Nanoparticle encapsulation to enhance seed treatment efficacy against *Fusarium graminearum*

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Plant Pathology

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2020

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ABSTRACT

Fusarium graminearum is a soilborne and seedborne pathogen widely distributed in agricultural areas all over the planet. It can cause serious damage on several economic crops, such as maize, wheat, and soybean. The main tactics to manage diseases caused by this fungus are to plant resistant varieties, apply fungicides, and to plant fungicide-treated seeds.

The importance of seed treatments has increased rapidly in the past decade, mainly due to their high efficacy controlling early-season pests and diseases, and due to their reduced environmental impact. If chemicals are applied as seed treatments, less pesticide is used and applied in the environment, and the selection pressure for the development of resistance in the pest population is much lower as well, compared to foliar or soil applications. However, the rapid dissipation of active ingredients after planting is associated with unpredictability about the effective duration of control, limiting the performance of this technology.

Polyanhydrides are carbon polymers that can be used to deliver active ingredients or pharmaceuticals in pathology systems. They can promote a steady release of active compounds, enhancing the treatment of diseases and pathogens. Therefore, this research addresses one of the main limitations of seed treatment technology by using polyanhydride polymers to encapsulate two fungicides commonly used against *F. graminearum*: fludioxonil and thiabendazole.

In order to study these two encapsulated fungicides, we performed two types of assays on maize and soybean: a rolled-towel assay (simulating a seedborne infection) and a delayed emergence assay (simulating a soilborne infection). The rolled-towel assay is similar to a germination test, with additional inoculation and fungicide treatment steps. Fifteen seeds per

replicate are placed on towels and incubated for seven days at 24°C. For the delayed emergence assay, ten seeds per treatment were planted in infested field soil and incubated in four different environments for four weeks. Each environment had a specific number of weeks at a non-emergence condition (10°C), to simulate a delayed emergence scenario.

Our results suggest no evidence of enhanced efficacy for the full rate nanoparticle-encapsulated treatments against this pathogen for the rolled-towel assay. However, the half rate nanoparticle-encapsulated treatments performed similarly to the full rate nanoparticle and full rate conventional fungicide. In the delayed emergence assay, the nanoparticle formulations performed better than conventional active ingredient formulations in the treatment with a 1-week delay in emergence only. For longer emergence delay treatments, nanoparticle and conventional fungicide treatments showed similar levels of control. Additionally, a storage assay was performed to assess the stability of nanoparticle formulation efficacy. Our results suggest a similar level of disease control compared to the conventional fungicides, measured over a 5-month storage period.

Release kinetics assays were conducted to assess the pattern of release of the nanoparticle-encapsulated treatments used in this research. Results showed a high burst in the first few hours, reaching above 40% of the total amount of active ingredient independently of the temperature.

In conclusion, polyanhydride nanoparticle encapsulation of fungicide seed treatments showed potential to provide enhanced efficacy and prolonged release of active ingredients when emergence is delayed due to cold temperatures. However, more research is needed on the dynamics of active ingredient release in soil under different environmental settings. Hence, more bioassays can be designed to evaluate employment of other chemicals and different types of pesticides, such as insecticides. The combination of different polyanhydride formulations carrying different

pesticides could revolutionize how integrated pest management is implemented through seed treatments.

CHAPTER 1: LITERATURE REVIEW

Production of high yields of maize or any other crop is very challenging and multi-dimensional. Optimizing management by choosing the best cultivar, best planting window, and proper scouting for pests is just a summary of decision-making processes that farmers are challenged to address. Among different types of disorders which can harm yield during the whole season, seedling diseases can have a critical effect on field productivity. Early infections are more harmful to plant yield than late infection due to the high impact of early infections on plant population per area. While late infections might not decrease plant population due to limited time until harvest, early infections have more time to develop and spread within the season, hence, can eliminate more plants when compared to the same pathogen as late infection. Furthermore, diseased surviving plants have their development and production impaired, decreasing the potential yield of the remaining plant population (da Silva *et al*., 2016).

Seedling diseases

Seedling diseases frequently are the result of the interaction of multiple organisms, including fungi, oomycetes, bacteria, and nematodes. In some cases, there is a synergistic effect between organisms in different kingdoms; for instance, wounds caused by direct feeding of a nematode predisposes plants to fungal or bacterial colonization (da Silva *et al*., 2016). In general, areas with a high incidence of soilborne pathogens, emergence, and plant development issues are frequent. Symptoms such as failure to emerge, wilting, yellowing, chlorosis, root rot, and slow growth are common.

Seed rots, seedling blights, and root rots typically are caused by several genera of fungi and oomycetes including *Fusarium, Aspergillus, Pythium, Rhizoctonia, Penicillium,* and *Trichoderma*. These genera are soil inhabitants and can harm seed germination and seedling development. Several of these genera, such as *Fusarium*, also can be seedborne. Cold soil conditions can promote seedling disease by slowing seed germination, although some seedling pathogens are favored by warmer soils. Soil temperatures under 12°C are favorable for fungal colonization by most seedling pathogens but not for seed germination, which increases the severity of seedling diseases in early plantings. These conditions are more common in no-till and reduced tillage systems (Robertson and Munkvold, 2009).

Fusarium is among the most important genera of seedling pathogens (Munkvold and O'Mara, 2002). Furthermore, *Fusarium* spp. are well known due to their diversified list of hosts (da Silva *et al*., 2016). *Fusarium graminearum* Schwabe [syn. *Gibberella zeae* (Schweinitz) Petch] is responsible for significant losses in several crops, causing Gibberella ear and stalk rot of maize and Fusarium head blight (FHB) of wheat, as well as root rots in several crops. Management of this pathogen is very complicated because crop rotation is not practical due to its wide host range. In 2013, Arias *et al.* investigated different *Fusarium* spp. assessing the aggressiveness and yield impact on soybean. Among nine different species, *F. graminearum* caused the most severe root rot symptoms.

Ecology of *Fusarium graminearum*

Fusarium is a member of the Ascomycota, and is one of the most heavily studied genera of plant pathogenic fungi due to its enormous relevance to agriculture. Species of this genus cause

substantial economic losses registered over the past centuries on crops such as maize, soybean, banana, and wheat. *Fusarium graminearum* as first described was a polyphyletic grouping of distinct fungal species; however, based on genealogical concordance, this species is now classified as a monophyletic complex with nine separate phylogenetic species, some of which are limited to different geographical regions (Munkvold, 2017). *F. graminearum sensu stricto* is the most important species of the *Fusarium graminearum* species complex (FGSC) and is the primary pathogen responsible for Fusarium head blight (FHB) of cereals and Gibberella stalk and ear rot of maize, which have been reported in most parts of the world (Agrios, 2005; Munkvold, 2017). The diversity of the *F. graminearum* complex is reflected in a large number of hosts that are colonized, its impact on grain yield and quality, and its production of mycotoxins that are hazardous to animals and humans (Rubella and Kistler, 2004).

Mycotoxin production

In addition to the impact of *F. graminearum* on crop yields, losses result from the production of mycotoxins, which contaminate the raw and processed grain, making it unfit for animal and human consumption. Mycotoxins are secondary metabolites that are toxic to animals, can suppress plant defenses, and provide a competitive advantage against other fungi. Members of the FGSC are known for the production of important mycotoxins such as Type B trichothecenes. *F. graminearum* is the most important species in the genus due to its broad host range (e.g., maize, wheat, barley, oats, rice, sorghum, potatoes, coffee, and legumes) and its diverse array of mycotoxins, producing important compounds such as deoxynivalenol (DON; deleterious for swine digestive system and other monogastric animals), zearalenone, fusarins, culmorins and others

(Munkvold, 2017; Schmale and Bergstrom, 2003). DON levels in wheat plants infected with FHB frequently reach values near 20 ppm, whereas the USDA recommendation for the maximum safe level is 1ppm (Schmale and Bergstrom, 2003). Sometimes, grains that are infected late in the season may not have apparent symptoms but are still contaminated. Infected seed can increase the risk of disease spread if the contaminated seed is planted in the subsequent season (Schmale and Bergstrom, 2003).

Field management

The management of this pathogen requires the employment of several methods: options include resistant or tolerant cultivars, proper agronomic practices, fungicide applications, and biological control. As the disease cycle starts with primary inoculum from infected seeds, as well as from perithecia, mycelia or chlamydospores on plant debris, and continues with macroconidia produced during the season as secondary inoculum, management practices must target both sources of primary inoculum and secondary spread.

Screening for maize-resistant hybrids has been ongoing for the past decades. Breeding focuses on reduced symptom development and low mycotoxin production (e.g., DON). Quantitative trait loci (QTL) for Gibberella ear and stalk rot were identified and can be exploited for resistance breeding (Kebede et al., 2015). Crop rotation and tillage may contribute to disease management by decreasing primary inoculum for the next season but should be associated with in-season tactics to manage secondary inoculum.

The employment of non-host crops in rotation sequences reduces the success of pathogen colonization and multiplication in the current season, thereby reducing the amount of primary

inoculum for the next season for diseases caused by this pathogen, such as FHB and Gibberella stalk and ear rot, especially when combined with application of fungicides (Schmale and Bergstrom, 2003). Fungicides can be used as seed treatments or as sprays during the growing season aiming for different outcomes. While seed treatments prevent seed transmission, spraying prevents infection when applying at early vegetative stages V5 to V6, and after pollination, VT to R3 (Munkvold and White, 2016). Additionally, timing for application to control FHB in wheat is also in early reproductive stages because is the most susceptible phase. Application immediately preceding flowering can achieve better results (Schmale and Bergstrom, 2003).

Generally, the management of diseases requires the combination of many tactics. Additionally, predictive models that use research on relationships between weather and disease development can help farmers to predict disease incidence. Models can be used to optimize the management of seedling and foliar diseases by combining cultivar resistance and other information to assess risk and support decision making. For instance, ScabSmart Management is a tool developed by the USDA, which provides information on key management aspects for grain crops affected by FHB. The tool relates cultivar resistance, scab forecasting based on weather and risk assessment, fungicide timing and availability, crop rotation recommendation, and other management strategies such as residue management, planting date, harvest practices, and seed treatment.

Seed treatment has been used as a strategy to manage fungi for centuries (Munkvold, 2009). Commercial use of seed treatments has been standard for decades. Treatment of seeds is being widely used to manage a range of diseases and pests; sharp increases in the value of the seed treatment market are due to many factors. Seed treatments are safer than aerial application. The amount of active ingredient required to perform a seed treatment is typically 90% less than if used

as a spray. The area impacted by the application is 99.5% less, which is directly related to environmental safety. Seed treatment therefore has a great environmental advantage over foliar application, for diseases and pests that can be managed through seed-applied technologies.

Control of diseases using seed treatments is mainly linked to the avoidance of colonization of seeds and seedlings in the first stages of development, during which the population of productive plants is established. For instance, there are many synthetic active ingredients such as captan, trifloxystrobin, azoxystrobin, and fludioxonil that are widely employed as seed treatments for maize (Broders *et al.,* 2017). Moreover, seed treatments also include biological control agents, such as yeasts (e.g., *Cryptococcus flavescens*) and bacteria (e.g., *Bacillus* spp.), which have shown promising results controlling *F. graminearum* and reducing mycotoxin production (Schmale and Bergstrom, 2003).

The use of seed-applied fungicides is one major management strategy to control seedling diseases; however, it is constrained by several factors including rapid dissipation of active ingredients, limiting uptake in plant tissues and absorption by target organisms. Therefore, our research investigates improving the efficacy of seed-applied active ingredients through the use of nanoparticles.

Nanoparticles

The nano- prefix is originally from Greek, meaning dwarf. A nanometer is 10^{-9} meters. Therefore, nanoparticles (NPs) are, by definition, particles with size within a range of 1-100 nm. Moreover, they are grouped into different classes based on their chemistry, size and shape, such as metal, ceramic, and polymeric. The main feature of NPs is a unique set of characteristics,

including a high surface by volume relation and high reactivity. Due to their unique chemistry, they are promising candidates for application in many endeavors, from the delivery of vaccines to synthetic chemicals (Khan et al., 2017; Bhatia, 2016).

Nanoparticles have been employed in a wide range of research areas. The use of nanotechnology in pharmaceutical science has shown successful improvement of conventional methods of drug delivery. Nanotechnology is an emerging branch in pharmaceutical sciences, providing new tools and opportunities, especially regarding treatment of diseases (Bhatia, 2016). Recent advancements have proven that the small size and unique physicochemical properties of nanoparticles can reduce toxicity, enhancing release, improving solubility and bioavailability when employed for drug delivery (Bhatia, 2016).

Polymeric NPs have been employed in research settings for the delivery of drugs and vaccines. Their size ranges from 10 to 100 nm, and special features as biodegradability and biocompatibility offer advantages for delivery of active compounds. Due to their controlled and sustained release patterns, stealth and modified surface, they can be used for active and passive delivery of bioactives. Within this class, polyanhydrides have been extensively studied as delivery platforms due to their specific surface erosion kinetics (Phanse et al., 2016; Bhatia, 2016).

Polyanhydride nanoparticles are polymers that have been studied extensively in biological systems to facilitate time release of active compounds. They have a surface erosion mechanism that promotes a sustainable and predictable release of encapsulated active ingredients. They are biodegradable and relatively hydrophobic; however, hydrolysis of the anhydride bond occurs easily, resulting in surface-eroding systems. Alteration of the proportion between monomer and

co-monomer provides a range of release duration, ranging from weeks to months (Phanse et al., 2016; Berkland et al., 2004).

In work conducted by Binnebose et al. (2015), polyanhydrides were used as drug carriers to kill filarial endoparasites. The polymer carrying antibiotics showed effective killing of *Brugia malayi* and its symbiotic bacteria *Wolbachia*. Experiments showed a great reduction (4,000-fold) in the amount of active ingredient required and a five-fold reduction in the time to death of the pathogen. Authors suggested that the high permeability of the particles delivering the drugs directly to both pathogens significantly enhanced effectiveness of the treatment (Binnebose et al., 2015).

To our knowledge, the first study involving the use of fungicides within polyanhydrides carrying fungicides as seed treatment was reported by Washington (2017). CPTEG:CPH formulation were used to encapsulate thiabendazole and fludioxonil against *F. graminearum*. Results suggested that encapsulated thiabendazole at half rate resulted in comparable protection against the pathogen in comparison with the same active ingredient at full rate (label rate). Based on this initial research, we decided to employ the same polymeric NPs, varying the ratio of CPTEG:CPH, and the loading of particles as an approach to improve the efficacy of treatments.

Our proposal is to use polyanhydride nanoparticles to encapsulate thiabendazole, a systemic fungicide, and fludioxonil, a contact fungicide, both of which are widely used as effective seed treatment ingredients. Additionally, we propose to use the NPs to encapsulate a novel compound, guanylhydrazone, reported to control *F. graminearum* infection of maize kernels (Woriedh *et al.*, 2011). One of the advantages of the use of nanoencapsulation is to facilitate employment of different types of compounds as active ingredients. Novel molecules, even

bioproducts or bioagents could be applied as seed treatments by encapsulating them in a nanopolymer. Therefore, we chose CNI-1493 due to its antimicrobial effect, and for its potential as an untested seed treatment active ingredient.

Guanylhydrazone (CNI-1493)

Known for its antimicrobial effect, guanylhydrazone (CNI-1493) is considered a promising drug for antimalarial therapy. It showed to be a potential candidate for the suppression of replication of human immunodeficiency virus type 1 (HIV-1) (Woriedh *et al.*, 2011). Due to its antiproliferative potential, it was studied as an agent against fungal virulence, more specifically, *F. graminearum*. CNI-1493 showed strong inhibition of the pathogen during infection of wheat and maize flowers. As it inhibits fungal DHS (deoxyhypusine synthase), it reduces pathogen virulence and development (Woriedh *et al.*, 2011). This activity against *F. graminearum* makes it an attractive candidate as a seed treatment component.

In summary, by reviewing possible benefits of reduced payloads of fungicides and controlled release aiming for extended control of diseases, we hope to provide an alternative method to deliver pesticides with higher efficacy in a more friendly alternative to the environment. Therefore, the objectives of this research were to determine if nanoencapsulation can extend the duration of efficacy of fungicide seed treatment into the season, to characterize the release of the fungicides from nanoparticle encapsulation under different temperatures, to assess efficacy of reduced rate nanoparticle-encapsulated treatments, to assess the stability of nanoparticle-encapsulated formulations in storage, and to determine the suitability of nanoparticle encapsulation for a novel crop protection compound.

CHAPTER 2: MATERIALS AND METHODS

Experiments were conducted to address two main hypotheses: i) nanoparticle encapsulation of seed treatment fungicides can prolong the release of active ingredients; ii) prolonged release can enhance the control of *Fusarium graminearum*. Additional experiments were conducted to characterize the release of active ingredients from nanoparticle encapsulation under different environments, and to assess the antimicrobial effect of a novel compound that could potentially be used as a nanoparticle-encapsulated seed treatment. In order to evaluate efficacy against seedborne inoculum using full and half rates of active ingredients, we conducted rolled-towel assays. Seeds were inoculated, treated and then placed on top of germination paper and incubated for seven days at 24°C. Additionally, to study the prolonged release effect, we conducted assays with a delayed-emergence scenario, where seeds were subjected to a low temperature, unfavorable for germination, prior to a higher temperature, favorable for germination. These treatments simulated early planting in cold soils. Seeds were planted in soil, held at 10°C for different periods, followed by an increase to 24°C until the termination of the experiment. In addition to influencing the efficacy of commonly used seed treatment active ingredients, the use of nanoparticles could also allow the employment of novel biological or synthetic compounds as treatments. To test this possibility, we conducted *in vitro* and *in vivo* experiments testing Guanylhydrazone as a potential candidate for the control of *F. graminearum*.

Seed preparation

Before each trial, seeds were externally disinfested by a two-step process. Seeds were soaked in a solution of bleach and then rinsed using deionized sterile water. For maize, we used a solution of 10% bleach, and a soaking period of five minutes. After the seeds were removed from the solution, seeds were rinsed at least five times until the bleach was completely removed. For soybean, the external disinfestation was performed with reduced bleach concentration (5%) and exposure period (1 minute), due to the high sensitivity of soybean seeds to physical and chemical damage. As bleach is commonly used to check the damage on soybean seed coats, we used this step to remove damaged seeds, such as swelled and cracked ones (VanUtrecht & Rukunudin, 2000). Finally, seeds were placed in labeled trays with paper towels inside a biosafety cabinet to dry before the next steps.

Isolate and inoculum preparation

The isolate of *F. graminearum* used for preliminary and primary experiments was FG27 (isolated in Iowa, U.S.A). It was grown for each experiment from stored silica gel pellets, dated from December 2016. We used two forms of inoculation: a spore suspension for rolled-towel assays, simulating a seedborne infection, and infested millet for experiments in field soil, simulating soilborne inoculum. The spore suspension was prepared by reviving the fungus from silica pellets on potato dextrose agar (PDA) (BD, Franklin Lakes, New Jersey) for ten days at ambient temperature (24-26°C). In order to promote sporulation, mycelial plugs from PDA cultures were transferred to Spezieller Nährstoffarmer Agar (SNA) (Leslie and Summerell 2008) plates. After approximately 15 days, spores from SNA plates were collected with sterile deionized

water and filtered through sterile cheesecloth. The concentration was adjusted to reach 1 x 10^5 spores/mL using a hemocytometer. Inoculation was finally performed by soaking batches of 100 seeds in 30 ml of a spore suspension for five hours. All containers were placed on a shaker at ambient temperature at 92 rpm. For soybeans, the soaking period was reduced to one minute. After the inoculation step, seeds were dried on paper towels inside a biosafety cabinet for twelve hours before application of the seed treatments.

Alternatively, to prepare inoculum for studies using field soil, we infested the soil using colonized millet. We pasteurized the millet by autoclaving it for 90 min at a temperature of 121°C. The millet remained in a temperature above 80°C for more than one hour after the autoclave cycle was finished. This process was done twice on two consecutive days. At the second day, after letting the bags containing the millet to cool down, PDA plates containing 15-day-old *F. graminearum* were blended and poured into aerated bags containing autoclaved millet seeds in a one-plate-per-bag ratio. These bags were kept in a growth chamber for 15-20 days until the mycelial growth covered all millet kernels. For the noninoculated control treatment, the colonized millet was autoclaved twice before mixing with soil. Millet inoculum and control millet samples were checked for viable *F. graminearum* by culturing on PDA. Millet was mixed into pasteurized field soil for all treatments, including controls. The percentage of inoculum in the soil (volume/volume) was 5%.

Seed treatment application

The traditional active ingredients chosen to be used in this research were fludioxonil and thiabendazole. Both fungicides are known to have a broad spectrum of action against fungi, and

are very effective against *Fusarium graminearum*. They are two components of the commercial formulation Maxim Quattro®, commercialized by Syngenta Crop Science. While the dosage of fludioxonil is only 0.0065mg/kernel (3.32% of the total slurry), thiabendazole has a much higher rate, 0.05mg/kernel (26.5%). The recommended dosages of each active ingredient were used with addition of a plantability polymer. The polymer used was Flo Rite® 1197 (BASF) following the manufacturer recommendation, approximately 0.44 ml per kernel. Besides the two traditional fungicides and their nanoparticle-encapsulated versions, CNI-1493 was used as a seed treatment as well. It was mixed with the plantability polymer and water similarly to what was done for the traditional fungicides. All compounds were then mixed with water (15 µL per seed) and vortexed before application on seeds. Similarly, for the nanoparticle formulations, we used the equivalent mass of the active ingredients. However, the total mass of active ingredient was adjusted to consider encapsulation efficiency and the total loading of the nanoparticles to ensure that the same active ingredient dosage would be delivered to each seed (Table 1). Nanoparticle treatments were formulated with a surfactant, Span80, but without the additional plantability polymer. Before the application, treatments were sonicated using a VCX 130PB sonicator with a CV138 tip (Sonics and Materials, Newton, CT) at 30% amplitude to reduce aggregation of particles.

Controls with no seed treatment with and without *F. graminearum* inoculation were added as well (Table 1). The treatments were applied by adding the slurry into plastic bags containing the specific number of seeds. After the application, the seeds were rubbed together to apply the solution evenly over the seeds. Seeds were dried again within a biosafety cabinet for 24 hours after treatment.

Table 1. Treatments employed in rolled-towel and delayed-emergence assays. All treatments included F. graminearum inoculation or infestation, except for CONTROL1.

Treatment codes	Treatment description
Non-inoculated	Water and plantability polymer without *F. graminearum* inoculation
Untreated	Water and plantability polymer with *F. graminearum* inoculation
FLD	Fludioxonil (0.0065 mg/kernel), water and plantability polymer
N-FLD	Polyanhydride nanoparticle encapsulated fludioxonil (0.0065 mg/kernel), water, and Span80
TBZ	Thiabendazole (0.05 mg/kernel), water and plantability polymer
N-TBZ	Polyanhydride nanoparticle encapsulated thiabendazole (0.05 mg/kernel), water and Span80.

Besides the treatments listed in the table above, additional treatments were included only in the second run of the rolled-towel assay. They consist of an unloaded nanoparticle (N-EMPTY), and half rates of each fungicide, i.e., HN-TBZ (half dosage of N-TBZ) and HN-FLD (half dosage of N-FLD).

Rolled-towel assay

Assessment of fungicide efficacy applied as seed treatment was performed using rolled germination paper as described by Ellis *et al*., 2011, except that seeds were inoculated prior to

"planting," as already described. These assays were conducted using maize seeds under favorable conditions of germination (23-25°C) for seven days.

For each replication, 15 seeds were placed on top of two layers of germination paper (Anchor Paper Co., St. Paul, MN) moistened with sterile water (Figure 1). A third sheet was placed on seeds, and they were rolled and placed inside a plastic bag containing sterile water. Each bag was placed inside buckets, which were covered with a second bag to maintain the moisture inside the bucket. They stayed inside a growth chamber at 24°C for one week, and then seven-day old seedlings were analyzed for root length, shoot length, disease severity, and total fresh weight. Disease severity was measured on a one-to-five scale (Cruz Jimenez, 2017), with five being the most severe disease, and one being completely healthy (Figure 1). Results of severity, root length, shoot length and plant weight were analyzed for analysis of variance and mean separation using Tukey's Honest Significant Difference test at $p \leq 0.05$. Planned contrasts were employed as well to address specific questions amongst all pairwise comparisons.

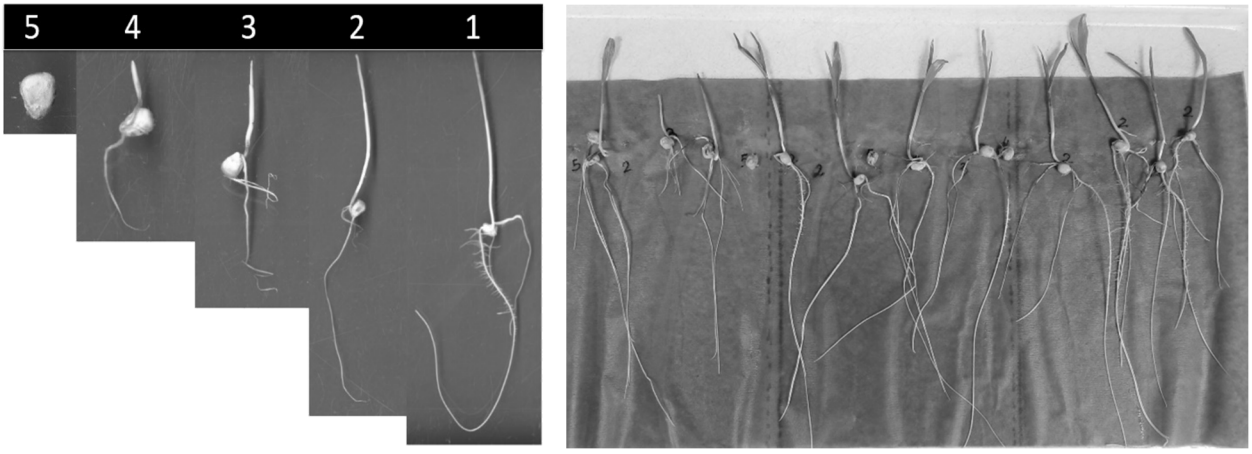

Figure 1. A – Disease severity scale; B – Seven-day old seedlings

Delayed Emergence assay

In this assay we assessed the efficacy of fungicides and their nanoencapsulated versions under conditions simulating an emergence delay due to cold soil temperatures. As the rapid dissipation of active ingredients is one of the most constraining factors of seed treatments, we employed this assay in order to evaluate if, in a delayed emergence situation, nanoparticle encapsulated fungicides would prolong the release of active ingredients, and hence improve and extend protection from the pathogen. Treatments consisted of four different temperature combinations, varying in the number of weeks of emergence delay (weeks after planting at 10° C). Treatments varied from no emergence delay to a three-week emergence delay (Table 2). This assay was repeated twice: first run was conducted using maize, and the second soybeans.

Table 2. Delayed emergence treatments for infested soil assay.

Treatments	Description
No delay	24°C for four weeks
1-week delay	10°C for one week followed by three weeks at 24°C
2-week delay	10°C for two weeks followed by two weeks at 24°C
3-week delay	10°C for three weeks followed by one week at 24°C

In order to assure correct temperatures for the duration of the experiments, data loggers (Spectrum Technologies, Aurora, IL) were placed in the growth chambers before the start to facilitate the temperature adjustment. Moreover, they remained through the entire experiment to monitor temperature fluctuations. Plants were uprooted after 28 days, and assessed for disease

severity, root and shoot length, and plant weight, identically to measurements used for the rolled-towel assays.

Data collected were analyzed by analysis of variance (ANOVA) in R version 3.6.1. Analysis of all main effects was conducted using all treatment combinations with Tukey's pairwise adjustments. Mean separations were done using Tukey's Honest Significant Difference test at $p \leq 0.05$, and orthogonal contrasts, where we combined the means of traditional fungicides *versus* nanoparticle encapsulated versions.

Storage assay

Stable efficacy of the nanoparticle-encapsulated compounds and active ingredients was assessed over time in storage. Maize and soybean seeds were treated and stored in a cold room simulating commercial conditions of seed storage (10°C and 50% RH) (Harrington, 1960). Batches of seeds were inoculated using a spore suspension and then treated following the aforementioned procedures. Approximately 180 seeds per treatment, per sampling date, were placed inside envelopes (8 cm x 14 cm) and then arranged in larger envelopes (23 cm x 30 cm) and stored in the cold room. Seeds were sampled every month for five months in storage. At each sampling date, continued efficacy of treatments was assessed by rolled-towel assays already described. Seedlings were analyzed for root length, shoot length, disease severity, and total fresh weight. Disease severity was measured on a one-to-five scale, with five being the most severe disease, and one being completely healthy (Figure 1). Results were analyzed for analysis of variance and mean separations using Tukey's Honest Significant Difference test at $p \leq 0.05$.

Antifungal activity assessment

Guanylhydrazone (CNI-1493) was synthesized *de novo* for assessment as a potential novel antifungal compound to be delivered as a nanoparticle encapsulated seed treatment. Activity of CNI-1493 against *F. graminearum* was assessed using three different approaches: fungal growth on CNI-1493-amended media, Kirby-Bauer test (the disc diffusion test), and a rolled-towel assay. Firstly, we grew *F. graminearum* on PDA for seven days. At day seven, we prepared modified PDA amended with the different treatments to be assessed, including 100 and 1000 ppm of CNI-1493, thiabendazole (1000 ppm) and non-modified PDA as controls. After the media were solidified, one-millimeter plugs of *F. graminearum* were placed on modified media and grown inside a growth chamber at 24°C for five days. Finally, fungal inhibition was measured by averaging two perpendicular colony diameters and dividing by the amount of growth on non-modified PDA. The result was multiplied by 100, to transform it to percentage.

Secondly, we used the Kirby-Bauer test (Bramhanwade *et al*., 2016), in which 1 ml of a spore suspension (10^5 spores/mL) was spread on the surface of PDA in petri dishes, and after 10 min, paper discs soaked in different solutions were placed on top of this fresh layer of spores. The discs were soaked in solutions of 100 ppm of CNI-1939, 100 ppm of thiabendazole, or water. The discs remained in the solutions for five minutes and were dried for one minute to remove the excessive moisture. This assay was used to evaluate to inhibition effect of the compound on the germination and development of the fungus. There were four plates for each treatment, and each plate had five soaked discs. The inhibition zones were assessed by measuring the size of the clear area surrounding the disc after seven days in a growth chamber at 24°C.

Lastly, we prepared a rolled-towel assay to evaluate the efficacy of CNI-1493 as a seed treatment. It was applied at 1000 ppm concentration (15 mg per kernel), mixed with the plantability

polymer similarly to what was done for the traditional fungicides. The treatments included full rate conventional thiabendazole, full rate of nanoencapsulated thiabendazole, and a third version with only half rate of nanoencapsulated thiabendazole (Table 3). The procedure was the same as described previously for rolled-towel assays.

Table 3. Treatments employed in the rolled-towel assay. All treatments included *F. graminearum* inoculation except for CONTROL1.

Treatment codes	**Treatment description**
Non-inoculated	Water and plantability polymer without *F. graminearum* inoculation
Untreated	Water and plantability polymer with *F. graminearum* inoculation
CNI-1493	Guanylhydrazone (1000 ppm), water and plantability polymer
TBZ	Thiabendazole (0.05 mg/kernel), water and plantability polymer
N-TBZ	The equivalent weight of 15% loaded conventional thiabendazole nanoparticle encapsulated, water and Span80.
HN-TBZ	Half dosage of the N-TBZ treatment

The CNI-1493 used on this research was synthetized by Dr. Joel Coats (professor) and James Klimavicz (graduate research assistant), Department of Entomology, Iowa State University of Science and Technology. The method is described in Appendix A.

Release Kinetics of Nanoparticles

In order to understand the release kinetics of the nanoparticle encapsulated formulations used in our experiments, we used HPLC (high-performance liquid chromatography) to estimate the mass of the fungicides released over time in an aqueous solution. As we want to understand the release trends for different potential soil conditions, we performed this assay under three different temperatures, 10°C, 15°C, and 24°C. The process started by weighing 9-11 mg of each nanoparticle formulation in triplicate and mixing with 0.5 mL of PBS (phosphate-buffered saline solution), pH 7.4. The particles were centrifuged, and the supernatant was collected, particles were washed with acetonitrile to extract the remaining fungicide. Fresh PBS was added, and the particles were re-dispersed by sonication. At the end of the aqueous (PBS) release, 40 mM NaOH was applied to rapidly breakdown the remaining particles, allowing estimation of the remaining encapsulated fungicide. This process was repeated daily for sixteen days, and each sampling was considered as one data point. 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.

Encapsulation efficiency was calculated by dividing the total encapsulated mass by the theoretical fungicide mass (based on the per tube particle mass and the nominal loading weight/weight ratio used during nanoparticle synthesis). Release kinetics are portrayed as fraction of active ingredient released, in which the cumulative mass of drug released is normalized by the total mass of encapsulated drug.

CHAPTER 3: RESULTS

Rolled-towel assay

For the first run, there were significant differences among treatments. *F*-test *p*-values were highly significant ($p < 0.0001$) for all the variables measured; i.e., severity (Figure 2), plant weight (Figure 3), root length (Figure 4) and shoot length (Figure 5). Thiabendazole (TBZ) and nanoparticle-encapsulated thiabendazole (N-TBZ) were not significantly different from one another for all variables. Each variable followed the same trend, where N-TBZ was slightly better, but not significantly different. However, conventional fludioxonil (FLD) and nanoparticle-encapsulated fludioxonil (N-FLD) were significantly different for all variables, with N-FLD demonstrating improved control of *F. graminearum*.

For the severity assessment (Figure 2), N-TBZ and TBZ presented similar results (1.24 and 1.33 respectively). On the other hand, N-FLD demonstrated lower levels of disease, reaching a severity of 1.36, while FLD averaged 2.36 ($p < 0.0001$), the highest value other than the untreated control. For plant weight (Figure 3), while N-FLD reached an average of 0.96 g, the mean for FLD was 0.76 g ($p < 0.0001$). FLD was also significantly different from the water control ($p = 0.0276$), where the average was 0.65 g. On the other hand, N-TBZ reached an average of 1.02, and TBZ 0.97 g ($p = 0.6768$).

Similarly, for root length (Figure 4), N-TBZ had the highest value, with an average of 217.9 mm, and TBZ averaged 210.3 mm. While conventional FLD averaged 169 mm, encapsulated fludioxonil reached 203.9 ($p = 0.0086$). Shoot length (Figure 5) was the only measurement where N-TBZ was slightly worse than TBZ (100.4 and 101.2 mm respectively), but not significantly

different. FLD and N-FLD were significantly different ($p = 0.0117$), averaging 75.9 and 92.1 mm, respectively.

The experiment was then repeated containing additional treatments. In this second run, the trend of results was different from the first one. When analyzing the data using *F*-tests, FLD and N-FLD results were not statistically different from one another for all variables; nor were there differences between TBZ and N-TBZ (Figures 6 through 9). Therefore, we decided to employ orthogonal contrasts to combine the two conventional ingredients and compare them against the two combined nanoencapsulated treatments (Figures 10 through 13).

The results showed no significant differences for root length ($p = 0.9556$) and plant weight ($p = 0.2053$). However, for severity and shoot length the *p*-values were 0.0450 and 0.0154, respectively. But this time, conventional formulations were significantly better than nanoparticle-encapsulated formulations. For severity, the combined value for the conventional ingredients was 1.49, while the nanoencapsulated combined average was 1.73. Conversely, for shoot length, nanoparticle-encapsulated formulations averaged higher than the conventional formulations. While conventional ingredients averaged 83.7 mm, the nanoparticle-encapsulated treatments reached 96 mm.

The non-inoculated and untreated control were statistically similar to several of the treatments that were inoculated but fungicide treated. The control that was inoculated and treated with only water (Untreated) was significantly different from all other treatments ($p < 0.0001$).

The two additional treatments that involved different rates of fungicides (half rates of nanoparticle-encapsulated thiabendazole and fludioxonil: HN-TBZ, HN-FLD) showed no significant differences compared to using full rates of the nanoparticle-encapsulated treatments for

all variables. Furthermore, there were no differences between using half rates of the nanoparticle-encapsulated fungicides and the full rate of conventional fungicides. The third additional treatment, nanoparticles alone (N-EMPTY), showed no difference compared to the untreated control, proving the effect of the treatment comes from the release of the encapsulated fungicide, without any antimicrobial effect of the polymer used.

Regarding the effect of inoculation in the water-treated controls, there was a significant difference for shoot length ($p = 0.0347$), severity ($p < 0.0001$) and plant weight ($p < 0.0001$). When comparing only the untreated control between the two runs, the severity level of the pathogen in the second run averaged 2.75, while for the first run, it was 3.33, showing a decreased aggressiveness of the pathogen in the second run.

Delayed emergence assay

In these experiments, we evaluated the efficacy of the seed treatments against soilborne *F. graminearum* under conditions simulating delayed emergence in cold soil. The plants were assessed after 28 days, following treatments that differed in temperature sequence, in order to evaluate whether nanoparticle encapsulation could prolong the effects of the active ingredients under different environments.

For the first run (maize), there were similar trends of results through different seed treatments and environmental treatments. Within the first environment (no delayed emergence) all pairwise comparisons showed no significant differences among seed treatments. Generally, N-TBZ and TBZ were slightly superior than FLD and N-FLD, but without statistical differences for all measurements.

In the second environment (1-week delay), pairwise comparisons showed statistical differences between seed treatments only for plant weight (Figure 20). While N-TBZ, TBZ and N-FLD were statistically different from the water-treated and inoculated control (Untreated), FLD was similar to the untreated control. For all remaining measurements, nanoparticle-encapsulated treatments were slightly superior, but without statistical differences. In the third environment (2-week delay), N-FLD was significantly different from remaining fungicides for shoot length (Figure 23), plant weight (Figure 24), and root length (Figure 25); N-FLD was statistically similar to the inoculated and water-treated control (Untreated). For severity (Figure 22), TBZ was the best treatment, statistically different from N-FLD and FLD, and similar to N-TBZ.

In the last environment (3-week delay), N-TBZ and TBZ had no statistical differences; however, N-TBZ was the only treatment with very low *p*-values when compared to the Untreated control for severity (Figure 26), shoot length (Figure 27), and plant weight (Figure 28). For root length (Figure 29), all treatments were similar to each other (*F*-test *p* was equal to 0.3243).

When employing contrasts to address our specific questions comparing only conventional ingredients *versus* nanoparticle-encapsulated treatments, clearer trends were evident. In this case, Figures 30-33 show all four environments for each measurement. For severity (Figure 30), only the 1-week emergence delay showed a low *p*-value (0.0760) for the treatment comparisons, but still not enough to be considered as statistically different. However, for shoot length (Figure 31) and plant weight (Figure 32), the trend was the same: statistical differences occurred only for the second environment (1-week delay), with the nanoparticle-encapsulated treatments performing better than the conventional fungicide treatments. For root length (Figure 33), there were no statistical differences for all four environments.

In the next experiment (soybeans), all pairwise comparisons for all environments showed the same trends: statistical differences were seen only when comparing seed treatments to the untreated control. All treatments, nanoparticle-encapsulated and conventional formulations, were similar to each other, with only one exception. In the second environment (1-week delay), N-FLD was statistically superior than TBZ (p = 0.0204). For all remaining comparisons among seed treatments, p-values were not significant. When analyzing the data with orthogonal contrasts, as used in the first run, we could see similar trends. Only in the second environment (1-week delay) were there statistical differences for two measurements. Plant weight (Figure 34) and shoot length (Figure 35) showed improved performance of the nanoparticle-encapsulated treatments compared to the conventional treatments. Severity (Figure 36) had a small p-value (0.0825) similar to the first run, but not small enough to be considered statistically different. For root length (Figure 37), and remaining environments, all contrasts showed no statistical differences.

Storage assay

Unlike the initial rolled-towel assay, in the storage experiment, the results showed superior control of the pathogen by the conventional formulations compared to the nanoparticle-encapsulated formulations for each sampling date (Figures 38 through 45). Across sampling dates, the trends differed among three main treatment groups: conventional formulations, nanoparticle-encapsulated formulations, and the water control. All results presented in this section are from the orthogonal contrasts from two complementary perspectives: within sampling dates and within treatments.

For severity (Figures 38 and 39), when analyzing the data among sampling dates within treatments, conventional formulations were very consistent, without any loss of efficacy through the whole duration of the experiment. Nanoparticle-encapsulated formulations showed a decrease in efficacy only between the first and second sampling dates ($p = 0.0002$), and then consistent efficacy until the end. The water control showed a small increase in severity until the third month, when it started to decrease. Severity of water control for the last sampling date was significantly different from the first ($p = 0.0068$), second ($p < 0.0001$) and third ($p < 0.0001$) sampling dates.

For root length (Figures 40 and 41), conventional formulations were very consistent until the fourth month in storage, with a loss in efficacy between the fourth sampling date and the last sampling date ($p = 0.0009$). For shoot length (Figures 42 and 43), more variation was observed. Conventional formulations were not consistent during the whole experiment. Second, fourth and fifth sampling dates had statistically inferior results compared to the first and third months. For plant weight (Figures 44 and 45), the analyses within treatments showed a steady result of conventional formulations until the third month in storage, with statistical decreases in the fourth and fifth months.

Antifungal activity assessment

In these experiments, we used different approaches to assess the antifungal activity of CNI-1493 (Figures 46 through 50). For all assays, the tested compound showed no antifungal activity against *F. graminearum*.

In the amended-medium assay (Figure 46), the compound promoted a faster growth of the pathogen in all different concentrations compared to the non-amended control. There was no negative effect of the compound on the growth and development of the pathogen in any repetition of this study. Pathogen growth was enhanced on the medium with 100 ppm or 1000 ppm CNI-1493 compared to the control. When compared to the positive control (pure PDA), the growth of the isolate was faster, reaching the board of the plates after four days for both concentrations (100 and 1000 ppm), while the control needed seven days to reach the same diameter.

Similarly, the Kirby-Bauer test results corroborated the results obtained from the amended-medium assay (Figure 47). There was no negative effect provided by the discs treated with CNI-1493 on the growth of *F. graminearum*. The pattern of growth of the CNI-1493 treated discs, and the water control was the same. The negative control (discs treated with thiabendazole) promoted inhibition towards the fungal growth.

When testing CNI-1493 in a rolled-towel assay, it showed an intermediate effect on control of seedling disease by *F. graminearum*; it was significantly different from the water control for shoot length ($p = 0.0005$), root length ($p = 0.0012$), and plant weight ($p = 0.0009$); however, it was statistically inferior to the remaining fungicide-based treatments. For severity, there was no significant difference compared to the water control. Compared to the positive control (TBZ), and other treatments (N-TBZ and HN-TBZ), CNI-1493 was less effective for all measurements ($p < 0.0001$).

For the three versions of thiabendazole, all variables showed the same trend, where there were no significant differences among the conventional formulation, nanoparticle-encapsulated full rate and nanoparticle-encapsulated half rate. For the control of *F. graminearum* in this assay,

the nanoparticle-encapsulated half rate was as effective as the nanoparticle-encapsulated full rate and the conventional formulation. For instance, while TBZ reached 1.45 grams, and N-TBZ 1.48, HN-TBZ averaged 1.40 grams for plant weight.

Release Kinetics of Nanoparticles

In these experiments, HPLC was used to evaluate the kinetics of the time-release of the active ingredients from nanoparticle encapsulation *in vitro* (Figure 51). Collaborators Dr. Balaji Narasimhan and Adam S. Mullis collected release kinetics data pertaining to the two different types of nanoparticles used; 20:80 CPTEG:CPH 20% fludioxonil, and 20:80 CPTEG:CPH 15% thiabendazole. The particles containing fludioxonil exhibited a large burst of fungicide release (~80% of total fludioxonil mass) within the first 24 hours. The remaining fludioxonil appeared to be released slowly through the end of the experiment. The release of 100% of the active ingredient was never reached. For thiabendazole loaded nanoparticles, the trend was similar; however, release occurred in a slower pattern. It exhibited a burst of fungicide of about 60% of the total after 24 hours, followed by a steady release of approximately 30% more of the fungicide until the end of the collection. Similarly, the total release never reached 100%. There were no significant differences among the three different temperature treatments (i.e., 10°C, 15°C, and 24°C).

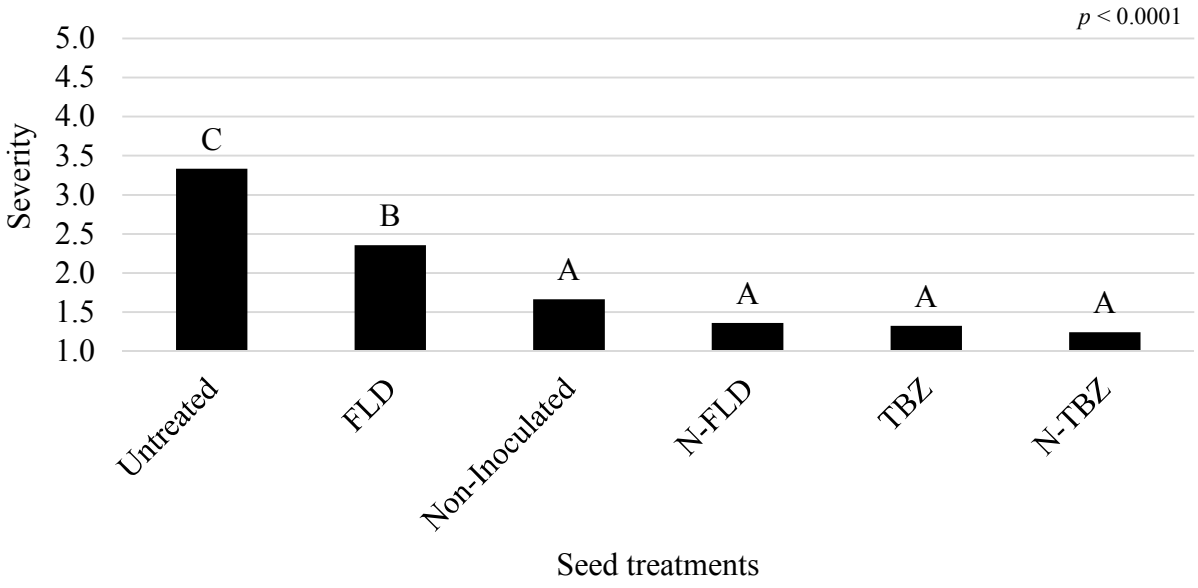

Figure 2. Average of severity for the first run of the rolled-towel assay. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

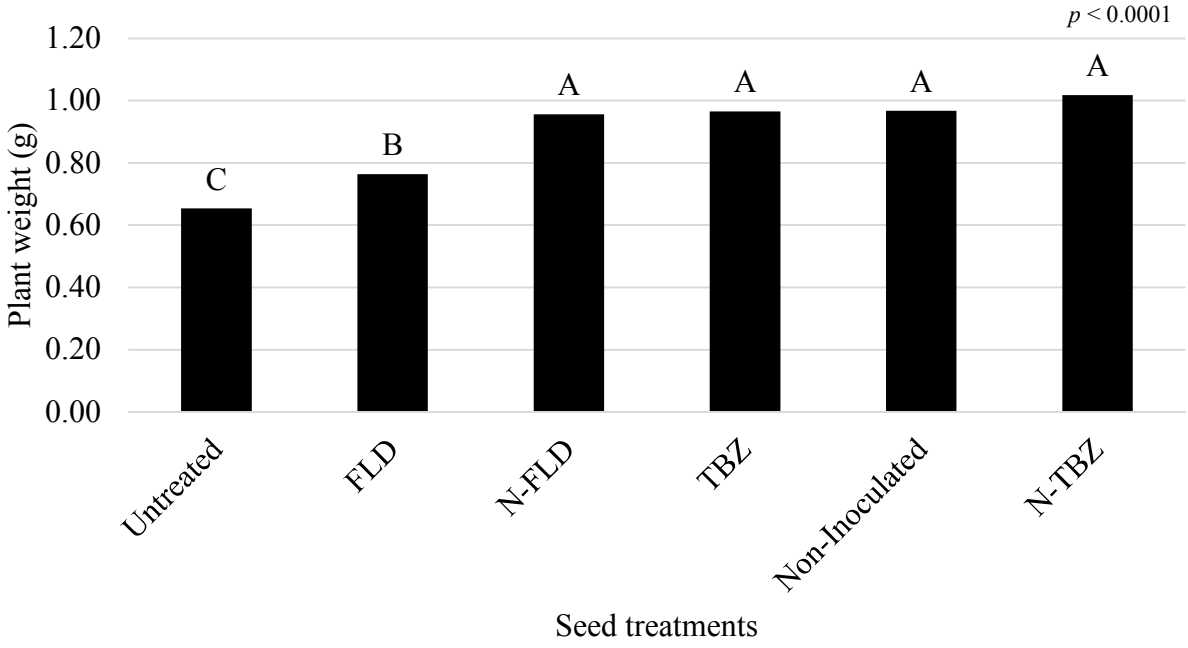

Figure 3. Average of plant weight for the first run of the rolled-towel assay. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

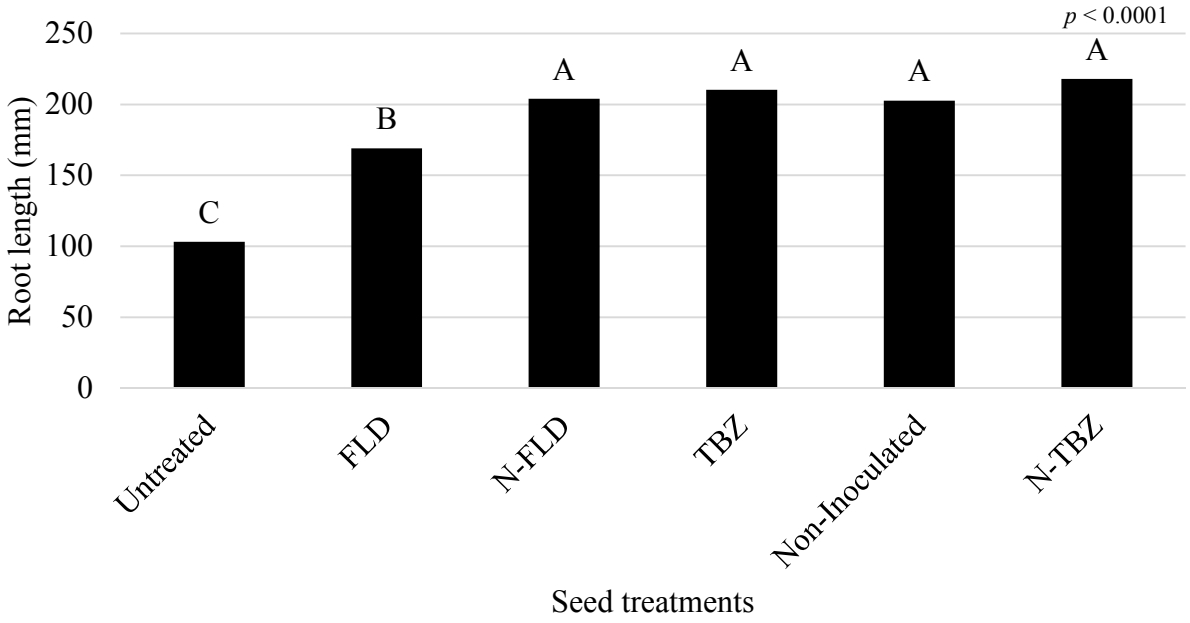

Figure 4. Average of root length for the first run of the rolled-towel assay. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

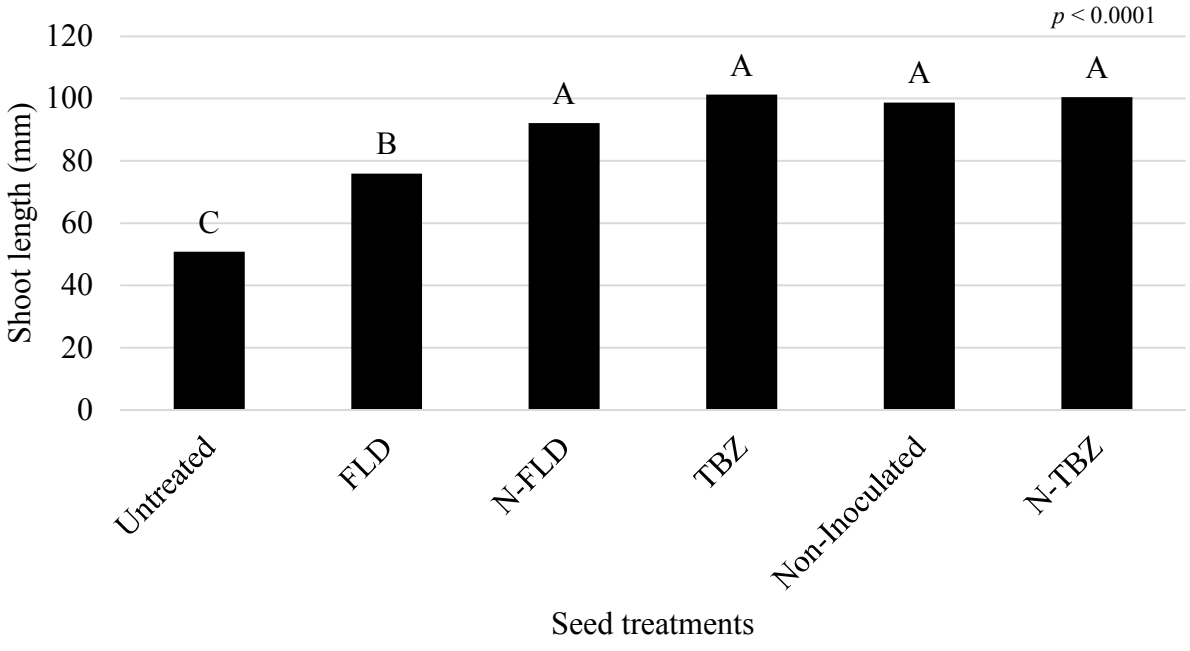

Figure 5. Average of shoot length for the first run of the rolled-towel assay. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

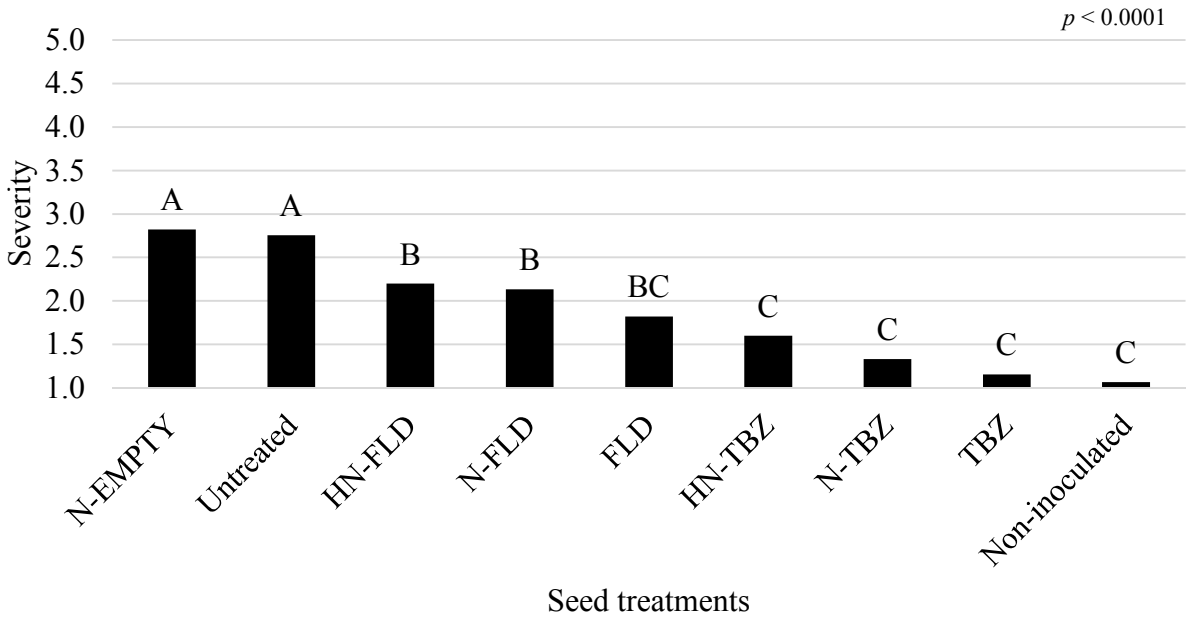

Figure 6. Average of severity for the second run of the rolled-towel assay. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

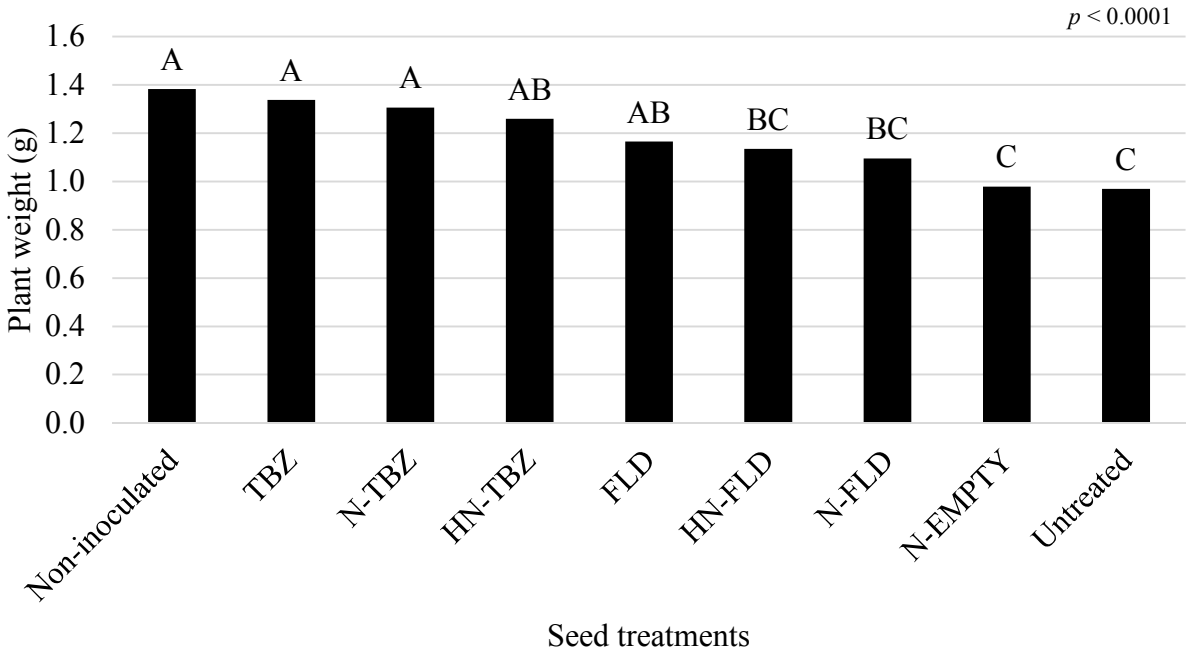

Figure 7. Average of plant weight for the second run of the rolled-towel assay. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

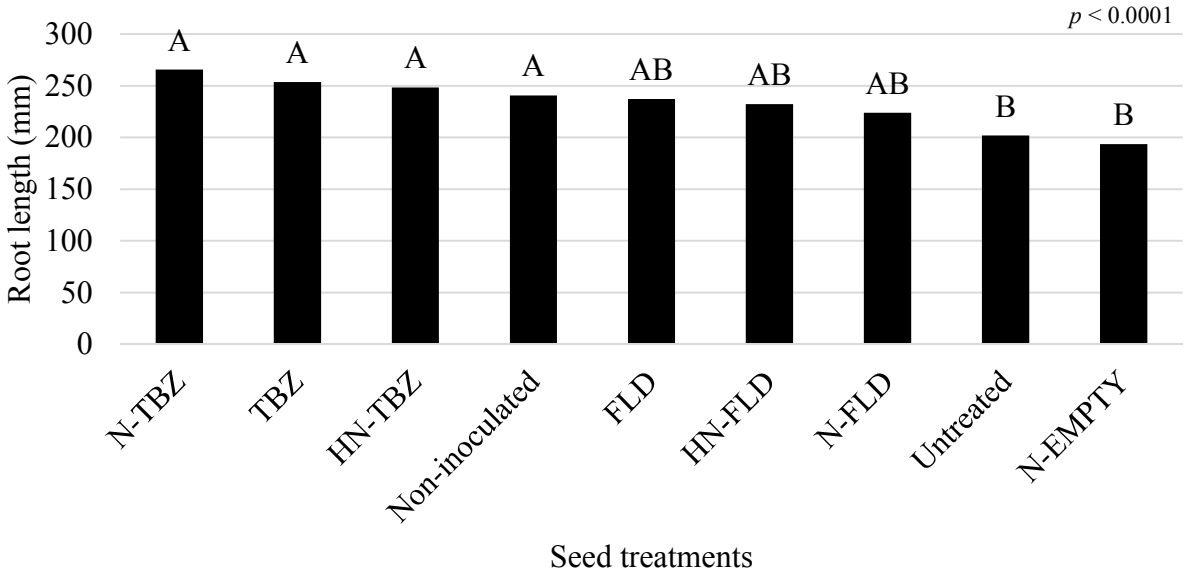

Figure 8. Average of root length for the second run of the rolled-towel assay. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

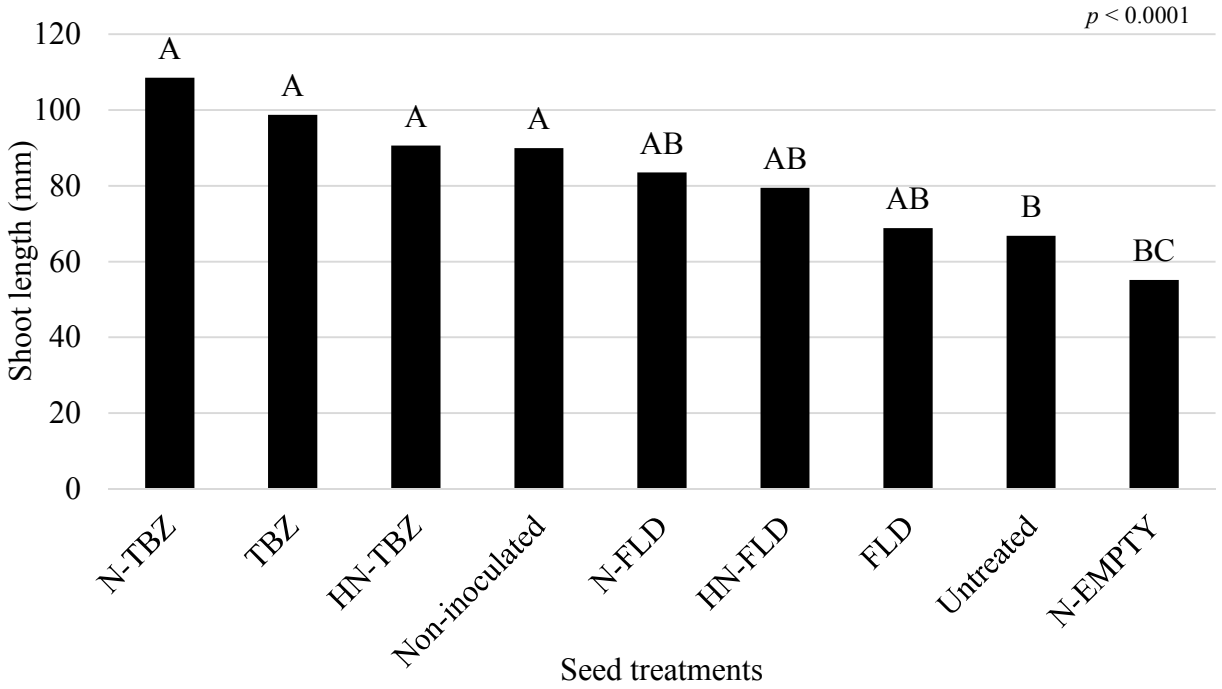

Figure 9. Average of shoot length for the second run of the rolled-towel assay. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

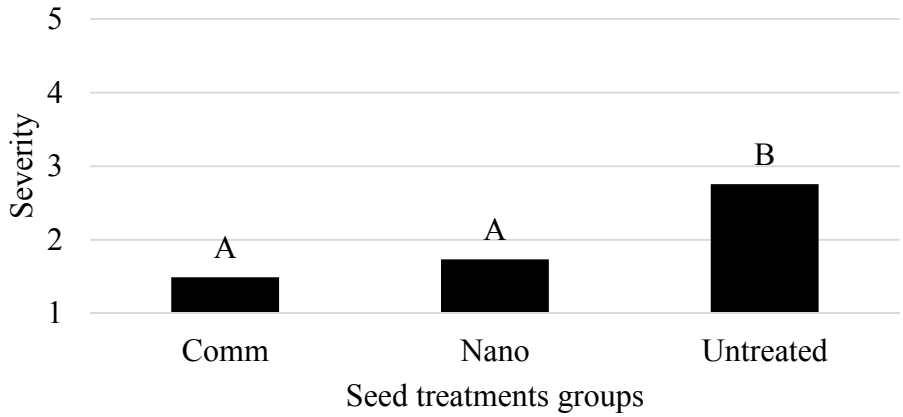

Figure 10. Average of severity for the second run of the rolled-towel assay using orthogonal contrasts comparing only nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

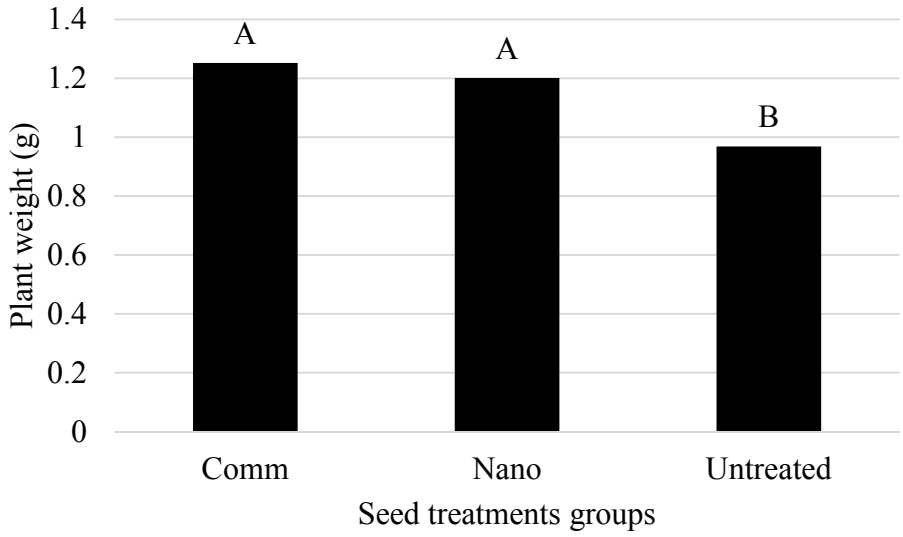

Figure 11. Average of plant weight (g) for the second run of the rolled-towel assay using orthogonal contrasts comparing only nanoparticle-encapsulated formulations versus conventional formulations. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

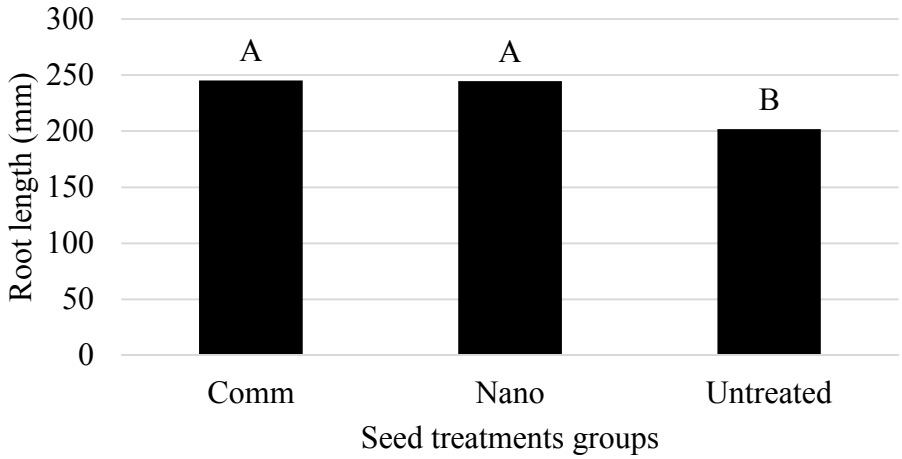

Figure 12. Average of root length (mm) for the second run of the rolled-towel assay using orthogonal contrasts comparing only nanoparticle-encapsulated formulations versus conventional formulations. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

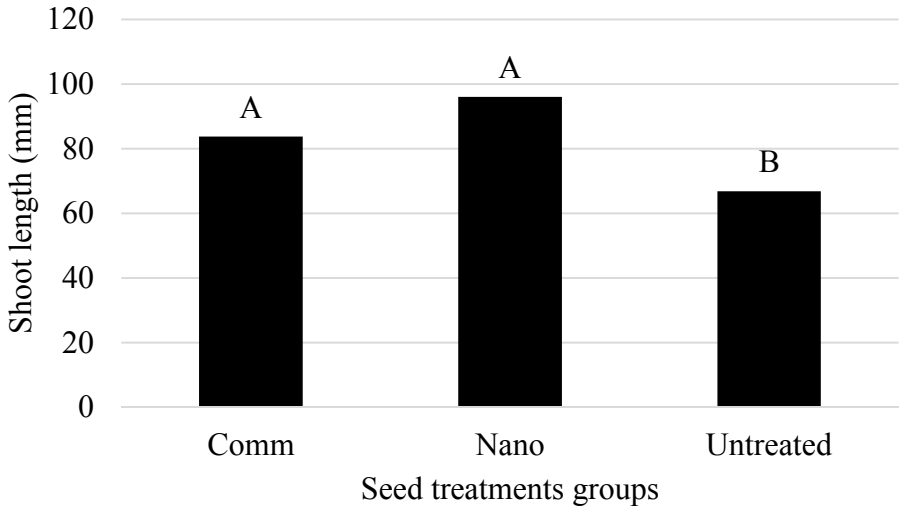

Figure 13. Average of shoot length (mm) for the second run of the rolled-towel assay using orthogonal contrasts comparing only nanoparticle-encapsulated formulations versus conventional formulations. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

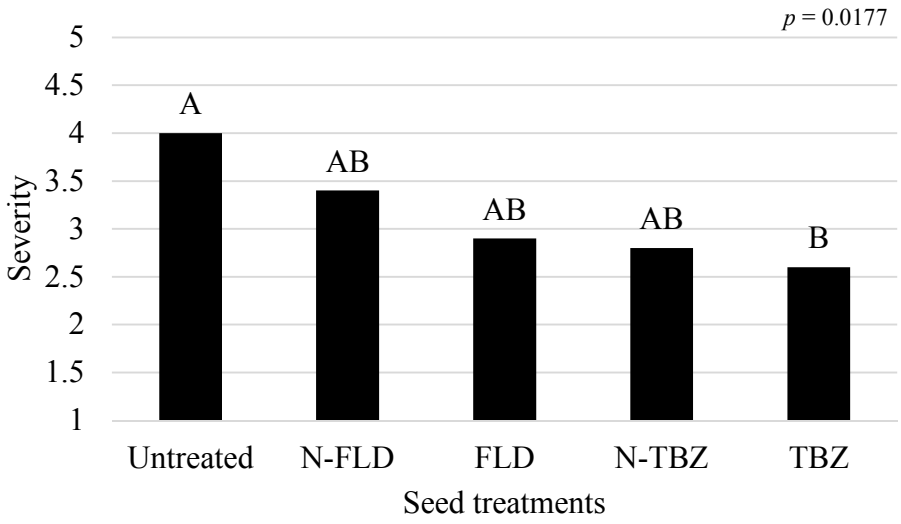

Figure 14. Average of severity for the first run of the delayed emergence assay for the first environmental condition (no delay in emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

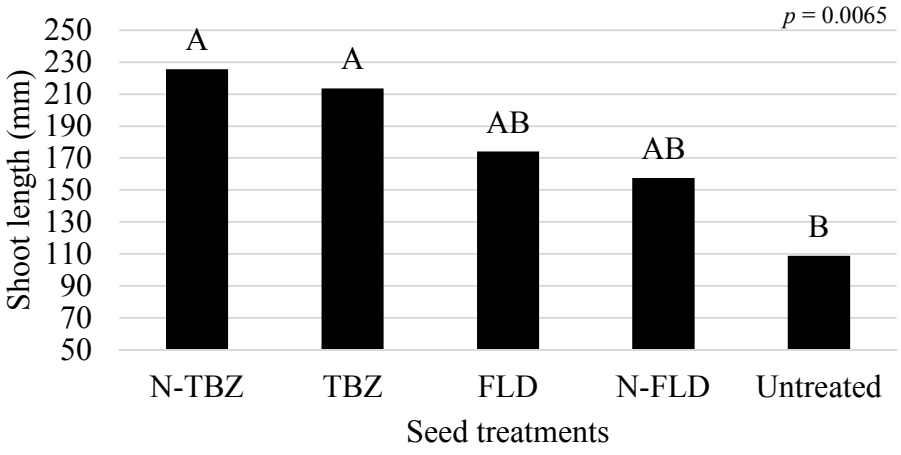

Figure 15. Average of shoot length (mm) for the first run of the delayed emergence assay for the first environmental condition (no delay in emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

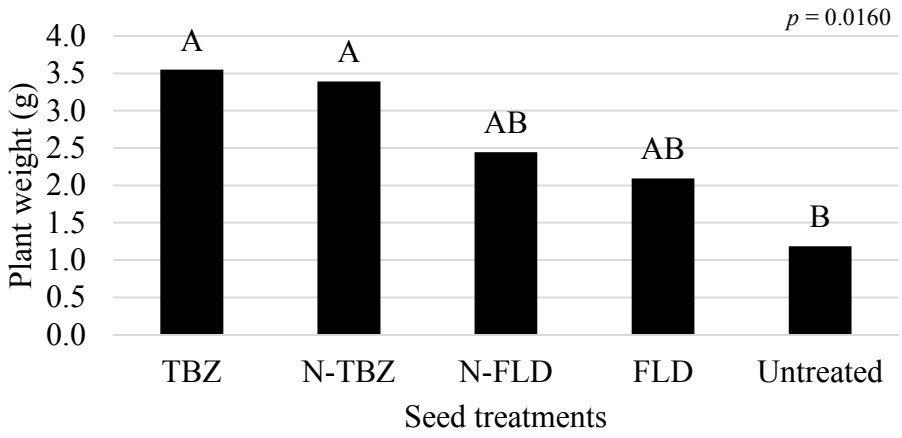

Figure 16. Average of plant weight (g) for the first run of the delayed emergence assay for the first environmental condition (no delay in emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

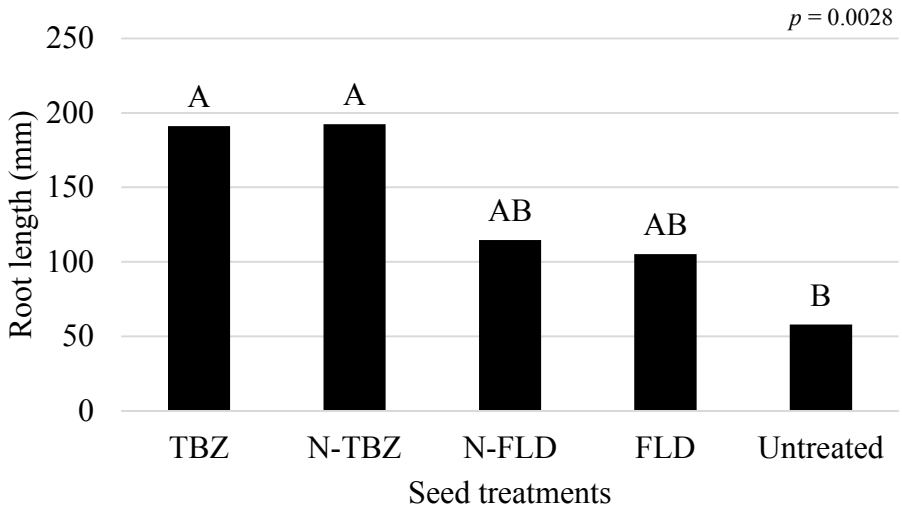

Figure 17. Average of root length (mm) for the first run of the delayed emergence assay for the first environmental condition (no delay in emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

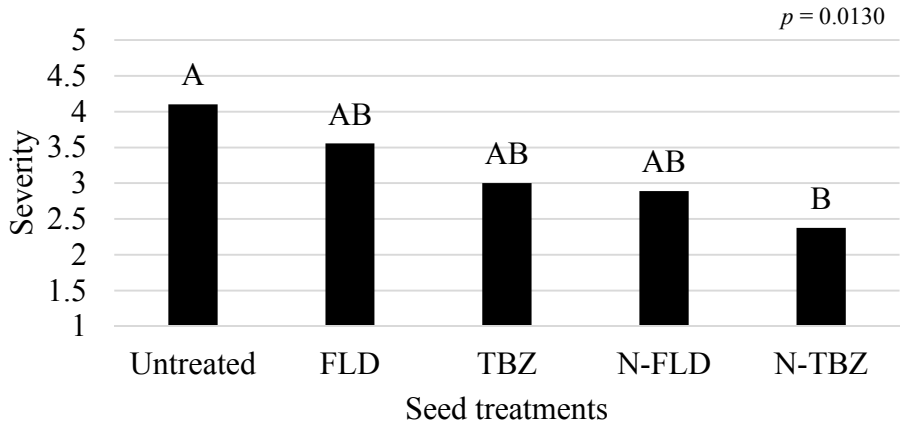

Figure 18. Average of severity for the first run of the delayed emergence assay for the second environmental condition (1 week of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

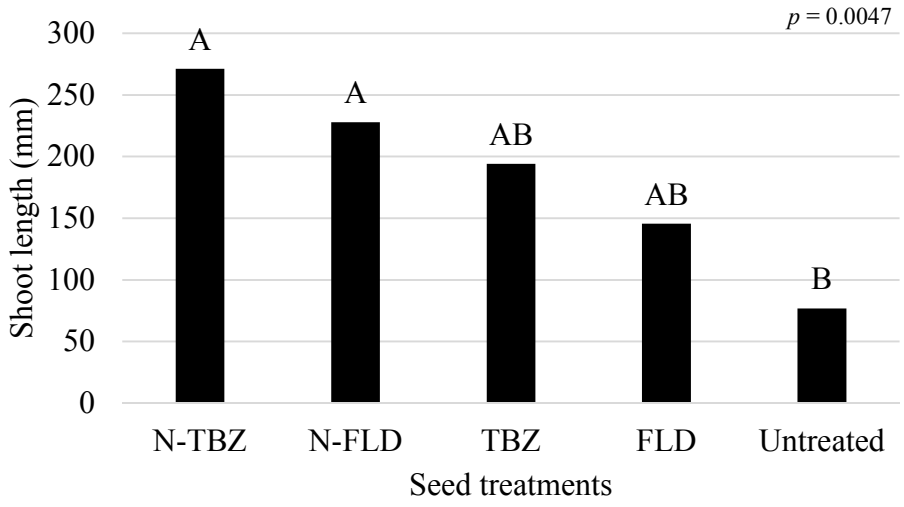

Figure 19. Average of shoot length (mm) for the first run of the delayed emergence assay for the second environmental condition (1 week of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

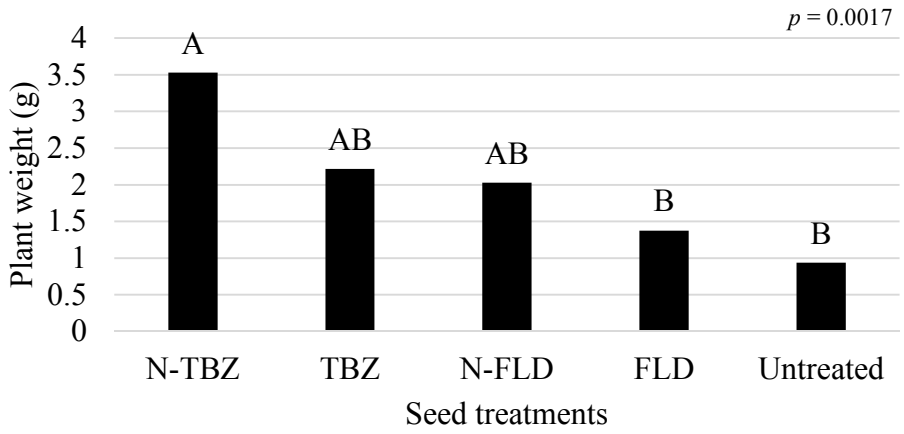

Figure 20. Average of plant weight (g) for the first run of the delayed emergence assay for the second environmental condition (1 week of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

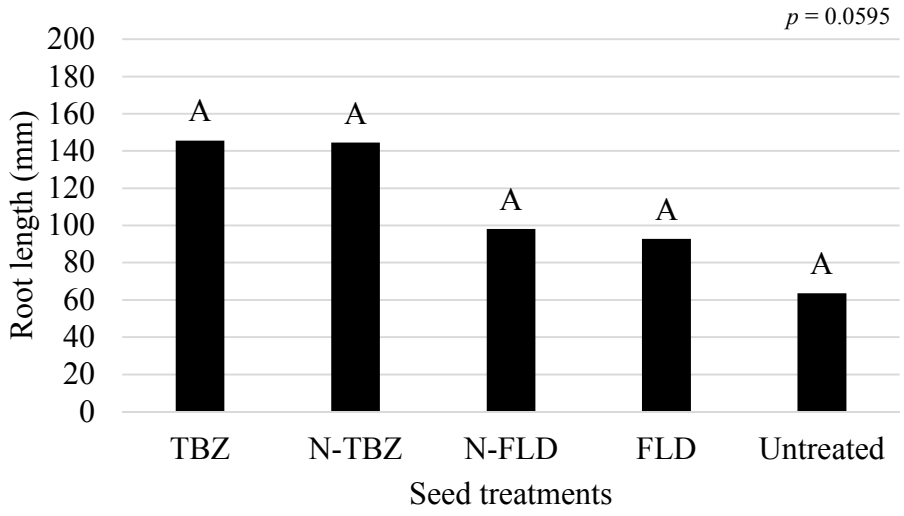

Figure 21. Average of root length (mm) for the first run of the delayed emergence assay for the second environmental condition (1 week of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

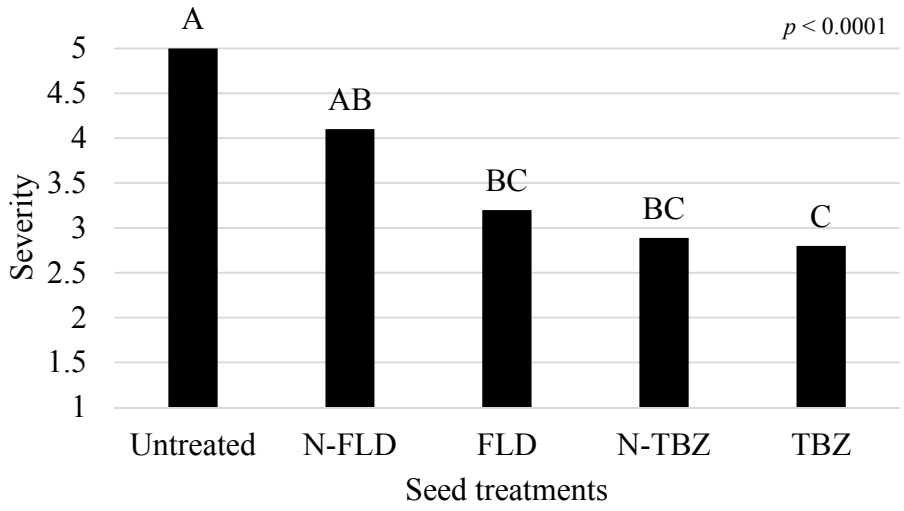

Figure 22. Average of severity for the first run of the delayed emergence assay for the third environmental condition (2 weeks of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

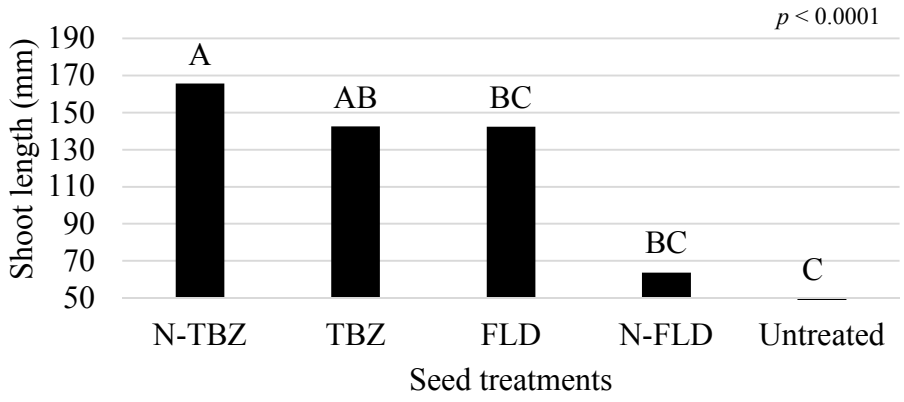

Figure 23. Average of shoot length (mm) for the first run of the delayed emergence assay for the third environmental condition (2 weeks of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

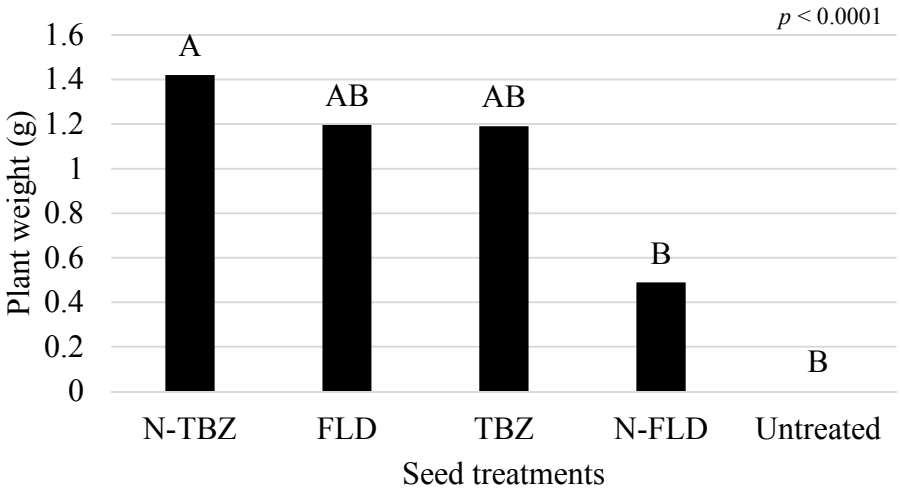

Figure 24. Average of plant weight (g) for the first run of the delayed emergence assay for the third environmental condition (2 weeks of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

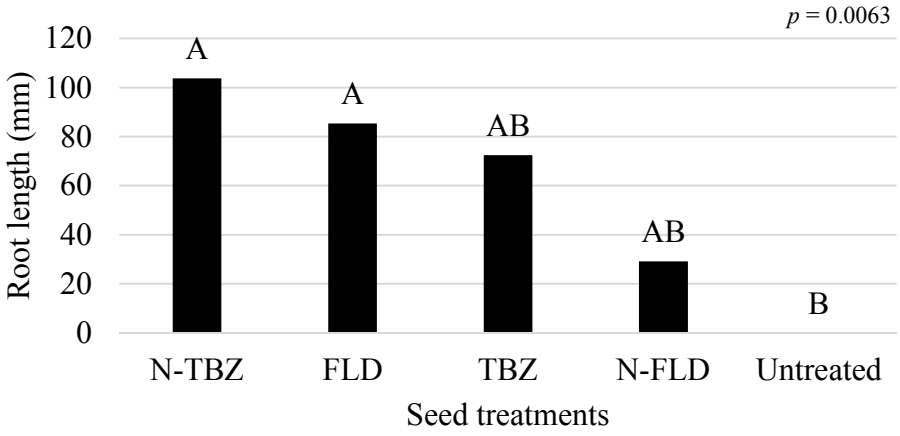

Figure 25. Average of root length (mm) for the first run of the delayed emergence assay for the third environmental condition (2 weeks of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

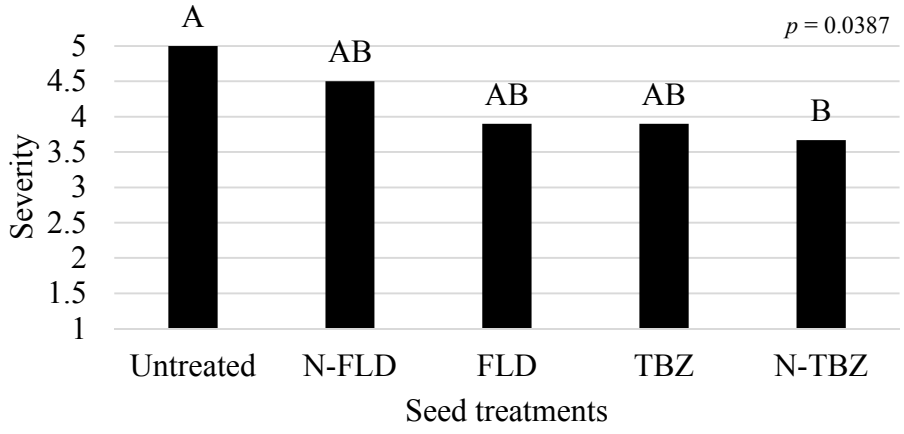

Figure 26. Average of severity for the first run of the delayed emergence assay for the fourth environmental condition (3 weeks of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

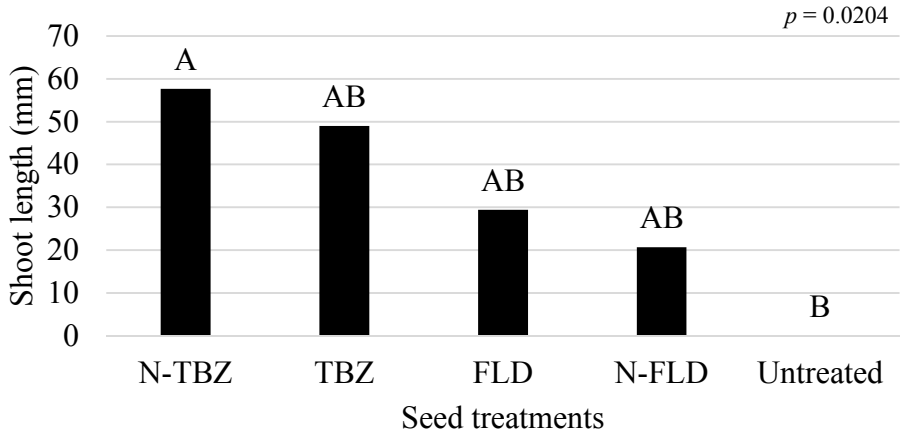

Figure 27. Average of shoot length (mm) for the first run of the delayed emergence assay for the fourth environmental condition (3 weeks of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

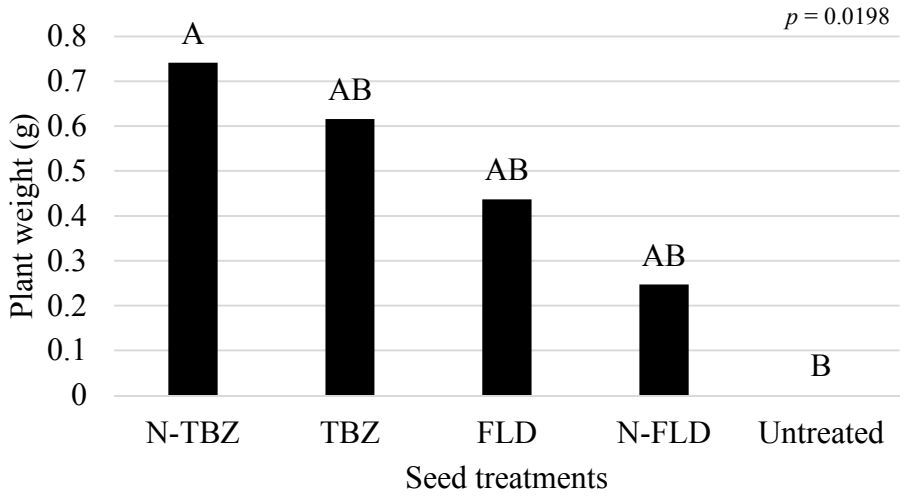

Figure 28. Average of plant weight (g) for the first run of the delayed emergence assay for the fourth environmental condition (3 weeks of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

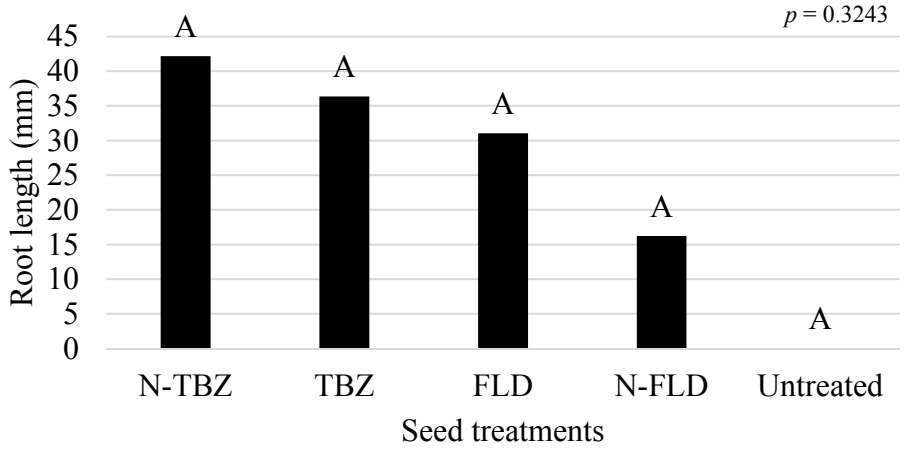

Figure 29. Average of root length (mm) for the first run of the delayed emergence assay for the fourth environmental condition (3 weeks of delayed emergence). The statistical analyses were conducted in each environment individually. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

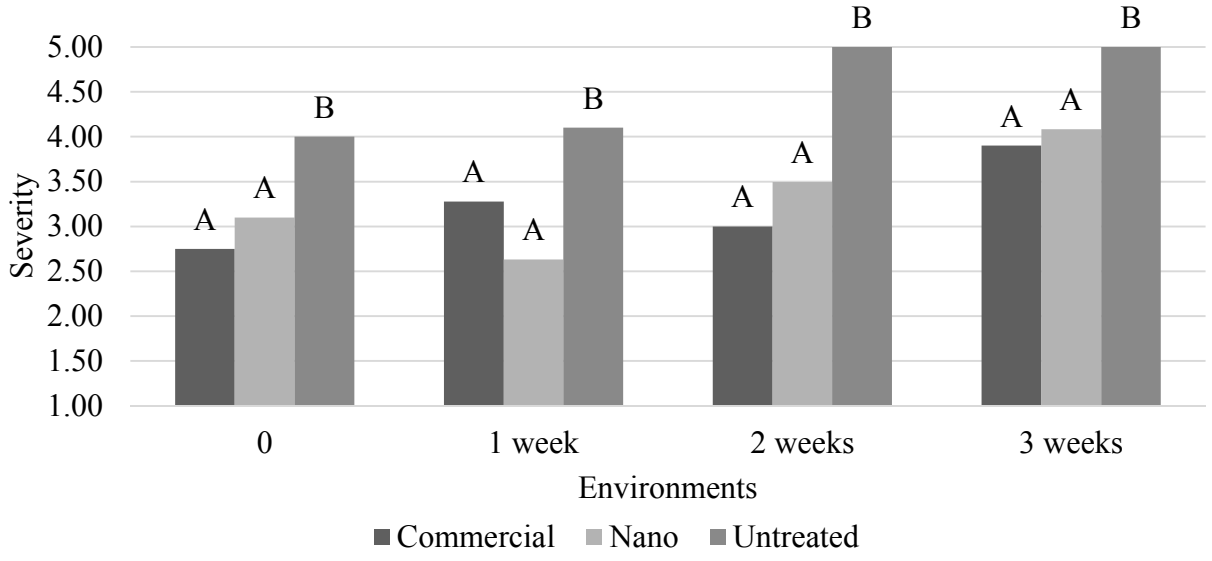

Figure 30. Average of severity for the first run of the delayed emergence assay. The statistical analyses were conducted using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

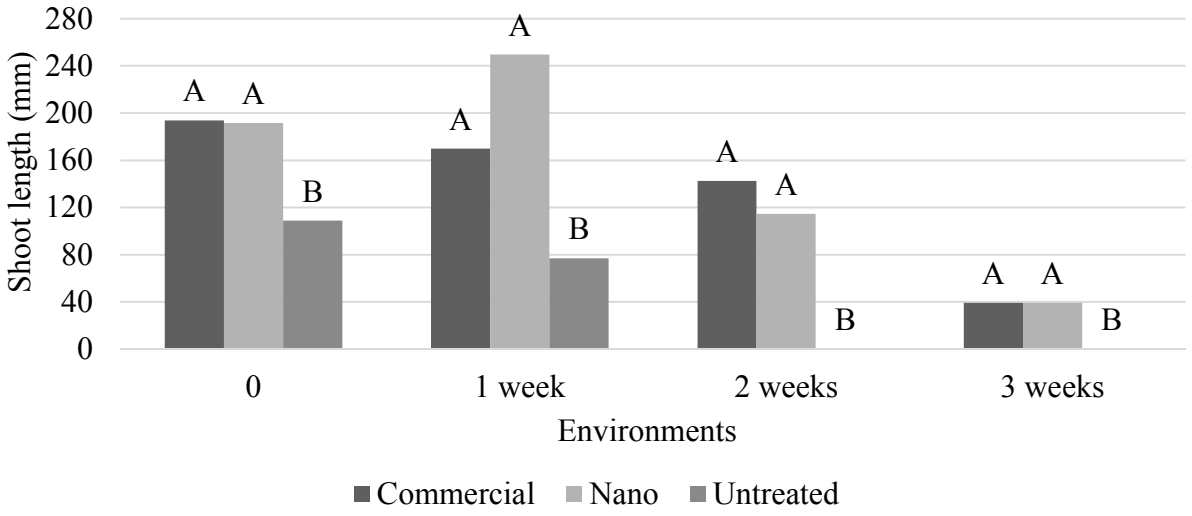

Figure 31. Average of shoot length (mm) for the first run of the delayed emergence assay. The statistical analyses were conducted using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

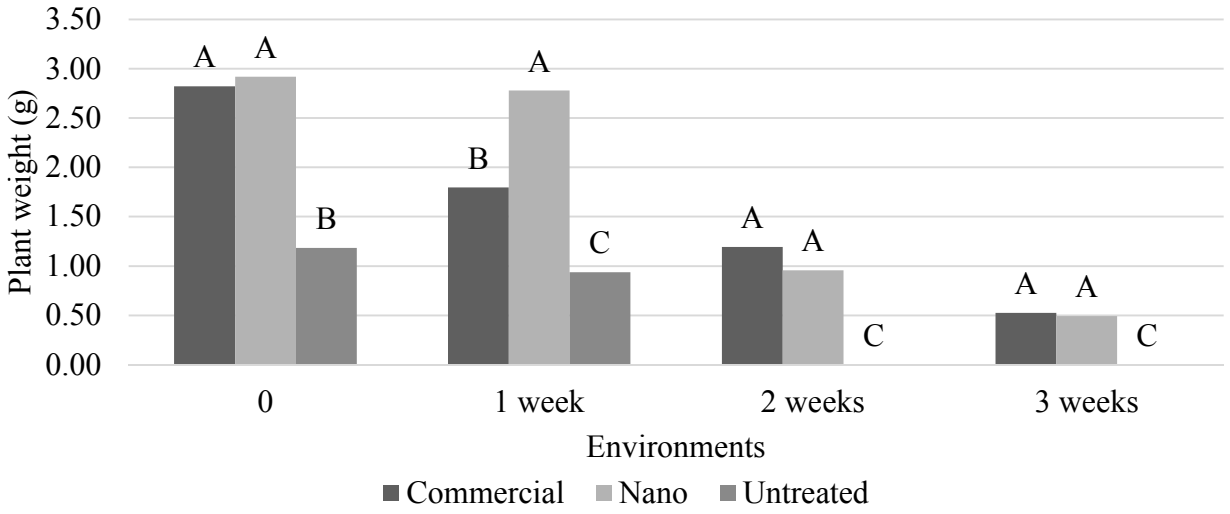

Figure 32. Average of plant weight (g) for the first run of the delayed emergence assay. The statistical analyses were conducted using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

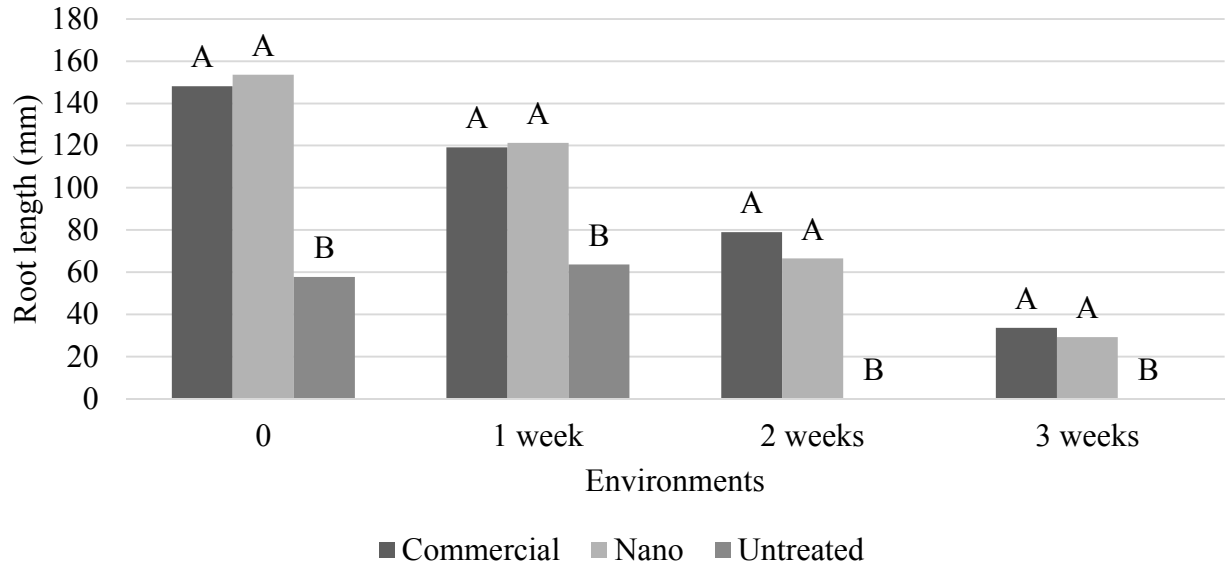

Figure 33. Average of root length (mm) for the first run of the delayed emergence assay. The statistical analyses were conducted using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

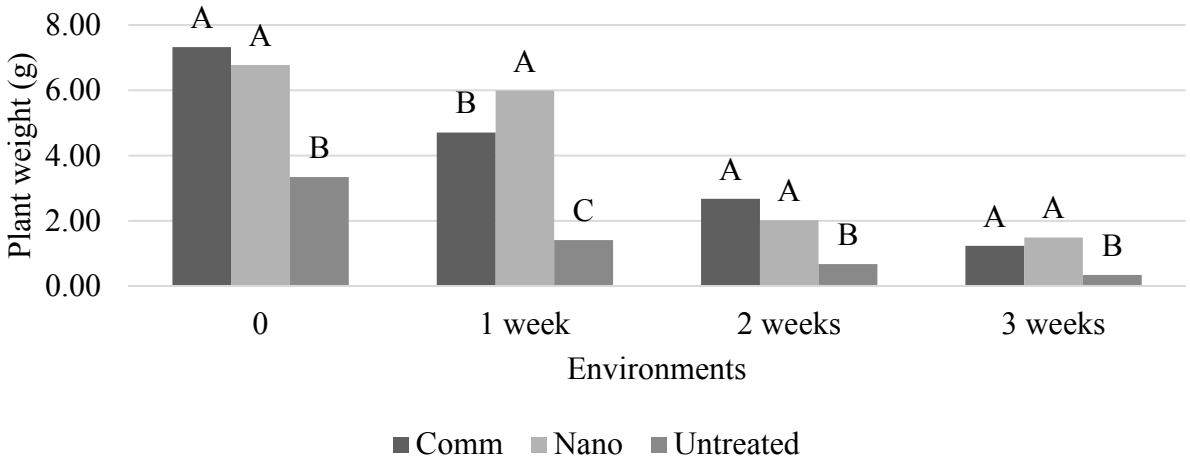

Figure 34. Average of plant weight (g) for the second experiment of the delayed emergence assay. The statistical analyses were conducted using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

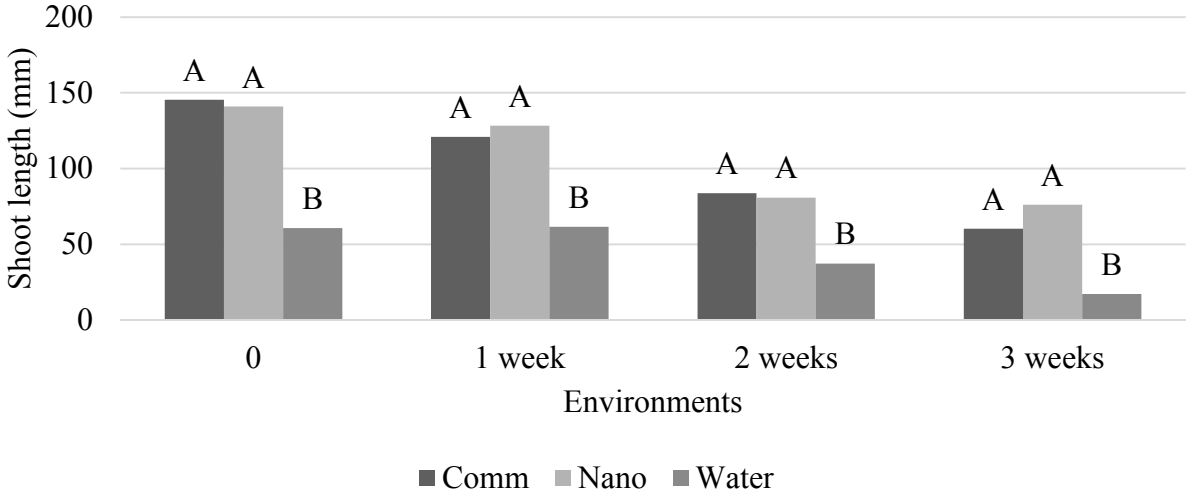

Figure 35. Average of shoot length (mm) for the second experiment of the delayed emergence assay. The statistical analyses were conducted using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

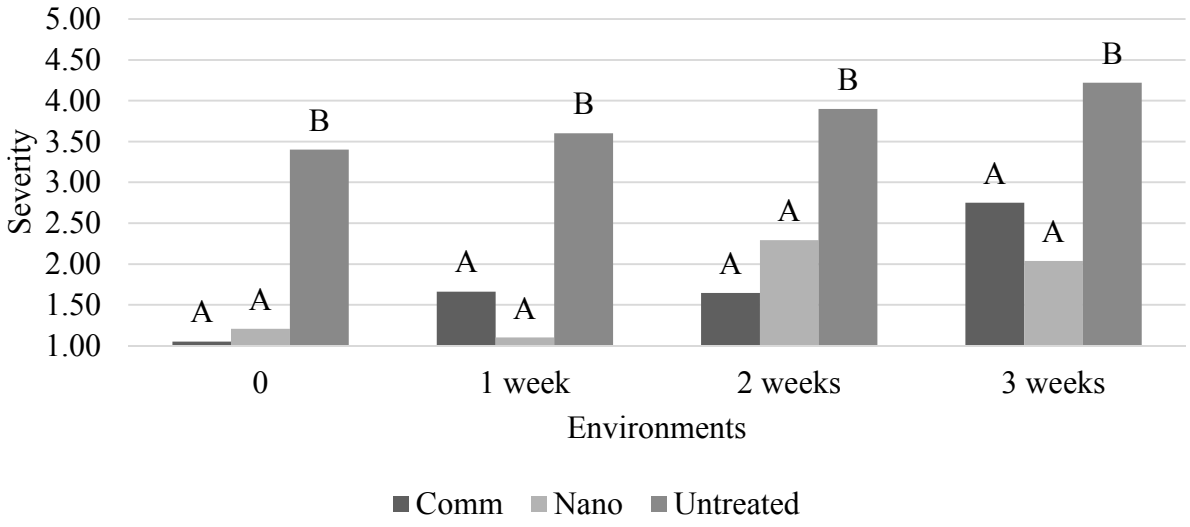

Figure 36. Average of severity for the second experiment of the delayed emergence assay. The statistical analyses were conducted using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

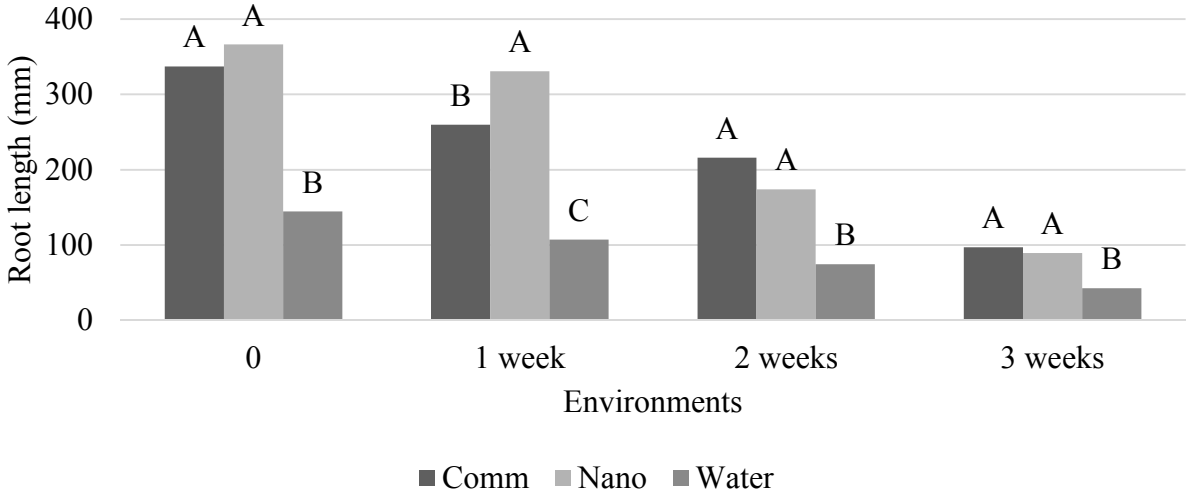

Figure 37. Average of root length (mm) for the second experiment of the delayed emergence assay. The statistical analyses were conducted using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

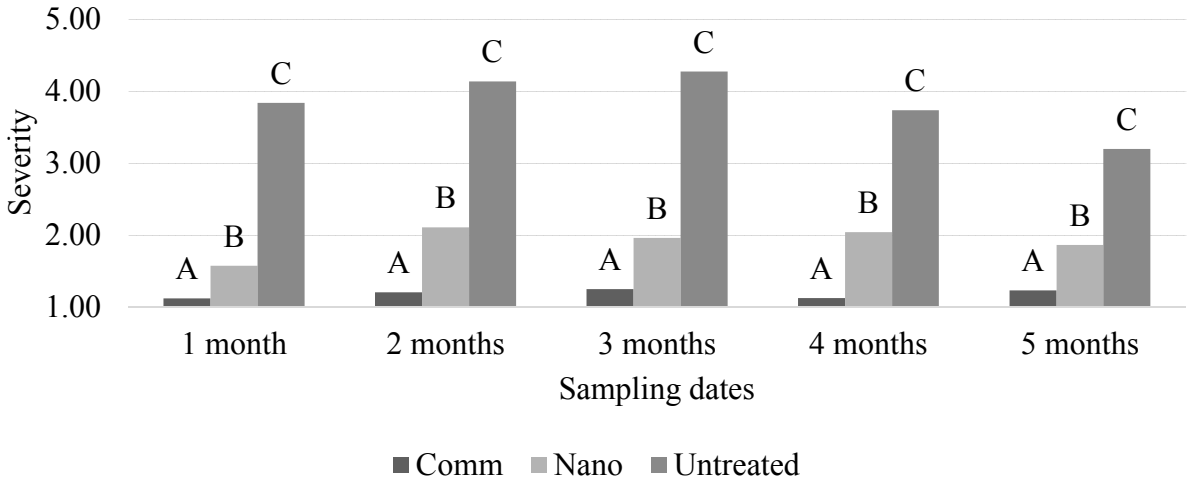

Figure 38. Average of severity for the storage assay. The statistical analyses were conducted within sampling dates using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

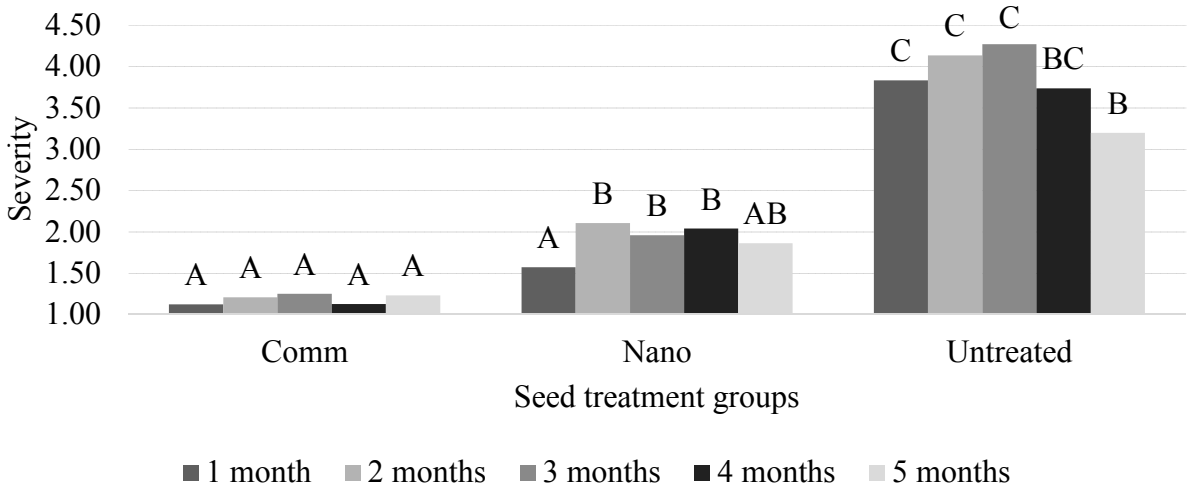

Figure 39. Average of severity for the storage assay. The statistical analyses were conducted within treatments using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

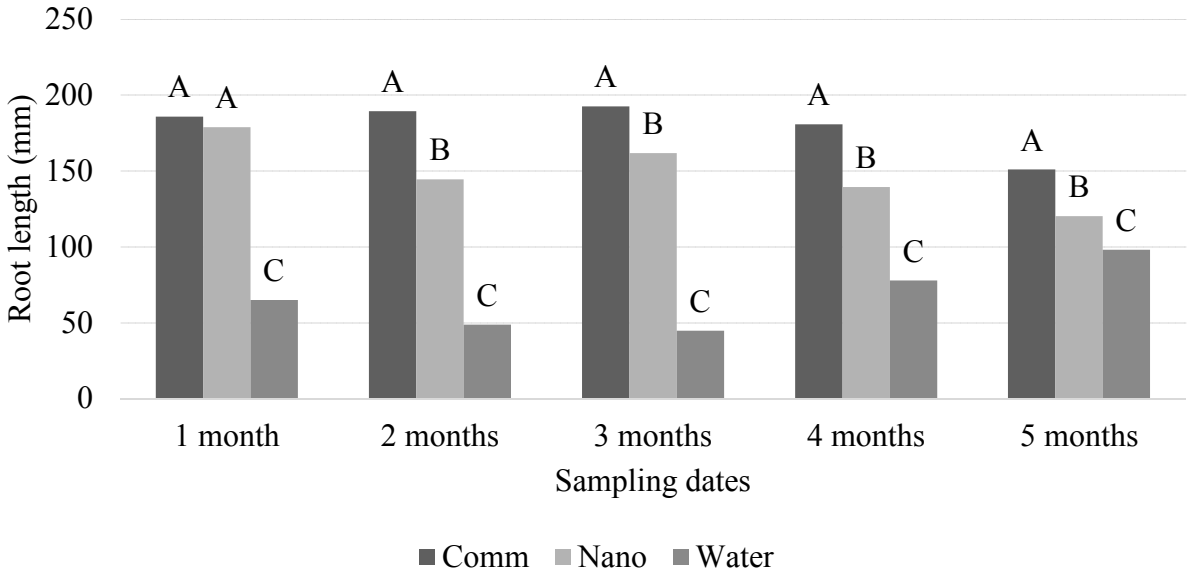

Figure 40. Average of root length (mm) for the storage assay. The statistical analyses were conducted within sampling dates using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

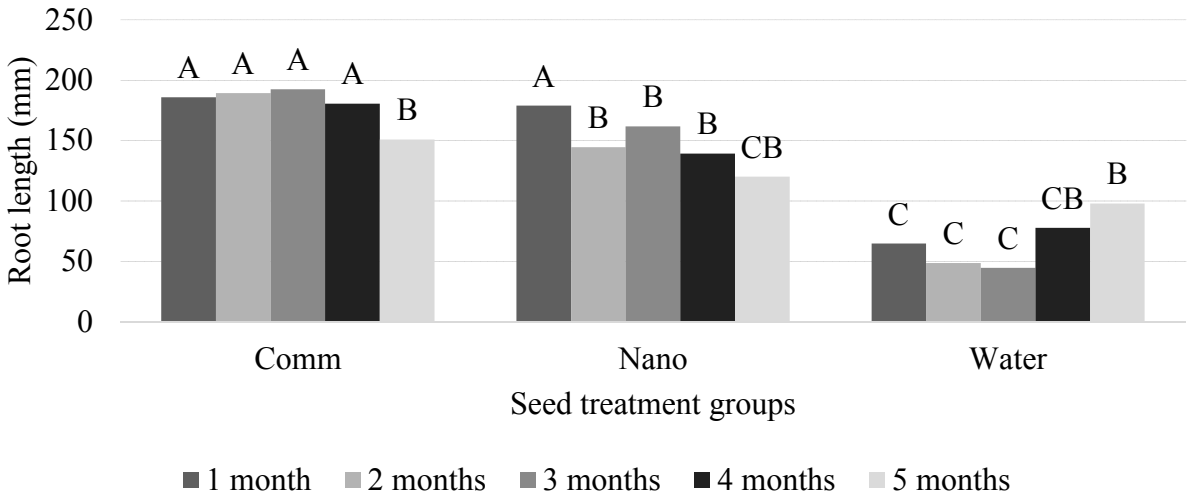

Figure 41. Average of root length (mm) for the storage assay. The statistical analyses were conducted within treatments using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

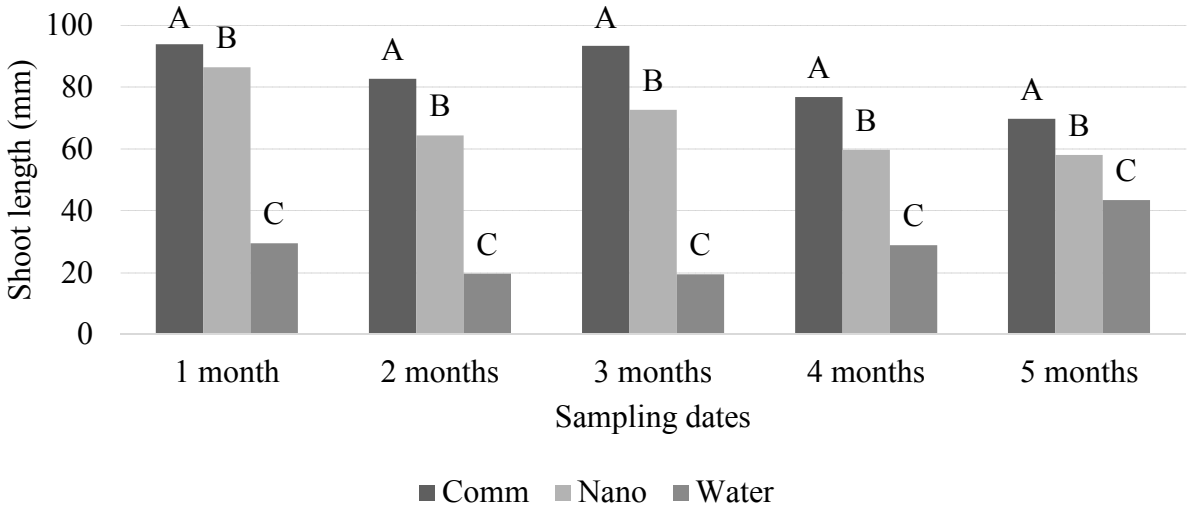

Figure 42. Average of shoot length (mm) for the storage assay. The statistical analyses were conducted within sampling dates using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

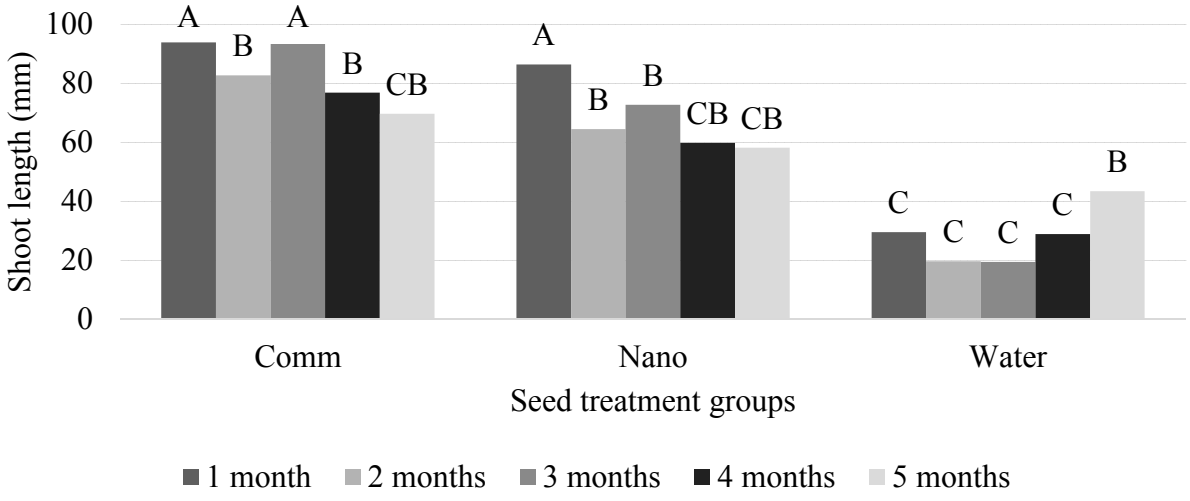

Figure 43. Average of shoot length (mm) for the storage assay. The statistical analyses were conducted within treatments using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

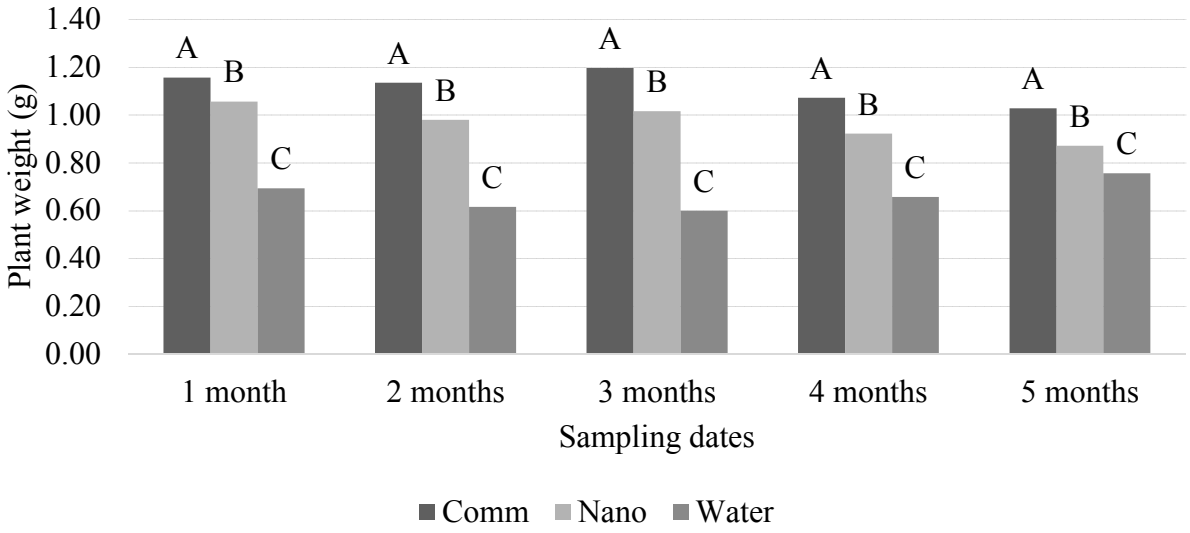

Figure 44. Average of plant weight (g) for the storage assay. The statistical analyses were conducted within sampling dates using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

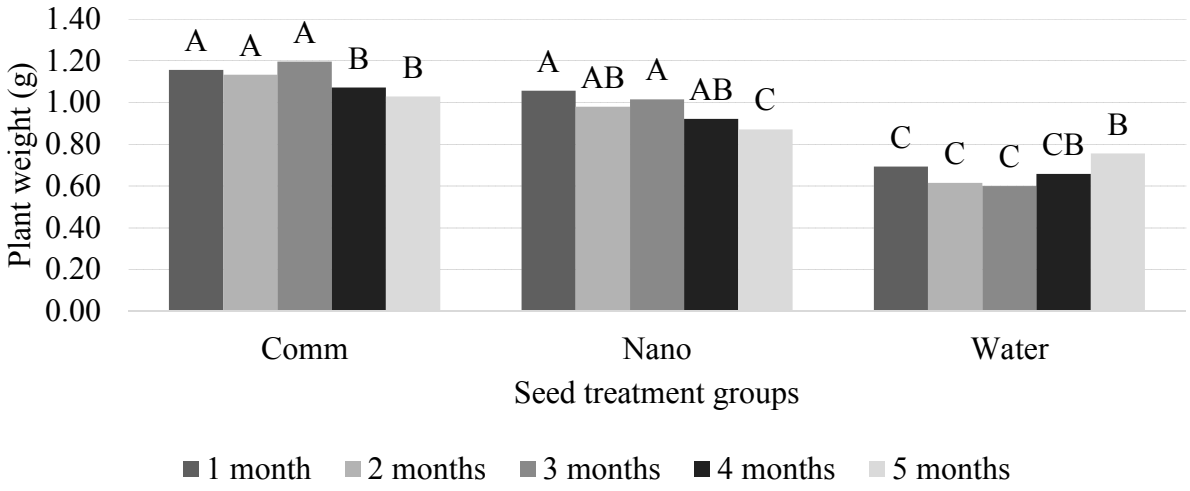

Figure 45. Average of plant weight (g) for the storage assay. The statistical analyses were conducted within treatments using contrasts comparing the two main groups, nanoparticle-encapsulated formulations versus conventional formulations, and an untreated control. Different treatments' letters indicate significant difference using contrasts and standard Tukey's adjustment.

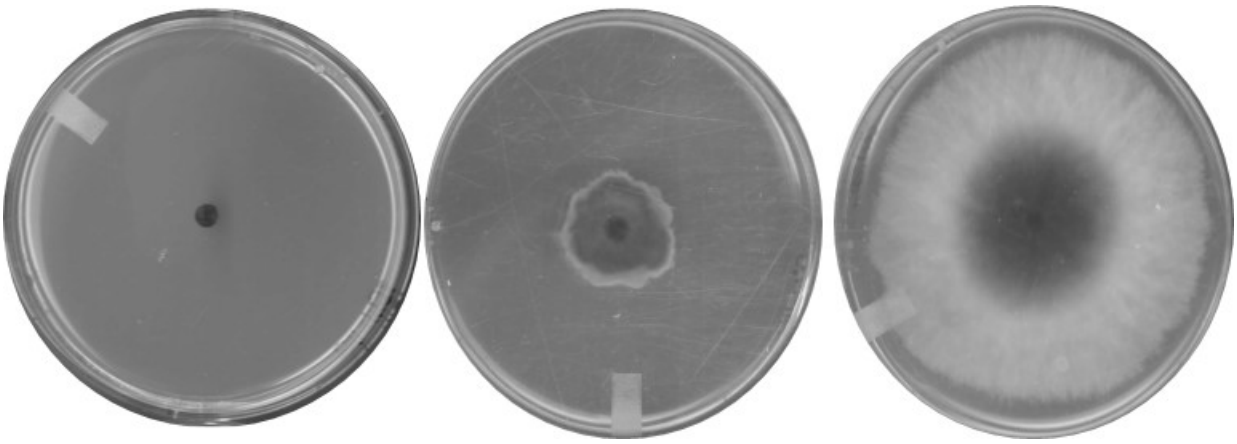

Figure 46. Growth of *F. graminearum* on different media. From left to right: FG27 disc from PDA at planting date; FG27 growth on pure PDA at day five; FG27 growth on media containing 100ppm of CNI-1493 at day five.

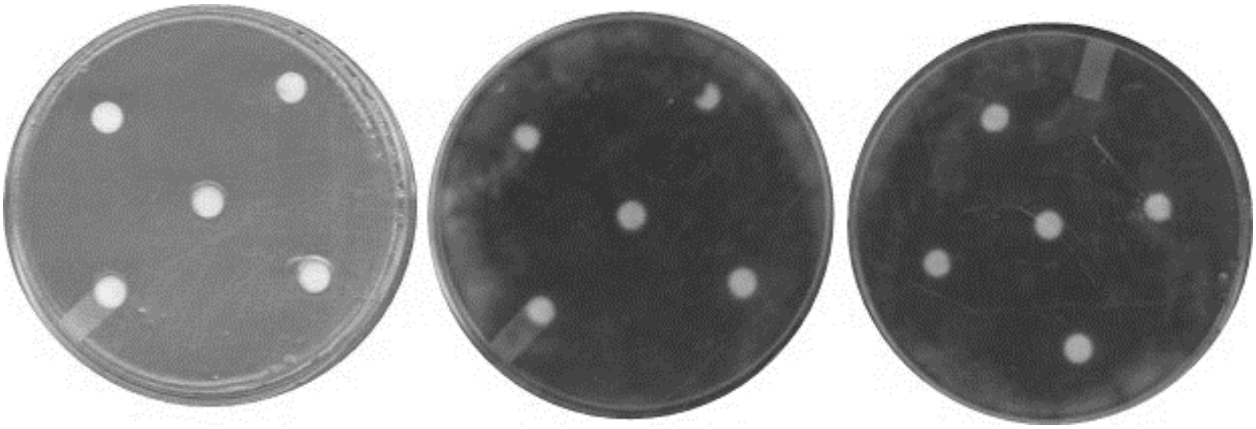

Figure 47. Growth of *F. graminearum* on PDA plates containing five soaked paper discs with different treatments. From left to right: soaked discs at first day; FG27 growth around discs soaked with water; FG27 growth around discs soaked with 100ppm of CNI-1493 at day five.

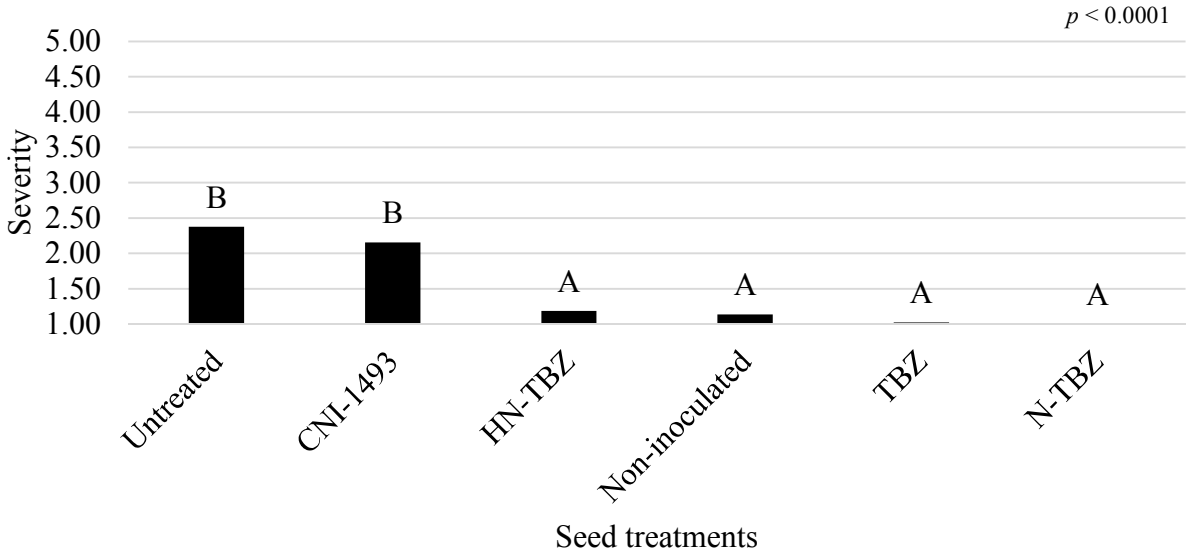

Figure 48. Average of severity for the rolled-towel assay assessing the efficacy of CNI-1493 against FG27. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

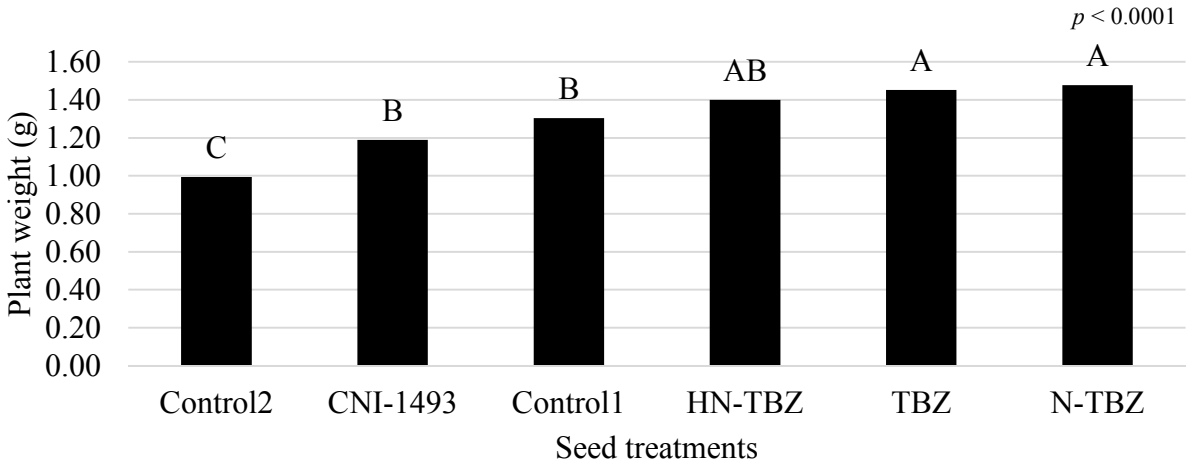

Figure 49. Average of plant weight (g) for the rolled-towel assay assessing the efficacy of CNI-1493 against FG27. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

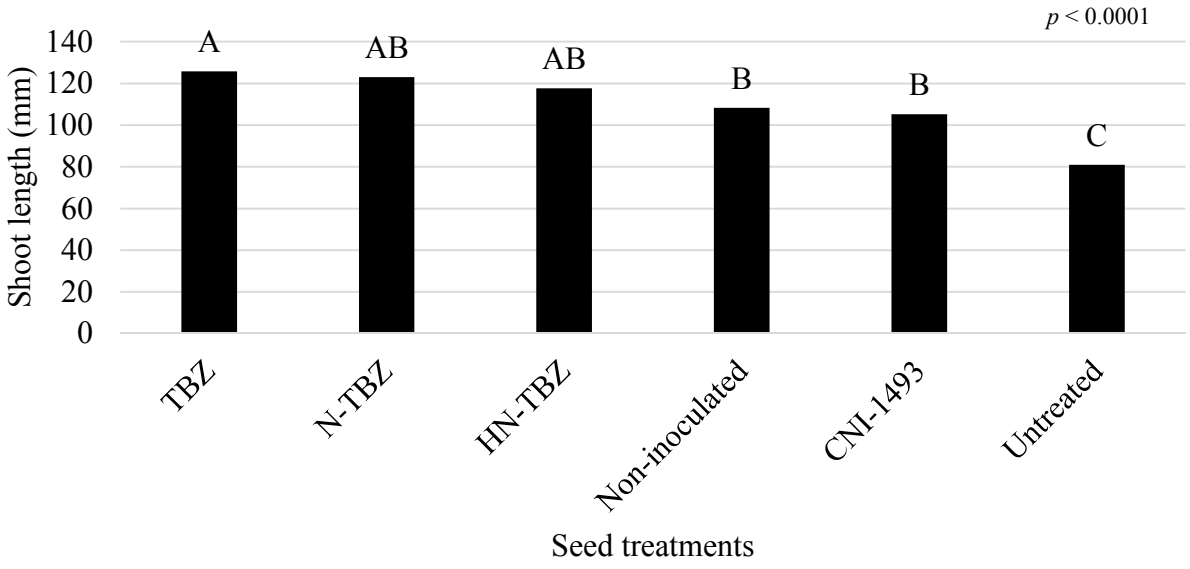

Figure 50. Average of shoot length (mm) for the rolled-towel assay assessing the efficacy of CNI-1493 against FG27. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

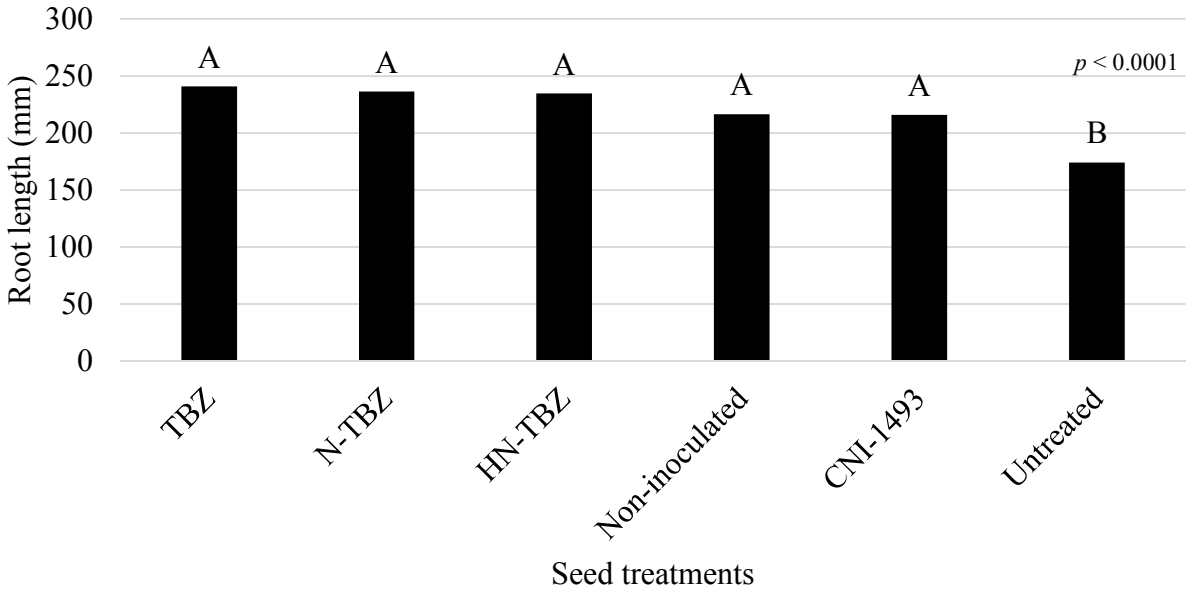

Figure 51. Average of root length (mm) for the rolled-towel assay assessing the efficacy of CNI-1493 against FG27. Different treatments' letters indicate significant differences by Tukey's Honest Significant Difference test.

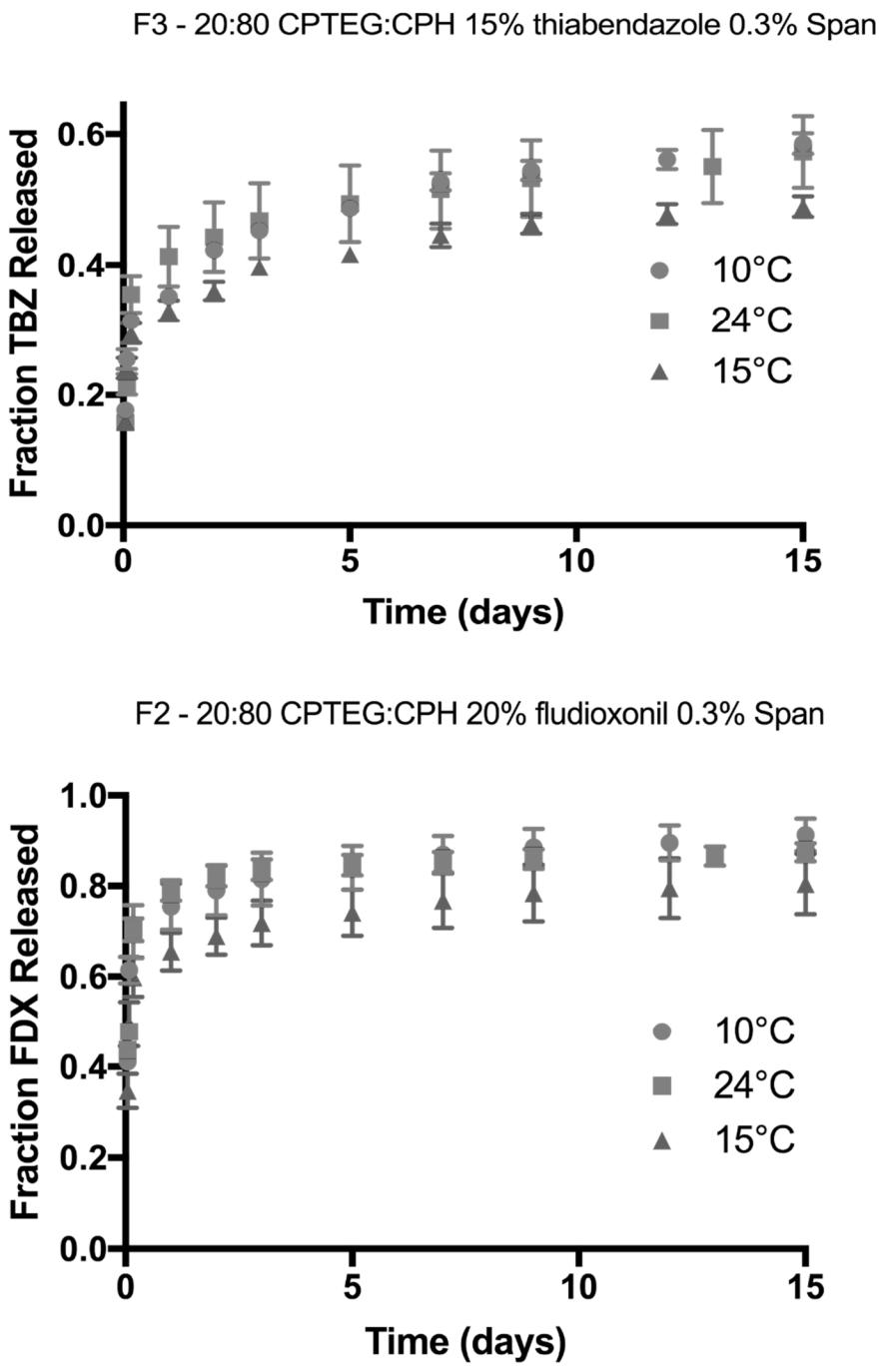

Figure 52. Release kinetics of thiabendazole (F3) and fludioxonil (F2) loaded CPTEG:CPH nanoparticles over 16 days at three different temperatures. Data were collected by Dr. Balaji Narasimhan (professor) and Adam S. Mullis (graduate research assistant), Department of Chemical and Biological Engineering, Iowa State University of Science and Technology, 2019.

CHAPTER 4: DISCUSSION

The results of this study showed that nanoparticle encapsulation of fungicides can provide some advantages for control of seedborne and soilborne inoculum of *F. graminearum*. In both delayed emergence experiments, nanoparticle-encapsulated formulations resulted in better disease management than conventional formulations when emergence was delayed for 1-week by low temperature. However, for the other environments, nanoparticle-encapsulated formulations and conventional formulations were statistically similar. These results showed a potential advantage to nanoparticle encapsulation when emergence was delayed for one week. This behavior could be related to the specific composition of the polymer used and its ratio of monomers. These results suggest that the nanopolymers used in this study are prolonging the release of the fungicides and hence the control of this pathogen only after one week of delayed emergence. Results may be improved using a mixture of different polymer compositions and ratios together in the same slurry to target different windows of active ingredient release. Moreover, these experiments showed a proof of the importance of seed-applied fungicides against seedling diseases. In both repetitions of the delayed emergence assay, (first run using maize and the second one with soybeans), the inoculated control had zero emergence when emergence was delayed for 2 and 3 weeks, while fungicide-based treatments were statistically superior.

For the rolled-towel results, nanoparticle-encapsulated formulations were superior only in the first experiment, which is related to the very low control provided by FLD, while TBZ and N-TBZ provided similar results. In the second experiment, when FLD was similar to N-FLD, the orthogonal contrasts showed similar results for all fungicide treatments. Additionally, the severity of the inoculated and water-treated control of the second experiment was lower than what was seen in the first experiment. Because in the second experiment the pathogen aggressiveness was lower,

the lack of significant differences could be related to smaller capability of detecting statistical differences. If the primary advantage of nanoparticle encapsulation is to prolong efficacy of the seed treatment through time-release, this benefit would not be evident in the rolled-towel assays, which only assess control in the first week of germination and seedling growth.

The rolled-towel assays did demonstrate that nanoparticle-encapsulated formulations can work as well or better than conventional formulations, even with reduced rates. The half-rate-nanoparticle-encapsulated formulations provided similar results when compared to the full rate of nanoparticle-encapsulated formulations and full rate of conventional formulations. Similarly, Washington (2017) observed equivalent levels of control using half rates. However, we did not test half rates of conventional formulations, which would strengthen our interpretations about the half rate results. Nevertheless, the results obtained by this study and Washington (2017) do not compare to the level of effective reduced rate results achieved by Binnebose *et al.*, (2015). Considering the differences in chemistry compatibility of fungicides and the antimicrobial used in the study by Binnebose *et al*., and the carbon polymers used by our collaborators, it may be possible to improve the reduction of the fungicides with more research on the chemistry area or with different fungicide active ingredients.

The results from the storage assay showed a relatively stable efficacy of nanoparticle-encapsulated formulations. For most of the variables measured, some of the efficacy was lost only after four or five months in storage. When comparing to the conventional formulations, which were tested exhaustively prior to launching, nanoparticles showed a similar behavior, which can be considered as adequate for the purpose of a seed treatment. Although maize seed is treated normally within one year before planting, this window of safe storage could be improved by more study on the chemistry of the polymers used in this research.

The results collected on the assessment of CNI-1493 as a potential ingredient to control *F. graminearum* did not support our hypothesis. Studied by Woriedh et al. (2011), this compound had shown a strong inhibition of the pathogen. Conversely, we observed enhanced growth by the fungus when in contact with CNI-1493. When applied in the media, the compound could be challenging the fungus in a low level, not enough to kill but to stimulate its metabolism. On the other hand, when used as a seed treatment, the compound promoted an intermediate level of control, but far from the levels obtained by the three versions of thiabendazole. CNI-1493 does not appear to be a good candidate as a seed treatment against *F. graminearum*.

The release study conducted by our collaborators showed no difference in time release profile for both chemicals under different temperatures. These *in vitro* results indicated that nanoparticle encapsulation did not greatly prolong active ingredient release. Additional experiments with different polyanhydride polymer formulations or combinations of formulation should be tested. Robust methods for measuring time release of active ingredients in soil need to be developed. Results of our experiments showing different outcomes in different delayed-emergence environments might be related with the interaction between the pathogen and the physiological development of the plants. Lower temperatures can give a competitive advantage to the fungus rather than the plant, and this interaction should be targeted by the employment of a seed treatment that can protect late-emerging seedlings. The results showing equivalent release profiles across temperatures can be advantageous to promote enhanced protection and prevent seed decay under various soil temperatures, including delayed emergence scenarios. This mechanism could be especially beneficial with a prolonged-release system, to avoid the rapid dissipation of the active ingredient before germination.

The use of seed treatments is currently one of the most important management tactics in many crops throughout the world. They might be applied due to many benefits and purposes; however, the most critical aspects are to avoid any kind of initial decay and introduction of pathogens and promote a competitive advantage to the plant in very diverse environments. Although, many cocktails of combined active ingredients are being used targeting different pathogens and abiotic stresses, there is an inconvenient limitation, the rapid dissipation of applied compounds.

Prolonging the efficacy of seed treatments is an important challenge that could greatly increase the value of this crop protection tactic. We hypothesized that the employment of nanoparticles could address the limitation of rapid release and promote a prolonged release, hence, an enhanced protection against pests. Our results partially support this hypothesis; even though our results of the several rolled-towel experiments suggest similar efficacy between nanoparticle-encapsulated formulations and conventional formulations, the results from the delayed emergence assays are promising. Nanoparticles presented improved control for a specific environment (1-week delay) when tested with two different crop species. Therefore, this trend could be pursued with potential to enhance the prolonged effect to levels that could increase seedling protection for several weeks after planting.

A better understanding of the chemical compatibility between the polymers and the active ingredient, as well as the ratio between polyanhydride monomers can bring more flexibility to the pattern of release of a specific chemical. Possibly, with more in-depth chemistry research, we could improve the release of these studied fungicides or other pesticides and enhance management of pests to a greater extent than what was observed in this research.

An important next step of research should address the improvement of methods to detect and measure the amount of chemical being released in soil under different environmental settings. It is very important to understand the release patterns for different ratios between monomers, and their behavior when applied in soil conditions. Additionally, other different types of chemicals should be used to understand which chemistry is most compatible when using polyanhydride polymers.

REFERENCES

1. Agrios, GN (2005) Plant Pathology. 5th Edition, Elsevier Academic Press, San Diego, 11: 534-538.
2. Arias, M. M. D., Leandro, L. F., & Munkvold, G. P. (2013). Aggressiveness of Fusarium species and impact of root infection on growth and yield of soybeans. Phytopathology, 103(8), 822–832. https://doi.org/10.1094/PHYTO-08-12-0207-R.
3. Berkland, C., Kipper, M. J., Narasimhan, B., Kim, K., & Pack, D. W. (2004). Microsphere size, precipitation kinetics and drug distribution control drug release from biodegradable polyanhydride microspheres. Journal of Controlled Release, 94(1), 129–141. https://doi.org/10.1016/j.jconrel.2003.09.011g.
4. Bhatia, S. (2016). Natural polymer drug delivery systems: Nanoparticles, plants, and algae. Natural Polymer Drug Delivery Systems: Nanoparticles, Plants, and Algae (pp. 1–225). Springer International Publishing. https://doi.org/10.1007/978-3-319-41129-3.
5. Binnebose, A. M., Haughney, S. L., Martin, R., Imerman, P. M., Narasimhan, B., & Bellaire, B. H. (2015). Polyanhydride nanoparticle delivery platform dramatically enhances killing of filarial worms. PLoS Neglected Tropical Diseases, 9(10). https://doi.org/10.1371/journal.pntd.0004173.
6. Broders KD, Lipps PE, Paul PA, Dorrance AE (2007) Evaluation of Fusarium graminearum associated with corn and soybean seed and seedling disease in Ohio. Plant Dis. 91:1155-1160.
7. da Silva MP, Tylka GL, Munkvold GP (2016) Seed treatment effects on maize seedlings coinfected with *Fusarium* spp. and *Pratylenchus penetrans*. Plant Dis. 100:431-437.

8. Ellis ML, Broders KD, Paul PA, Dorrance AE (2011) Infection of soybean seed by Fusarium graminearum and effect of seed treatments on disease under controlled conditions. Plant Disease 95:401-407.
9. Khan, I., Saeed, K., & Khan, I. (2017, March 18). Nanoparticles: Properties, applications and toxicities. Arabian Journal of Chemistry. Elsevier B.V. https://doi.org/10.1016/j.arabjc.2017.05.011.
10. Kebede AZ, Woldemariam T, Reid LM, Harris LJ (2015) Quantitative trait loci mapping for Gibberella ear rot resistance and associated agronomic traits using genotyping‐by‐sequencing in maize. Theor Appl Genet (2016) 129:17–29.
11. Munkvold, GP (2017) Fusarium species and their associated mycotoxins Ch. 4 (pp. 51-106). In: Moretti A, Susca A (eds.), Mycotoxigenic Fungi: Methods and Protocols, Methods in Molecular Biology, vol. 1542, DOI 10.1007/978-1-4939-6707-0_4, © Springer Science+Business Media LLC 2017.
12. Munkvold, GP (2009) Seed pathology progress in academia and industry. Annu. Rev. Phytopathol. 2009. 47:285–311.
13. Munkvold GP and O'Mara JK (2002) Laboratory and growth chamber evaluation of fungicidal seed treatments for maize seedling blight caused by Fusarium species. Plant Dis. 86:143- 150.
14. Nganje WE, Bangsund DA, Leistritz FL, Wilson WW, Tiapo NM (2002) Estimating the economic impact of a crop disease: the case of Fusarium head blight in U.S. wheat and barley. National Fusarium Head Blight Forum Proceedings. East Lansing: Michigan State University, pp. 275–281.
15. Parikh L, Kodati S, Eskelson MJ, Adesemoye AO (2018) Identification and pathogenicity of Fusarium spp. in row crops in Nebraska. Crop Protection 108, 120–127.

16. Paul PA, Lipps PE, Hershman, DE, McMullen, MP, Draper MA, Madden LV (2008) Efficacy of triazole-based fungicides for Fusarium head blight and deoxynivalenol control in wheat: A multivariate meta-analysis. Phytopathology 98:999-1011.

17. Phanse, Y., Lueth, P., Ramer-Tait, A. E., Carrillo-Conde, B. R., Wannemuehler, M. J., Narasimhan, B., & Bellaire, B. H. (2016). Cellular internalization mechanisms of polyanhydride particles: Implications for rational design of drug delivery vehicles. Journal of Biomedical Nanotechnology, 12(7), 1544–1552. https://doi.org/10.1166/jbn.2016.2259.

18. Robertson, A., and Munkvold, GP (2009) Check general root and mesocotyl health when assessing corn stands. Iowa State University Integrated Crop Management Newsletter. Online publication. http://www.extension.iastate.edu/CropNews/2009/0519robertson.htm.

19. Reynolds, GA, and Hauser, CR (1950) Orp.Synth., s, 70-72.

20. Rubella, SG, and Kistler, HC (2004) Heading for disaster: *Fusarium graminearum* on cereal. Molecular Plant Pathology 5 (6), 515-525.

21. Schmale, III, DG, Bergstrom, GC (2003) Fusarium head blight in wheat. The Plant Health Instructor. DOI:10.1094/PHI-I-2003-0612-01 Updated 2010.

22. Schröder, M., Kolodzik, A., Windshügel, B., Krepstakies, M., Priyadarshini, P., Hartjen, P., … Meier, C. (2016). Linker-region modified derivatives of the deoxyhypusine synthase inhibitor CNI-1493 suppress HIV-1 replication. Archiv Der Pharmazie, 349(2), 91–103. https://doi.org/10.1002/ardp.201500323.

23. Trail, F (2009) For blighted waves of grain: Fusarium graminearum in the postgenomics era. Plant Physiology, January 2009, Vol. 149, pp. 103–110.

24. Washington, LA (2017) "Utilization of polyanhydride nanoparticle encapsulated fungicide seed treatments against seedborne and soilborne Fusarium graminearum on maize". Graduate Theses and Dissertations. 15639. https://lib.dr.iastate.edu/etd/15639.

25. Woriedh, M., Hauber, I., Martinez-Rocha, A. L., Voigt, C., Maier, F. J., Schröder, M., … Schäfer, W. (2011). Preventing fusarium head blight of wheat and cob rot of maize by inhibition of fungal deoxyhypusine synthase. Molecular Plant-Microbe Interactions, 24(5), 619–627. https://doi.org/10.1094/MPMI-03-10-0068+

APPENDIX. Guanylhydrazone SYNTHESIS

5-nitroisophthaloyl dichloride (**3**): In a modification of literature procedure (Schröder *et al*., 2016), 5-nitroisophthalic acid (**2**) (21.11 g, 100 mmol) was added to thionyl chloride (29.0 mL, 400 mmol) in a 250 mL round-bottom flask with stirbar. Five drops of dimethylformamide were added, and the suspension was refluxed for five hours to produce a pale-yellow solution. The excess thionyl chloride was distilled off at atmospheric pressure, and the crude pale-yellow solid was triturated with heptane, which was removed under vacuum to give a yellow solid that was sufficiently pure for the next step.

1,3-diacetyl-5-nitrobenzene (**4**): Similar to a known procedure (Reynolds and Hauser, 1950), In a 500 mL two-neck round-bottom flask equipped with reflux condenser and addition funnel was added magnesium turnings (5.4 g, 0.22 mol). Ethanol (5 mL, 0.85 mmol) and 1,2-dibromoethane (0.3 mL) were added, at which point an exothermic reaction commenced, along with the formation of numerous bubbles emanating from the magnesium. After 15 minutes, tetrahydrofuran (150 mL) was added. Diethyl malonate (35.2 g, 220 mmol) was dissolved in tetrahydrofuran (25 mL) and ethanol (20 mL, 0.34 mol), and this solution was added at such a rate that reflux was maintained; the reaction became gray during this addition. After the completion of addition, reflux was maintained for two hours, by which point the magnesium is nearly completely consumed. While the reaction was still refluxing, a solution of **3** (24.8 g, 100 mmol) in tetrahydrofuran (50 mL) was added over 20 minutes, and the now yellow solution was refluxed for four hours and then cooled to room temperature. 25% sulfuric acid (100 mL) was added, and the bulk of the tetrahydrofuran was removed under vacuum. The crude product was extracted with diethyl ether (3x75 mL). The combined organic layers were washed with water (75 mL) and brine.

The ether was removed under vacuum, and to the thick liquid was added glacial acetic acid (60 mL), water (40 mL), and concentrated sulfuric acid (7.6 mL). The biphasic mixture was vigorously stirred at reflux for four hours, after which the bulk of the acetic acid was removed under vacuum. Crushed ice (200 g) was added, causing crude product to solidify. The solid is filtered off, and the filtrate is extracted with ethyl acetate (2x75 mL). The crude solid is then dissolved in the combined organic layers, which is then washed with water, then 0.5 M sodium hydroxide, and then brine. The organic layer is then dried over anhydrous magnesium sulfate, and the solvent removed under vacuum. The crude solid was recrystallized from toluene/heptane to give pale yellow platelets (10.21 g, 49% over two steps). ^{1}H and ^{13}C NMR are in agreement with literature values.[1]

***N,N'*-bis(3,5-diacetylphenyl)decanediamide** (**5**): A solution of **4** (1.5 g, 7.24 mmol, 2.1 equiv.) dissolved in ethyl acetate (100 mL) was added to a 250-mL round-bottom flask equipped for hydrogenation. The solution was deoxygenated by sparging with argon for 15 minutes, after which 10% palladium on carbon (91.7 mg catalyst, 96 μmol palladium, 0.025 equivalent) was added. Hydrogen was bubbled through the solution with vigorous stirring for four hours, during which time the solution changes from colorless to bright yellow; the reduction is somewhat exothermic and was kept around room temperature using a water bath. After completion of the reaction, as monitored by TLC (1:1 ethyl acetate:hexane), the reaction was flushed with argon, and pyridine (0.69 mL, 8.62 mmol, 2.5 equivalent) was added in one portion by syringe. Decanedioyl dichloride (0.74 mL, 824 mg, 3.45 mmol, 1 equivalent) was added dropwise over 5 minutes, followed by stirring at room temperature for two hours. Water (20 mL) was added, and the mixture was filtered through Celite to remove the Pd/C catalyst. The organic layer was washed with 1 M hydrochloric acid (50 mL), then 1 M sodium hydroxide (50 mL), and then brine (50 mL), and the

dried over magnesium sulfate. The solvent was removed under vacuum, and the crude product was recrystallized from hot methanol/water to give a white solid (0.91 g, 51%). NMR spectra matched literature values.[1]

CNI-1493 (**1**): To 9:1 ethanol:water was added **5** (500 mg, 0.96 mmol). Aminoguanidine hydrochloride (584 mg, 5.28 mmol, 5.5 equivalent) was added, and the suspension was stirred at 80 °C for three hours. The reaction was cooled and placed in a freezer at -20 °C overnight. The precipitated solid was filtered and washed with 200 proof ethanol to give an off-white solid (0.753 g, 88%). NMR spectra matched literature values (Schröder *et al*., 2016).