Description
Control	Plantability polymer and water
TBZ	Thiabendazole, water, and plantability polymer
FLD	Fludioxonil, water, and plantability polymer
ENP	Full rate empty nanoparticle, water
TNP	Equivalent weight of commercial thiabendazole encapsulated in nanoparticle with surfactant, water
FNP	Equivalent weight of commercial fludioxonil encapsulated in nanoparticle with surfactant, water

Delayed emergence environments simulated four different planting situations. These environments included: three weeks of delayed emergence at 13°C (followed by 1 week at 24°C), two weeks of delayed emergence at 13°C (followed by 2 weeks at 24°C), one week of delayed emergence at 13°C (followed by 3 weeks at 24°C), or ideal emergence conditions (~24°C) for four weeks. Including environmental parameters, there were a total of forty eight treatments, and a total of four-hundred and eighty plants.

Plants were watered as was necessary to keep the soils moist for the duration of the experiment, usually once every other day. Plants were collected four weeks after planting. Their roots were washed before being placed into labeled bags to await measurements and root scanning.

Data loggers (Spectrum Technologies, Aurora, IL) were placed in the growth chambers for one week before the experiments took place to determine whether the chambers were at appropriate temperatures to begin the experiments. The data loggers remained in the growth chambers during the four-week study in order to monitor any potential environmental fluctuations.

After 28 days, plants were assessed for disease severity, root length, shoot length, and fresh weight. Roots were then cut from the plant, patted dry and scanned using an Epson 1000X scanner in 24-bit color. After scanning, roots were placed into labeled envelopes and dried in an oven at 90°C for twelve hours. They were then weighed to ascertain dry weights.

Image analysis of the root scans were performed using WinRhizo and assessed for the number of forks, surface area, overall length, and root tips. All data were then evaluated using SAS 9.4 with a two-way factorial analysis and mean separations were made using Tukey's honestly significant difference test at $P \leq 0.05$.

Release Kinetics

Nanoparticles were evaluated for their release kinetics by weighing out ~10mg of each type of fungicide loaded nanoparticle (thiabendazole and fludioxonil). Particles were placed then into 1.5mL tubes with 0.5mL PBS in each tube and incubated at 37°C with constant agitation. This set up was done in triplicate. At regular time points, the supernatant was removed and centrifuged. After removal, fresh PBS was added to the

1.5mL tube. At the end of the aqueous (PBS) release, 40mM NaOH was utilized to degrade the remaining polymer to allow for release of any remaining fungicide from the nanoparticle.

Aqueous and base extraction samples were examined via HPLC to obtain the mass of drug release at each time point. Total fungicide loading was calculated by summing the amount of fungicide release for each individual formulation over all time points. Encapsulation efficiency was calculated by dividing the total drug loading, by the theoretical drug loading (amount of fungicide used during synthesis). Fraction release was considered to be the cumulative drug released at a time point divided the total drug loading.

CHAPTER 3 RESULTS

Rolled Towel Assay

Given that there was no significant treatment by run interaction detected for root length (P=0.2658), shoot length (P=0.4076), severity (P=0.5336), or fresh weight (P=0.216), the two runs were combined for analysis. In this experiment, seed treatment, seedborne fungal infection, and their interaction significantly affected (P<0.0001) fresh weight, disease severity, as well as shoot and root length of the maize seedlings 7 days after planting (7 d.a.p).

Non-encapsulated thiabendazole (TBZ) (0.05 mg/seed), non-encapsulated fludioxonil (FLD) (0.0065 mg/seed) and half rate (0.025 mg/seed) thiabendazole-loaded nanoparticle (HTNP) treatments were not significantly different from one another (Fig. 1). The mean root length of the FLD treatment was not significantly different from either the full or half rate encapsulated thiabendazole (TNP and HTNP). Neither empty nanoparticles (ENP) nor fludioxonil-loaded nanoparticles (both rates) were significantly different from the non-treated control.

For shoot length, the TBZ treatment displayed the highest mean and was significantly different from all other treatments (Fig. 2). HTNP performed similarly to FLD and TNP in terms of shoot length. Neither the ENP nor fludioxonil-loaded nanoparticles (both rates) were significantly different from the non-treated control.

For fresh weight, only the FLD and TBZ treatments were significantly different from the non-treated control (Fig. 3).

Non-encapsulated seed treatments (FLD and TBZ), as well as HTNP and TNP, performed significantly better than the inoculated control for root and shoot length, but not for fresh weight.

Thiabendazole treated seeds were observed to have lower disease severity in comparison to all other treatments (Fig. 4). However, other fungicide treatments, excluding encapsulated fludioxonil nanoparticles, performed significantly better than the untreated and empty nanoparticle control. HFNP and FNP performed similarly to the inoculated untreated control in all parameters (Fig. 1, 2, 3, and 4).

No significant differences were observed among the non-inoculated controls. Inoculated maize seeds treated with thiabendazole performed similarly to the non-inoculated controls in all parameters. HTNP and FLD also performed similarly to the non-inoculated controls in terms of root length.

Delayed Emergence Assay

There were significant interactions between experimental run and treatment factors for several variables, so data were analyzed separately by run. No significant interaction was observed between treatment and delay in emergence for any parameter in the first run, however this interaction was significant for all parameters except shoot length in the second run (Table 3 and 4). Similarly, the interaction between treatment, inoculation, and delay was significant only for disease severity and shoot length in the second run (Table 4).

Thiabendazole mean root length was highest amongst treatments in most environmental parameters in the second experimental run, but only significantly different from the other treatments when emergence was not delayed. During the first experimental

run, roots development was severely impaired in all inoculated treatments and no significant differences in root length were observed.

Mean shoot length was highest in FLD, TBZ, and TNP in most environmental parameters in both runs (Fig. 6). TNP and TBZ were significantly different from the inoculated control in both runs when there was no delay in emergence. However, in the second run FLD was also significantly different from the inoculated control, which was not observed in the first run.

In the second run, FLD and TBZ were observed to possess longer shoots for all emergence delays except the two-week delay (Fig. 6). Similarly, TNP was observed to also have longer shoots in the second run, except that it was not significantly different from the untreated inoculated control for environmental conditions where emergence was delayed for one week. Similarly, in the first run TBZ treated seeds possessed longer shoots in comparison to all other treatments when emergence was delayed for three weeks, while FLD treated seeds were only significantly different from the untreated control during the two-week delay in emergence.

Disease severity was at or near the maximum possible value in all treatments except for FNP with no delay in emergence in both runs (Fig. 7). FLD and TBZ were observed to have the greatest root dry weight and were significantly different from the inoculated control but not from one another in the second run (Fig. 8). These results are mirrored in the fresh weights observed in the same run (Fig. 9). In both instances of fresh and dry weight, there were no significant differences among any of the seed treatments or the control in the first run (data not shown).

The only seed treatment that performed similarly to the non-inoculated controls during the second experimental run was TBZ in terms of shoot length, when there was no delay in emergence and two weeks delay in emergence. Similarities in root length for the first experimental run were observed between the FLD treated seeds in infested soil and TNP in non-inoculated controls when emergence was delayed for two weeks. Significant differences were inconsistent among non-inoculated controls across delays of germination and runs (data not shown). For example, root and shoot length of seeds treated with thiabendazole were significantly shorter than other treatments when germination was delayed for one week during the first run; however, these differences were not observed among germination delays in the second run. Similar trends were observed in water polymer control, FLD, TNP, TBZ, and ENP treatments regardless of delay or run.

Release Kinetics

Collaborators Dr. Balaji Narasimhan and Adam S. Mullis collected release kinetics data pertaining to the two different types of nanoparticles used; 65.6% (+/-3.9%) loaded fludioxonil CPTEG:CPH nanoparticles and 60.6% (+/-0.4%) thiabendazole loaded CPTEG:CPH nanoparticles. Fludioxonil loaded nanoparticles exhibited a large burst of fungicide (~90%) within the first 24 hours (Fig. 12). Thiabendazole loaded nanoparticles had a medium initial burst of fungicide (~40%) followed by a steady release of approximately 30% fungicide over the next 16 days (Fig. 11).
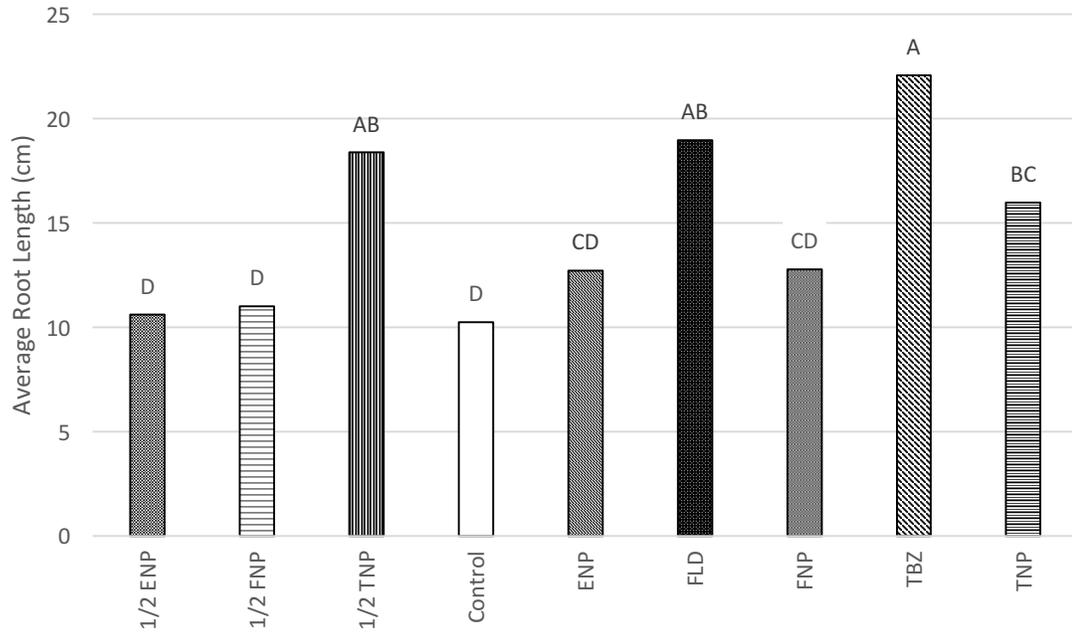
Figure 1. Average root length 7 days after planting of seed treated maize inoculated with *Fusarium graminearum*. Treatments sharing letters are not significantly different by Tukey's Honest Significant Difference test.

Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer
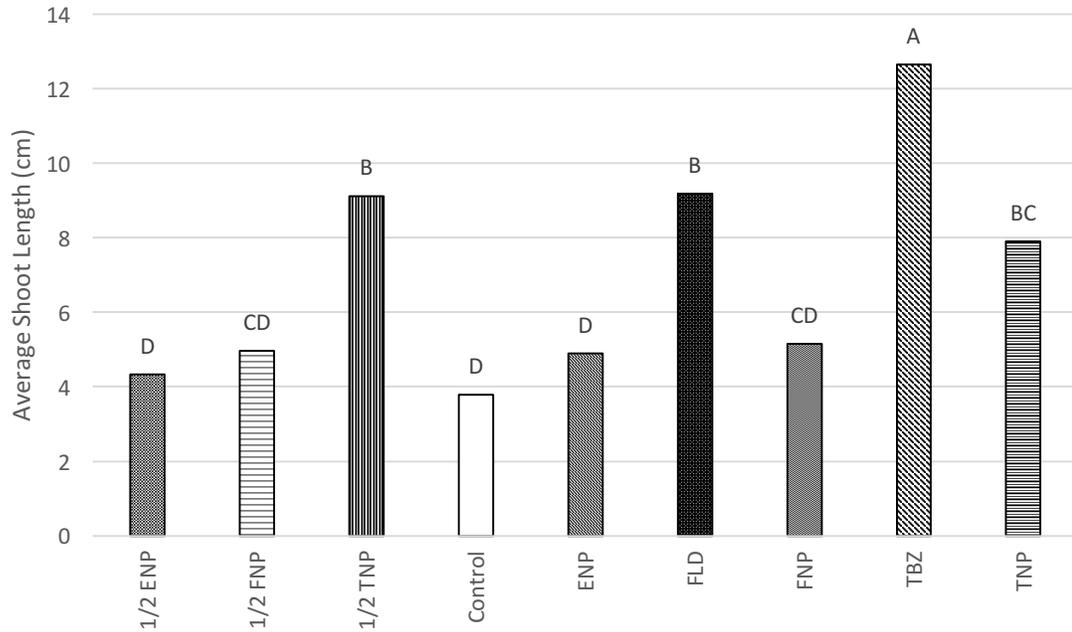
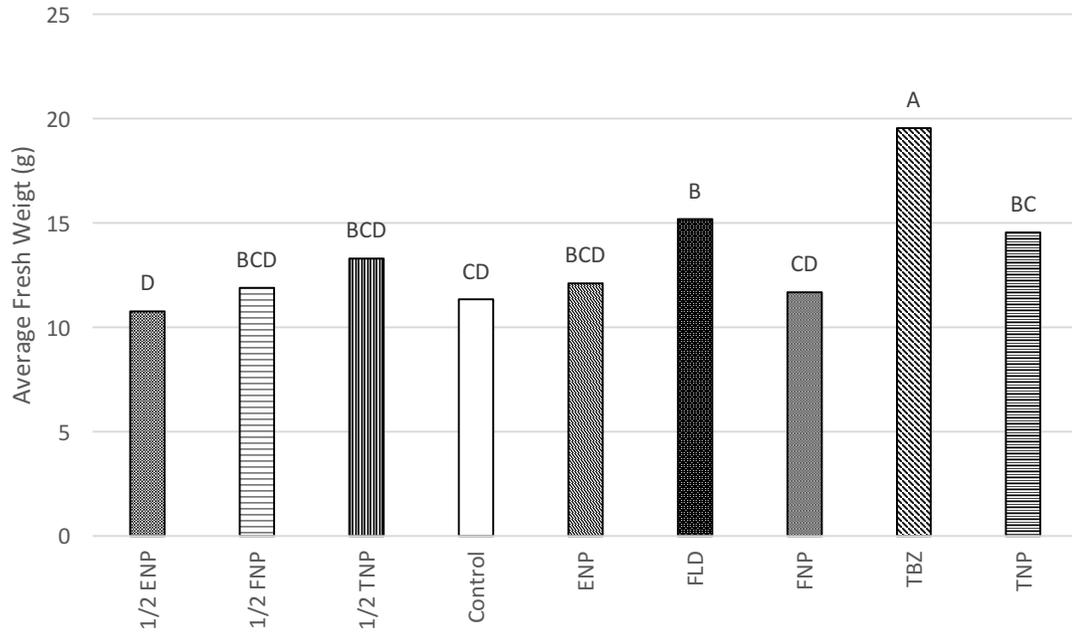1/2ENP-half rate of nanoparticle, surfactant and water

ENP-full rate empty nanoparticle surfactant, and water

HTNP-half rate of nanoparticle encapsulated thiabendazole, surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

HFNP-half rate of nanoparticle encapsulated fludioxonil, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Figure 2. Average shoot length 7 days after planting of seed treated maize inoculated with *Fusarium graminearum*. Treatments sharing letters are similar by Tukey's Honest Significant Difference test.

Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer

1/2ENP-half rate of nanoparticle, surfactant and water

ENP-full rate empty nanoparticle surfactant, and water

HTNP-half rate of nanoparticle encapsulated thiabendazole, surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

HFNP-half rate of nanoparticle encapsulated fludioxonil, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Figure 3. Average fresh weight 7 days after planting of seed treated maize inoculated with *Fusarium graminearum*. Treatments sharing letters are similar by Tukey's Honest Significant Difference test.

Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer

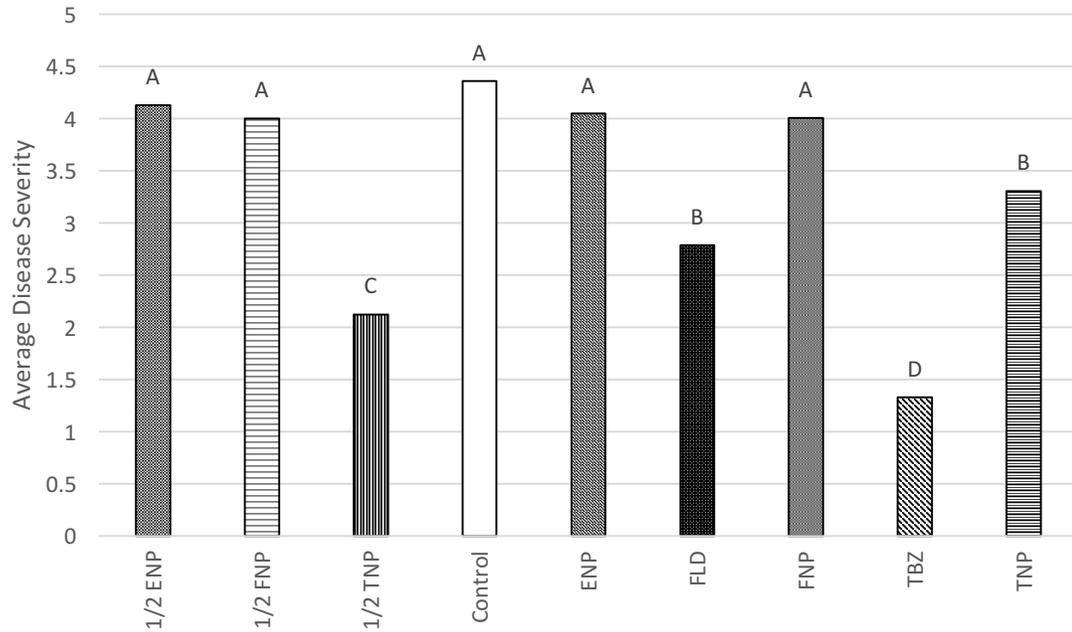1/2ENP-half rate of nanoparticle, surfactant and water

ENP-full rate empty nanoparticle surfactant, and water

HTNP-half rate of nanoparticle encapsulated thiabendazole, surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

HFNP-half rate of nanoparticle encapsulated fludioxonil, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Figure 4. Average disease severity 7 days after planting of seed treated maize inoculated with *Fusarium graminearum*. Treatments sharing letters are similar by Tukey's Honest Significant Difference test.

Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer

1/2ENP-half rate of nanoparticle, surfactant and water

ENP-full rate empty nanoparticle surfactant, and water

HTNP-half rate of nanoparticle encapsulated thiabendazole, surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

HFNP-half rate of nanoparticle encapsulated fludioxonil, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Table 3. P-values from the first run of delayed emergence ANOVA table for effect of seed treatment, delay, and soilborne fungal inoculation utilizing PROC GLIMMIX.

Source	df	DS[1] P>F	SL P>F	RL P>F	FW P>F	DW P>F
Trt[2]	5	0.0076	0.2705	0.6405	0.0076	0.0076
Fg[3]	1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Trt*Fg	5	0.0034	<0.0001	0.0987	0.0034	0.0034
Delay	3	0.0101	0.1214	0.0046	0.0101	0.0101
Trt*Delay	15	0.1169	0.5772	0.1448	0.1169	0.1169
Fg*Delay	3	<0.0001	0.0456	0.0050	<0.0001	<0.0001
Trt*Fg*Delay	15	0.3749	0.4186	0.3413	0.3749	0.3749

[1]DS = disease severity, SL = shoot length, RL = root length, FW = fresh weight, DW = dry weight.

[2]Trt = seed treatment.

[3]Fg = *Fusarium graminearum*.

Table 4. P-values from the second run of delayed emergence ANOVA table for effect of seed treatment, delay, and soilborne fungal inoculation utilizing PROC GLIMMIX.

Source	df	DS[1] P>F	SL P>F	RL P>F	FW P>F	DW P>F
Trt[2]	5	<0.0001	<0.0001	0.0433	<0.0001	<0.0001
Fg[3]	1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Trt*Fg	5	0.0490	<0.0001	0.5846	0.0467	0.0467
Delay	3	<0.0001	0.1574	0.0204	0.0196	0.0196

Table 4. continued

Trt*Delay	15	<0.0001	0.0904	0.0008	0.0004	0.0004
Fg*Delay	3	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 4 continued

| Trt*Fg*Delay | 15 | 0.0002 | 0.0219 | 0.0662 | 0.1239 | 0.1239 |

[1]DS = disease severity, SL = shoot length, RL = root length, FW = fresh weight, DW = dry weight.

[2]Trt = seed treatment.

[3]Fg = *Fusarium graminearum*.
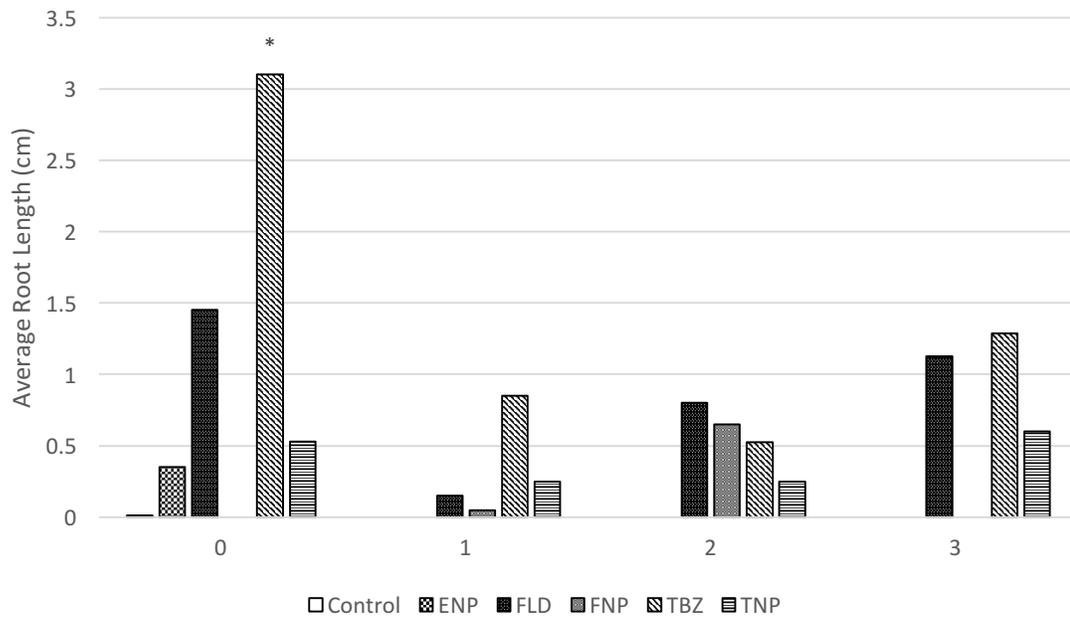
Figure 5. Average root length 28 days after planting of seed treated maize in infested soil with *Fusarium* (second run). Treatments were placed in a 13°C chamber for 0, 1, 2, or 3 weeks to delay emergence before being transferred to a 24°C chamber for the remaining duration of the study. Treatments with an asterisk represent those that were significantly different from the inoculated control in their respective environmental parameters by Tukey's Honest Significant Difference test.
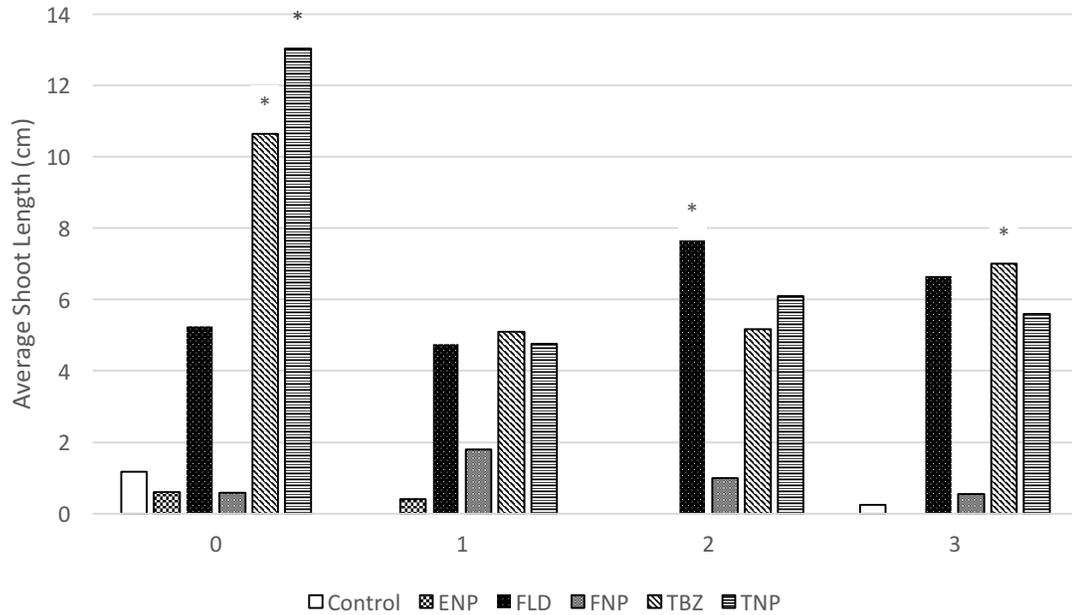
Figure 6. Average shoot length from run 1, 28 days after planting of seed treated maize in infested soil with *Fusarium.* Treatments were placed in a 13°C chamber for 0, 1, 2, or 3 weeks to delay emergence before being transferred to a 24°C chamber for the remaining duration of the study. Treatments with an asterisk represent those that were significantly different from the inoculated control in their respective environmental parameters and run by Tukey's Honest Significant Difference test.

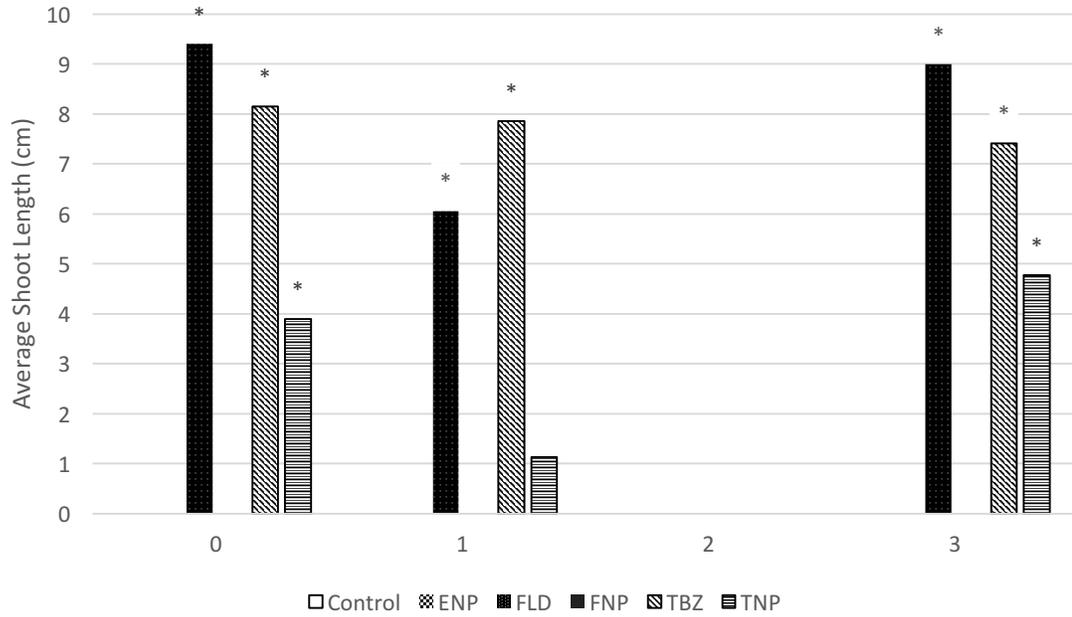Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer

ENP-full rate empty nanoparticle surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Figure 7. Average shoot length from run 2, 28 days after planting of seed treated maize in infested soil with *Fusarium.* Treatments were placed in a 13°C chamber for 0, 1, 2, or 3 weeks to delay emergence before being transferred to a 24°C chamber for the remaining duration of the study. Treatments with an asterisk represent those that were significantly different from the inoculated control in their respective environmental parameters and run by Tukey's Honest Significant Difference test.

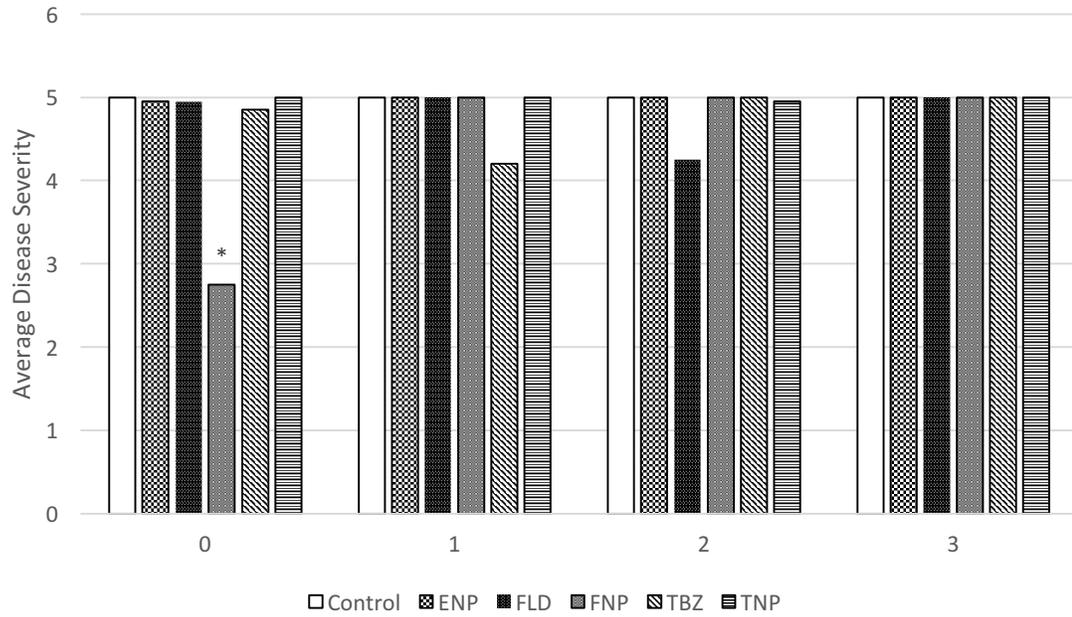Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer

ENP-full rate empty nanoparticle surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Figure 8. Average disease severity from run 2, 28 days after planting of seed treated maize in infested soil with *Fusarium.* Treatments were placed in a 13°C chamber for 0, 1, 2, or 3 weeks to delay emergence before being transferred to a 24°C chamber for the remaining duration of the study. Treatments with an asterisk represent those that were significantly different from the inoculated control in their respective environmental parameters by Tukey's Honest Significant Difference test.

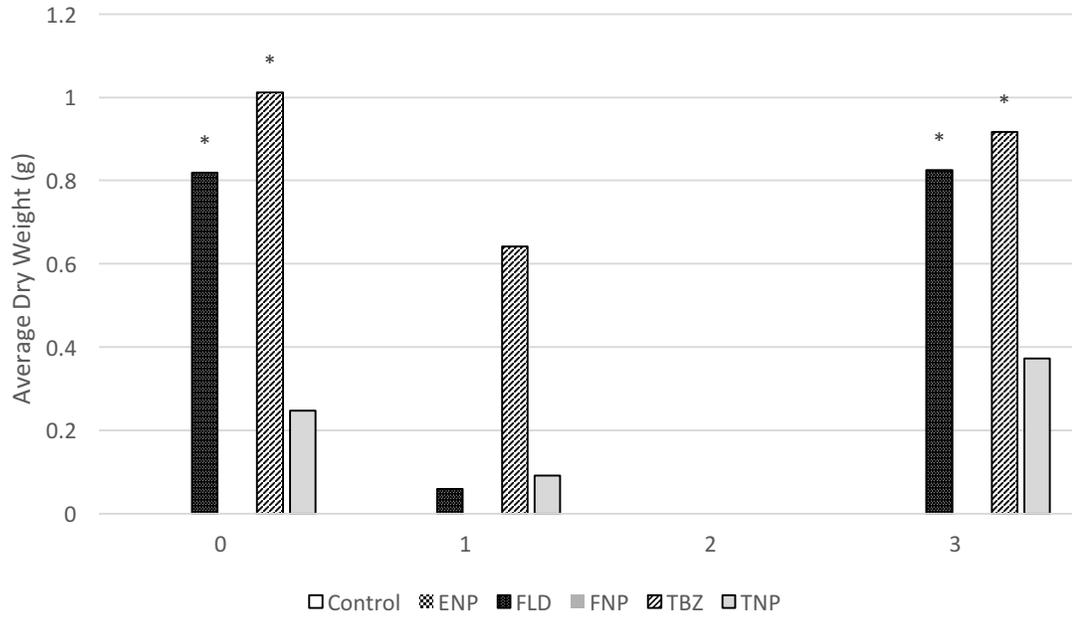Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer

ENP-full rate empty nanoparticle surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Figure 9. Average dry weight from run 2, 28 days after planting of seed treated maize in infested soil with *Fusarium graminearum.* Treatments were placed in a 13°C chamber for 0, 1, 2, or 3 weeks to delay emergence before being transferred to a 24°C chamber for the remaining duration of the study. Treatments with an asterisk represent those that were significantly different from the inoculated control in their respective environmental parameters by Tukey's Honest Significant Difference test.
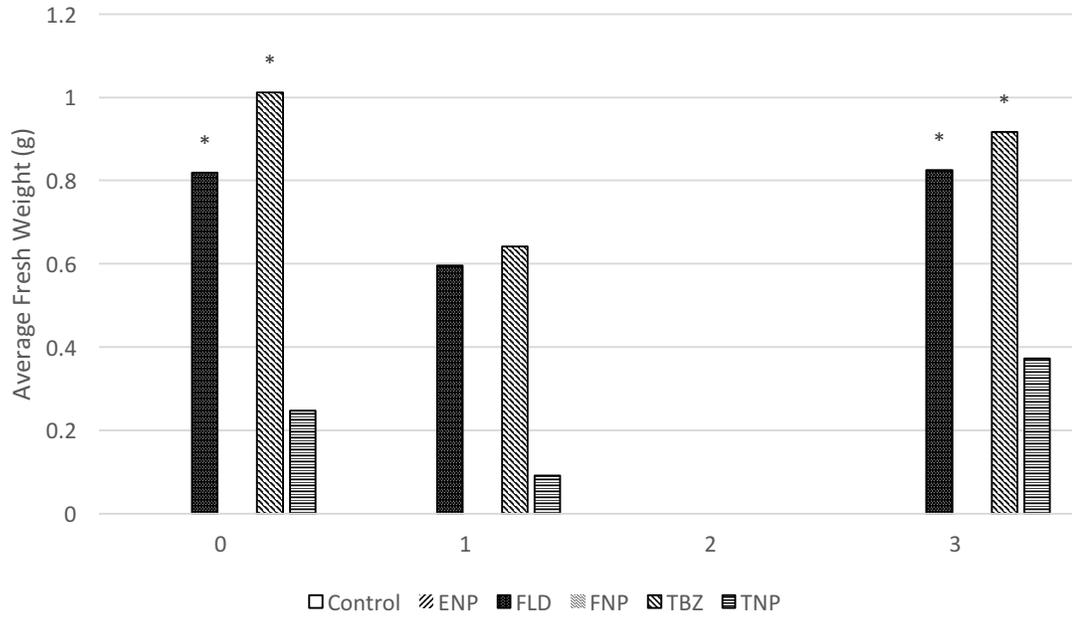
Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer

ENP-full rate empty nanoparticle surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Figure 10. Average fresh weight from run 2 28 days after planting of seed treated maize in infested soil with *Fusarium.* Treatments were placed in a 13°C chamber for 0, 1, 2, or 3 weeks to delay emergence before being transferred to a 24°C chamber for the remaining duration of the study. Treatments with an asterisk represent those that were significantly different from the inoculated control in their respective environmental parameters.
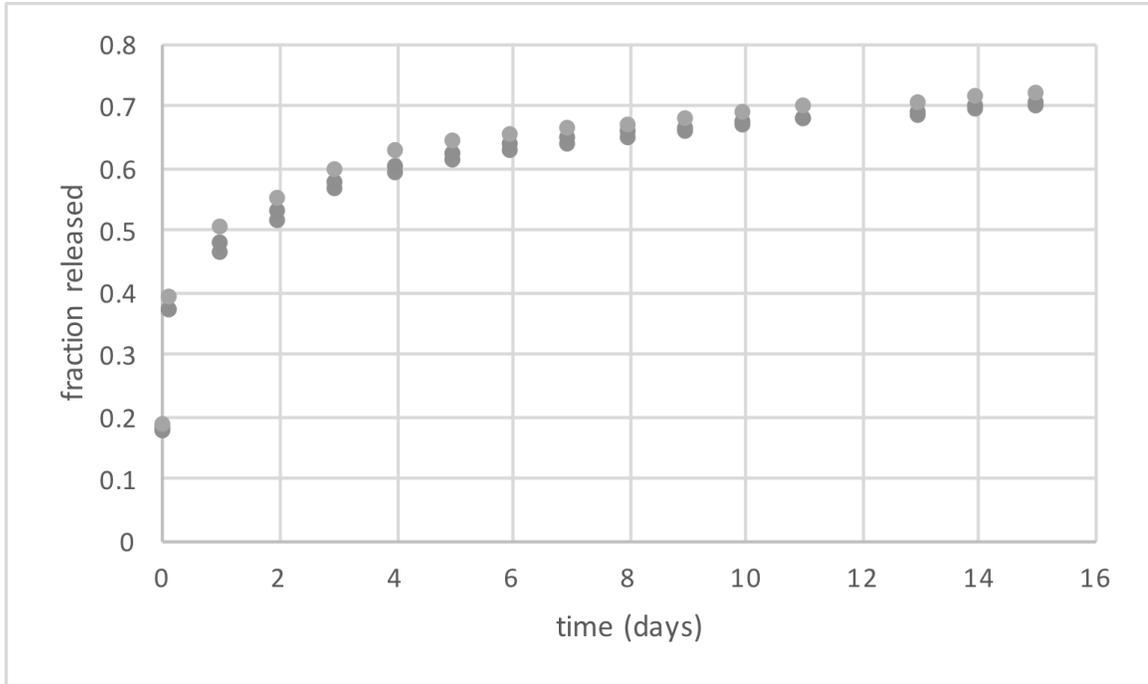
Control-plantability polymer and water

TBZ-thiabendazole, water, and plantability polymer

FLD-fludioxonil water and plantability polymer

ENP-full rate empty nanoparticle surfactant, and water

TNP-full rate of nanoparticle encapsulated thiabendazole, surfactant, and water

FNP- full rate of nanoparticle encapsulated fludioxonil, surfactant, and water

Figure 11. Release kinetics of thiabendazole loaded CPTEG:CPH nanoparticles over 16 days at 37°C. Data were collected by Dr. Balaji Narasimhan (professor) and Adam S. Mullis (graduate research assistant), Department of Chemical and Biological Engineering, Iowa State University of Science and Technology, 2017.
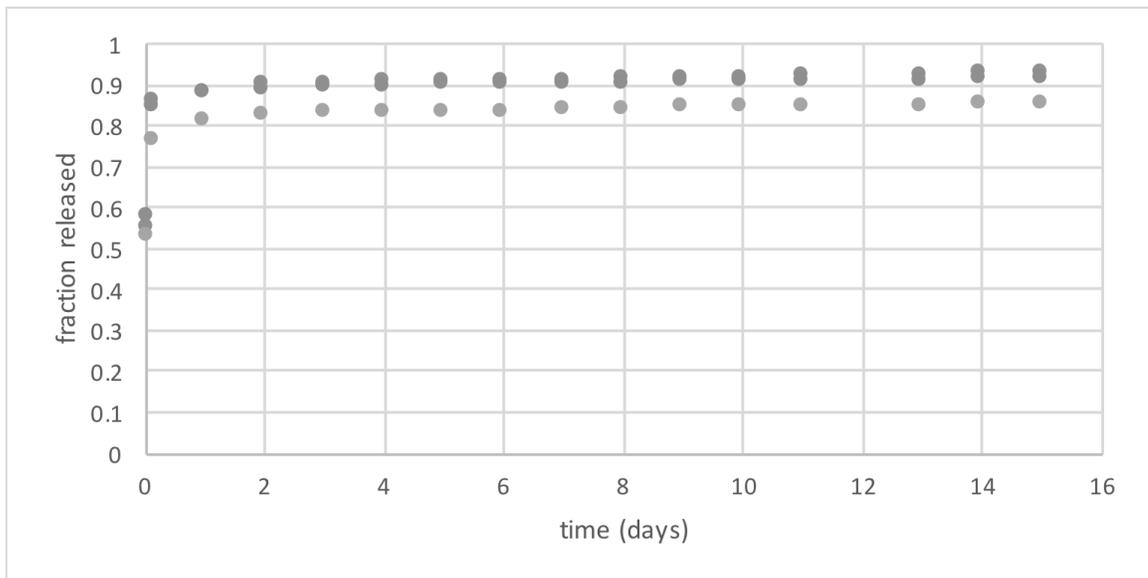
Figure 12. Release kinetics of fludioxonil loaded CPTEG:CPH nanoparticles 16 days at 37°C. Data were collected by Dr. Balaji Narasimhan (professor) and Adam S. Mullis (graduate research assistant), Department of Chemical and Biological Engineering, Iowa State University of Science and Technology, 2017.

CHAPTER 4 DISCUSSION

The results of this study show that nanoparticle encapsulated fungicides may be a viable seed treatment technique to protect against *Fusarium* maize seedling blight in the future. Despite the wide spread use of seed treatments on maize, seedling blight plays a significant role in yearly losses in every maize producing state (Mueller, 2015). To our knowledge, no other study has been conducted utilizing polyanhydride nanoparticle encapsulated fungicides as a seed treatment.

In delivery systems for other bioactive compounds, an advantage of polyanhydride nanoparticle encapsulation has been the ability to use reduced rates of active ingredients (Binnebose et al., 2015). We saw some evidence for that in this study. Within the rolled towel experiment, half-rate nano-encapsulated thiabendazole (HTNP) was statistically similar to thiabendazole (TBZ) and fludioxonil (FLD) in protecting the root length of maize root systems against *F. graminearum*. However, the HTNP treatment did not perform as well as the TBZ treatment for all variables. The thiabendazole treatment had statistically longer shoots and greater fresh weight than FLD and HTNP, while FLD and HTNP were still statistically similar.

The similarity of protection conferred by HTNP and TBZ during the rolled towel experiment is encouraging, given that the actual rate of active ingredient was lower than the nominal rate (0.025 mg/seed). This occurred due to variation in nanoparticle loading efficiency. Formulation application rates were calculated with the assumption that the nanoparticle formulation was 12% active ingredient (either FLD or TBZ); however, subsequent testing indicated that in fact they were 7.2% thiabendazole and 7.6%

fludioxonil. With this knowledge, the fact that HTNP did comparably well to TBZ in terms of mean root length and was similar in shoot length, is promising for potentially being able to reduce the amount of thiabendazole used in seed treatments in the future. Loading efficiency may require an optimization process for each active ingredient, in order to attain higher and more consistent levels of active ingredient in the final formulation.

Conversely, there was no evidence for a rate-reducing advantage when fludioxonil was the active ingredient. Given that non-encapsulated fludioxonil was able to confer protection to infested maize seedlings, the observed lack of protection by fludioxonil loaded nanoparticles may be due to the fact that they have a high initial release rather than a sustained release of active ingredient. This issue of release kinetics may be solved by investigating different polymer chemistries that vary in the rate and type of bulk and surface erosion (Carillo-Conde et al., 2015).

Seed-to-seed variability in coating uniformity may have contributed to inconsistency of performance for the nanoparticle formulations. Variable results were occasionally observed per towel and per seed within the rolled towel assay as well as per cone in the delayed emergence for the FNP and TNP treated seeds exposed to *Fusarium graminearum*. This may be due to uneven coating of seeds when the nanoparticle water suspension was pipetted over the seeds during plastic bag treatment for both the FNP and TNP treatments. Further work should be done to verify whether this method of seed coating is effective for nanoparticle treatments by perhaps utilizing imaging of the seeds before planting and varying the amount of water used in suspension.

Another potential advantage of nanoparticle encapsulation is controlled release of active ingredients, which can prolong the duration of activity (Li et al., 2005). This was

investigated in the delayed emergence experiment, which involved soilborne rather than seedborne inoculum. These experiments were designed to mimic field conditions that are sometimes too cold for rapid maize seedling emergence, when prolonged seed treatment activity would be desirable. Incubation of planted seeds at 13°C was intended to postpone germination, but treatments held at 13°C for longer than two weeks began to emerge before being transferred to the 24°C chamber. In comparison seeds in the 24°C chamber emerged after approximately forty-eight hours. A lower temperature may be used in future experiments, in order to achieve the intended emergence delays.

As in the rolled-towel experiments, TBZ performed the best of the fungicide treatments in terms of root and shoot length, as well as fresh and dry weight; however, disease severity was not significantly reduced by any treatment amongst the delays of emergence, with the exception of FLD when there was no delay in emergence. The pathogen isolate was extremely virulent and the high disease severity may have masked the treatment effects of the fungicides and nanoparticles used in this experiment. Adjustments to the inoculation procedure may be needed in order to better assess treatment effects under disease conditions that more closely resemble field observations.

Nanoparticle-encapsulated fludioxonil did not perform better than the untreated inoculated control in either type of experiment. This may be due to the fact that the release kinetics of FNP were different than that of TNP (Narasimhan and Mullis, 2017; (Fig. 11 and 12). While both formulations exhibited an initial burst of their active ingredient, the burst of FLD was more than double that of TBZ, making the fungicide available immediately rather than providing a prolonged release of active ingredient, which may have an effect on its efficacy. This release kinetic study, however, was performed at 37°C

and not performed in soil, which may impact the actual release profiles that would be observed in a field situation. Furthermore, the chemistries of each active ingredient differ and may have an impact on their respective loading and release efficiencies within the nanoparticles. As observed in Carrillo-Conde et al. (2015) different polymer ratios and chemistries can alter the amount of active ingredient released as well as release kinetics after the initial release. In order to better understand the results and optimize the nanoparticle formulations, it would be advisable to conduct release kinetics studies in soil under a range of temperature and moisture conditions with a variety of different nanoparticle chemistries in order to determine their range of capability.

CHAPTER 5 CONCLUSIONS

To our knowledge, this is the first study involving polyanhydride nanoparticle encapsulated fungicides as seed treatments. We observed that by encapsulating thiabendazole and applying it at half the label rate, it can confer relatively comparable protection against *Fusarium graminearum* seedling blight in comparison to a traditional full rate application. However, this benefit requires further confirmation and may not be applicable to other active ingredients.

Controlled release of seed treatment active ingredients is an important potential benefit of nanoparticle encapsulation, but the severity of disease within the delayed emergence experiments masked the potential effects of the seed treatments and should be investigated further, utilizing a different isolate or inoculation protocol. A key aspect of this potential benefit is the understanding of release kinetics of nanoparticle-encapsulated active ingredients in soil. Either in conjunction with, or separately from a future study, the release kinetics of the respective nanoparticle formulations to be used should be studied in soil and with a range of different temperatures comparable to those that would be found in the field.

In future, creating nanoparticle encapsulated fungicide cocktails may allow for decreased dosage of fungicide applications to seeds. Further research should be done to investigate the ability of these nanoparticles to release fungicides at a prolonged rate and their impact on persistence in plants and adsorption in various soil types and weather conditions.

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