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6-1-2023

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Recommended Citation

Bonser, C., Borgatta, J., White, J., Astete, C., Sabliov, C., & Davis, J. (2023). Impact of zein and lignin-PLGA biopolymer nanoparticles used as pesticide nanocarriers on soybean growth and yield under field conditions. *Agrosystems, Geosciences and Environment, 6* (2) https://doi.org/10.1002/agg2.20350

Received: 13 December 2022 | Accepted: 18 January 2023

DOI: 10.1002/agg2.20350

Agrosystems, Geosciences & Environment OPEN ACCESS

ORIGINAL ARTICLE

Agrosystems

Impact of zein and lignin-PLGA biopolymer nanoparticles used as pesticide nanocarriers on soybean growth and yield under field conditions

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Assigned to Associate Editor Raju Bheemanahalli.

Funding information
U.S. Department of Agriculture, Grant/Award Number: 2019-67021-29449; Louisiana Soybean and Grain Research and Promotion Board; National Institute of Food and Agriculture, Grant/Award Numbers: 1008750, 1021546

Abstract

Nanoparticles are being utilized in agriculture as fertilizers, pesticides, and agrochemical-carriers. Designed to be biocompatible and degradable, biopolymer nanoparticles were developed as an alternative to metallic nanoparticles, and though safe-by-design, polymeric nanoparticles must be field-tested prior to largescale use. Several field studies were conducted to observe detrimental effects of biopolymer nanoparticles on plant growth and yield using soybean, *Glycine max* (L.) Merr., as a model system. Biopolymer nanoparticles made from lignin or zein were applied as seed treatments to soybean seeds or as foliar sprays (zein only) to soybean plants. Studies using biopolymer nanoparticle seed treatments (nano-STs) measured the germination rates and seedling growth were evaluated in the laboratory, while stand counts, plant height, growth stage, yield, and hundred-seed weight were measured in the field. Foliar treatments assessed nanoparticle impact on flower abortion and pod production. To ensure nano-STs would not compromise the plant's defensive capabilities, herbivore feeding was assessed using a leaf bioassay for defoliators and a seed damage index for pod feeders. Growth rate, percent germination, or root length were not impacted by nano-STs. In the field, nano-STs had no impact on stand counts, heights, growth stage, yield, and hundred-seed weights. Leaf feeding assays and damage indices indicate plant susceptibility to herbivore attack was not increased due to nano-STs. Foliar applications of zein nanoparticles did not increase flower abortion or decrease pod set. These results indicate that biopolymer nanoparticles have no negative effects on growth, yield, and herbivore susceptibility and should be suitable for use in agriculture.

Abbreviations: DAP, days after planting; LNP, lignin biopolymer nanoparticles; LNP(AZO), lignin biopolymer nanoparticles loaded with azoxystrobin; nano-STs, biopolymer nanoparticle seed treatments; PLGA, poly(lactic-co-glycolic acid); RH, relative humidity; ST, seed treatment; (+)ZNP, positively charged zein biopolymer nanoparticles; (+)ZNP(AZO), positively charged zein biopolymer nanoparticles loaded with azoxystrobin.

1 | INTRODUCTION

Due to concerns over an ever-increasing human population and the negative environmental impacts of the Green Revolution, novel technologies are being adopted to assist agricultural food production (Ndaba et al., 2022). Nanotechnology developments in the form of nanoparticles as novel fertilizers, pesticides, agrochemical-carriers, and protectors against abiotic and biotic stress are now entering the application phase of development (Roco et al., 2011). Examples abound in the literature; from silver nanoparticles increasing yield by 35% in *Zea mays* L. (Poales:Poacaea) (Berahmand et al., 2012) and 23% in *Allium cepa* L. (Asparagales:Amaryllidaceae) (Fouda et al., 2020) to in vitro fungicide studies showing metal nanoparticles (copper, silver, and zinc) successful inhibition of mycelial growth, spore germination, and suppression of gray mold (*Botrytis cinerea* Pers.) (Helotiales:Sclerotiniaceae) (Malandrakis et al., 2019).

However, the use of some metal nanoparticles under specific conditions comes with an environmental risk. The accumulation of metal nanoparticles or their transformed products in the environment can increase the risk of negative environmental impacts (Nel et al., 2006; Nowack, 2009; Sawicki et al., 2019). Concerns also exist over the possible biotransformation of these particles in vivo (Doolette et al., 2015; Singh et al., 2018) and the documented ability of metal nanoparticles to cross the blood-brain barrier (de Simone et al., 2018; Lasagna-Reeves et al., 2010; Sawicki et al., 2019; Węsierska et al., 2018; Zhang et al., 2021). Biopolymer nanoparticles are a viable alternative to metal nanoparticles due to their greater degradability, biocompatibility, and digestibility (Banu et al., 2019; Patel et al., 2012).

Seed treatments (STs) are common in agricultural production, in the form of chemical or biological substances that are used as nutritional supplements for growing plants (Seagraves & Lundgren, 2012) or seed protection against arthropods, fungi, bacteria, and nematodes (Bailey et al., 2015). Polymer STs are currently used to bind active chemical product to the seed, improving coverage and creating a protective layer, while also minimizing abrasion during handling and storage (CLF, 2013). With research indicating that nanoparticles delay active ingredient release times (Couvreur, 2013; Farokhzad & Langer, 2009), nano-scaled biopolymers could ensure continued or controlled release of ST pesticides or nutrients for extended periods of time. In addition, nanoparticle carriers (nanocarriers) of pesticides have the potential to increase efficacy of STs by delaying active ingredient release times and improving pesticide translocation from roots to leaves (Couvreur, 2013; Farokhzad & Langer, 2009; Mendez et al., 2022). For example, zein nanoparticle STs loaded with essential oils increased repellency against mites while demonstrating no phytotoxicity on test plants under greenhouse conditions (de Oliveira et al., 2018).

Core Ideas

- A multiyear field study evaluated the agronomic effects of biopolymer nanoparticle seed treatments on soybean.
- As seed treatments, biopolymer nanoparticles have no negative effects on the growth or yield of soybean.
- As a foliar application, zein nanoparticles had no deleterious effects on the soybean flowers or pod formation.

However, prior to large-scale use in agriculture, biopolymer nanoparticles need to be field tested. Bonser et al. (2022) showed that zein biopolymer nanoparticles do not have generational effects on *Chrysodeixis includens* (Walker) (Lepidoptera:Noctuidae) in the laboratory; similarly, lignin nanoparticle STs had no adverse impact on soybean (*Glycine max* [L.] Merr.) (Fabales:Fabaceae) and insect growth under laboratory settings (Kacsó et al., 2022). However, the impact of these biopolymer nanoparticles on yield and growth under field conditions is still uncertain. Therefore, using soybean as a model system, we conducted several studies using biopolymer nanoparticles applied as STs (lignin or zein) or as foliar sprays (zein only). Seed germination assays were conducted in the laboratory to measure germination rates and root and stem growth using different formulations of biopolymer nanoparticles. For biopolymer nanoparticle seed treatments (nano-STs) in the field, we evaluated stand counts, plant height, growth stage, yield, and hundred-seed weight. Foliar treatments assessed nanoparticle impact on plant stress using flower abortion and pod production as a metric. To ensure nano-ST would not compromise the plant's ability to protect itself from insect attack, we assessed herbivore feeding rates using a leaf bioassay for defoliators and a seed damage index for pod feeders. This study provides important information on the sustainable use of novel biopolymer agrochemical carriers in agriculture.

2 | MATERIALS AND METHODS

2.1 | Preparation of nanoparticle solutions

The creation of zein biopolymer nanoparticles for spray followed standards prescribed in Bonser et al. (2022). Empty positively charged zein biopolymer nanoparticles, (+)ZNP, were formed by the emulsion-diffusion method. The organic phase was first created by dissolving 1000 mg of zein powder in 30 mL of a 4:1 (v/v) acetone:water solution. The organic

TABLE 1 Summary table detailing treatments used and their corresponding experiments.

Treatment abbreviation	Definition	Experiment
(+)ZNP	Empty positively charged zein biopolymer nanoparticles.	Seed germination study/foliar application/seed treatment field study
(+)ZNP(AZO)	Positively charged zein biopolymer nanoparticles loaded with azoxystrobin, a fungicide.	Seed treatment field study
(+)ZNP(MFZ)	Positively charged zein biopolymer nanoparticles loaded with methoxyfenozide, an insecticide.	Seed germination study/foliar application
Dynasty	Commercial fungicide. Active ingredient is azoxystrobin.	Seed treatment field study
Intrepid 2F	Commercial insecticide. Active ingredient is methoxyfenozide.	Seed germination study/foliar application
LNP	Lignin biopolymer nanoparticles.	Seed treatment field study
LNP(AZO)	Lignin biopolymer nanoparticles loaded with azoxystrobin, a fungicide.	Seed treatment field study

Abbreviations: LNP, lignin biopolymer nanoparticles; LNP(AZO), lignin biopolymer nanoparticles loaded with azoxystrobin; (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(AZO), positively charged zein nanoparticles loaded with azoxystrobin; (+)ZNP(MFZ), positively charged zein nanoparticles loaded with methoxyfenozide.

phase was added to the aqueous phase, which consisted of 300 mL of low resistivity water (Thermo Fisher Sci.) and 173 mg of didodecyldimethylammonium bromide (DDAB) (MilliporeSigma). The emulsion was run through a microfluidizer (M-110P; Microfluidics International Co.) thrice at high pressure (206,840 kPa) prior to solvent evaporation. The acetone was evaporated using a rotary evaporator (Rotavapor R-300; BÜCHI Labortechnik) under vacuum at 33˚C for 60 min. The zein biopolymer nanoparticles loaded with methoxyfenozide, (+)ZNP(MFZ), followed the same protocol but the with the addition of 100 mg of methoxyfenozide (Fisher Thermo Scientific) in the organic phase. The stock concentration of zein to methoxyfenozide used in the experiments (2000:124 ppm) was adjusted by dilution with low resistivity water. All nanoparticle solutions were used within 1 month of synthesis. Due to limited funding, not all experiments used the same biopolymer nanoparticle formulations. A summary of all treatments used in the following experiments is listed in Table 1.

Biopolymer nanoparticles for the ST study were synthesized as described in (Kacsó et al., 2022). The entrapment of the tested antifungal agents used in this study, technical grade azoxystrobin (MilliporeSigma) and Dynasty (Syngenta), was performed in the organic phase of nanoparticle synthesis in a ratio of 10 mg per 100 mg of biopolymer. Zein biopolymer nanoparticles were prepared through nanoprecipitation using the surfactant, DDAB. The organic phase was created first by mixing 3778 mg of zein in 120 mL of an acetone/water solution (80:20 [v/v]) and stirring at room temperature under dissolved water. Next, the aqueous phase was created by combining 394 mg of the surfactant DDAB and 682 mL of deionized water and mixing at room temperature for 10 min. Then, both the organic phase and aqueous phase were combined and stirred at room temperature until the suspension was homogenous. The suspension was then passed through a microfluidizer thrice at 206,840 kPa. Lastly, the acetone was evaporated with a rotary evaporator until the solution had a final concentration of 10,000 ppm. Lignin biopolymer nanoparticles were synthesized by an emulsion evaporation technique, created from a grafted lignin-poly(lactic-co-glycolic acid) (PLGA) biopolymer, which was obtained using an acylation reaction (Astete et al., 2020). The organic phase was created by dissolving 150–500 mg of lignin-PLGA in 5 mL of ethyl acetate at room temperature and stirring. The organic phase was added to the aqueous phase, which consisted of 50 mL of distilled water. The suspension was mixed for 10 min, and then passed through a microfluidizer four times at 30,000. The suspension was evaporated in a rotary evaporator until the solution had a final concentration of 10,000 ppm.

2.2 | Insect colony

The following experiments used an in-house colony of *Chrysodeixis includens* (Walker), LSU1, which has been maintained since 1976, when it was originally collected from south Louisiana (Newsom et al., 1980). The colony was maintained as described in Bonser et al. (2022). In summary, *C. includens* larvae were reared at 26˚C with 50% Relative humidity (RH) and 14:10 (L:D) h photophase on artificial meridic diet (Southland Products Inc.), made following manufacture's protocols. While warm, liquid diet was dispensed into 30-mL soufflé cups and allowed to cool and dry overnight. Following eclosion from eggs, *C. includens* larvae were placed into cups with diet using a nylon paintbrush.

Once the insects reached the pupal stage, pupae were removed from the cups and placed in a bed of vermiculite at the bottom of a 5.7-L round food storage container (Parade Plastics). Individual containers were lined with brown single-fold paper towels used for adult female oviposition. A solution of 90:10 distilled water–honey solution in a 30-mL cup filled with cotton wadding was used for adult feeding. At every 2 days, egg sheets were collected and placed into plastic bags. Those eggs that were collected had larvae eclose in about 2–3 days, allowing the process to restart.

2.3 | Soybean seed germination and root growth

A series of laboratory experiments were conducted to understand the impact biopolymer nanoparticles as nanocarriers of pesticides on soybean seed germination and root growth. Treatments consisted of positively charged zein biopolymer nanoparticles that were empty (unloaded), loaded with technical grade methoxyfenozide (AdipoGen Life Sciences, Fisher Scientific), and the commercially available methoxyfenozide product, Intrepid 2F (Corteva Agriscience). All zein biopolymer nanoparticles were applied at concentrations of 0, 10, 100, 1000, and 2000 ppm with corresponding concentrations of active ingredient methoxyfenozide at 0, 0.62, 6.2, 62, and 124 ppm. The germination assay was performed in sterile Petri dishes (100 × 15 mm) that had a layer of filter paper (9.0-cm diameter) (VWR International) acting as germination paper. Soybean seeds (Var.: Seedranch; Seedranch, Odessa, FL) were first surfaced sterilized with 10% bleach (Clorox Company) and 1% Tween 20 solution (Sigma-Aldrich) for 10 min. Next, the bleach solution was decanted, and the seeds were rinsed with distilled water. Seeds were then placed onto filter paper, 10 per plate, and the experiment was replicated four times. Nanoparticle suspensions of 4 mL were added to the germination paper and a consistent amount was reapplied to all Petri dishes to maintain a moist environment. Seedling growth and germination was recorded daily, and counts were determined by counting seedlings and extracting the root lengths using ImageJ (Schneider et al., 2012). The following equation was used to calculate the germination rate:

$$\text{Germination rate} = \sum \frac{G_t}{D_t},$$

where G_t is the number of seeds on day t and D_t is the number of days that have elapsed by day t. The root growth length was calculated using the following equation:

$$\text{Root elongation} = \frac{\text{Length}_2 - \text{Length}_1}{\text{Day}_2 - \text{Day}_1},$$

where Length_2 is the root length on the final day of the experiment and Length_1 is the root length on the first day of the experiment.

2.4 | Nanoparticle seed treatment field study

The following experiment was conducted in 2021 and 2022. In 2021, soybean variety UA5414RR, maturity group V (University of Arkansas) was used and in 2022, a maturity group IV variety, Terral REV 48A76 (Terral Seed Inc.) was used. Soybean seeds were treated with one of four following treatments (1) Dynasty empty (+)ZNP, (2) (+)ZNP with nano-entrapped azoxystrobin [(+)ZNP(AZO)], (3) empty grafted lignin-PLGA nanoparticles (LNP), and (4) grafted lignin-PLGA nanoparticles with nano-entrapped azoxystrobin (LNP[AZO]). All concentrations of biopolymer nanoparticles were at 10,000 ppm, while all concentrations of Dynasty and azoxystrobin were at 500 ppm. The zein biopolymer nanoparticles used DDAB as a surfactant and were positively charged, while the grafted lignin-PLGA nanoparticles used no surfactant and were negatively charged. For treatment, soybean seeds were immersed for 5 min in freshly prepared nanoparticle colloids using 1 mL for every 1 g of seed. Treated seeds were dried overnight on a sieve at room temperature, after which they were stored at 4°C.

Test plots of soybeans were planted in Baton Rouge, Louisiana at the Doyle Chambers Central Research Station (30°22′04″ N, 91°09′59″ W) from 2021 to 2022. Seeds were planted at eight seeds per 0.30 m on 0.76 m centers using a four-row cone planter (Almaco). Plots consisted of two 9.14 × 0.76 m^2 rows, which were arranged in a randomized complete block design with four replicates per year ($n = 24$). No insecticides were applied except for the treatments and all plots were managed according to best agronomic practices for the area (LeBlanc et al., 2005).

2.5 | Impacts of biopolymer nanocarriers applied as seed treatments on plant growth and yield

The effect nano-STs on soybean planted in the field was measured by stand counts, plant heights, and differences in growth stage. Establishment of stands were determined by counting the number of emerged plants in each two-row plot 14 days after planting (DAP) to determine how nano-STs affected germination under field conditions. Soybean plant heights were measured at intervals of 14, 21, and 28 DAP using a meter stick and rounding to the nearest half centimeter. Soybeans were staged based upon standards set by Fehr and Caviness (1977). For plant height and stage, five random soybean plants were selected within each of the 24 plots.

Once plants had reached harvest maturity (R8) (Fehr & Caviness, 1977), five whole plants per plot were randomly selected and destructively sampled. Plants were placed in a walk-in propane plant dryer at the Doyle Chambers Central Research Station, Baton Rouge, LA and dried at 43˚C for 5–7 days until seed moisture reached 13%. Moisture content of samples was determined by a GAC 500 XT Moisture Tester (Dickey-john, TSI Incorporated). Once dried, pods were threshed using a Small Bundle Thresher (Almaco). Soybean seed was sorted based upon a soybean grading mat (Seedburo) using a USDA approved soybean dockage sieve set (Seedburo). Three subsamples of 100 seeds per plot were randomly sampled and weighed to the nearest hundredth of a gram using a Scout balance (Ohaus Corporation).

2.6 | Impacts of biopolymer nanocarriers applied as seed treatments on herbivore susceptibility

To ensure nano-ST would not compromise the plant's ability to protect itself from insect attack, herbivore feeding rates using a leaf bioassay for defoliators and a seed damage index for pod feeders were conducted. Soybean leaves were randomly sampled at 28 DAP, with at least 15 random leaves sampled from each blocked treatment. Leaves were immediately brought back to the laboratory and placed into a sterile Petri dish (100×15 mm^2) lined with dampened qualitative filter paper 410 (9.0-cm diameter), one leaf per dish. Individual *C. includens* neonates were transferred to leaves using a small nylon paintbrush. Petri dishes with their respective leaves and neonates were placed into a rearing room with conditions at 26˚C, 50% RH, and 14:10 (L:D). Petri dish filter paper was checked and moistened daily. Insect larval weights and mortality were assessed 7 days following placement. Larval weights were measured using a Mettler-Toledo XS105 scale and mortality was assessed following procedures described in (Bonser et al., 2022), in which the specimen would be considered dead upon failure to respond from prodding using a small nylon paintbrush.

Previously harvested soybean seeds were evaluated based upon stink bug feeding and the presence of peck. Damage indices (DI) were recorded for each maturity group and developed by classifying soybean into one of categories (0, no damage; 1, light damage; 2, moderate damage; and 3, heavy damage) (McPherson et al., 1979). Light damage was defined as the presence of only peck. Moderate damage was defined as the presence of shriveling. Heavy damage was defined as the seeds that passed into the bottom sieve, or the presence of both peck and shriveling.

2.7 | Impacts of foliarly applied biopolymer nanocarriers on flower abortion and pod set

In 2022, two-row plots (9.14×0.76 m^2) of soybean variety AG3555 were sprayed at R2 using a CO_2 backpack sprayer calibrated to deliver 140.2 L ha^{-1} at 241.3 kPa using four TeeJet 80015VS flat-fan nozzles (TeeJet Technologies). Each plot was sprayed with 250 mL with one of the following treatment solutions: distilled water (untreated check), (+)ZNP at 2520 ppm, (+)ZNP(MFZ) at 2520 ppm zein and 200 ppm methoxyfenozide, and Intrepid 2F at 200 ppm. Sprayer equipment was thoroughly rinsed with 1 L of water before and after spraying each treatment solution. Plots were arranged in randomized complete block design with four replicates. Fifteen random plants from each row (30 random plants per plot) were selected and had their flowers counted. Flower counting was conducted at pre-spray, 3 days after, and 7 days after. Soybean plots were sampled again at the beginning seed fill stage (R5) to measure pod numbers.

2.8 | Statistical analysis

All data were analyzed in R 4.2.1 using IDE RStudio (R Core Team, 2022; RStudio Team, 2022) or Microsoft Excel version 2010 (Microsoft Corporation). Data for soybean germination in nanoparticle solutions were analyzed using a two-way analysis of variance (ANOVA), which compared the interaction between formulations and their concentrations to the cumulative germination rates of soybeans and root elongation. A Fisher's least significant difference post hoc test was used to compare differences in cumulative germination and root elongation. Nanoparticle seed treatment plant growth and development data were analyzed between treatments by either a one-way ANOVA (stand counts to treatments) or a two-way ANOVA (plant heights to treatments and DAP) using R package *lme4* with year treated as a random effect. Means were separated using R package *emmeans* with an adjusted Tukey's pairwise comparison at $p < 0.05$ (Lenth, 2022). Weight data of *C. includens* fed nano-ST leaf tissue were analyzed using a one-way ANOVA, with year treated as a random effect in the model. Comparisons between treatments were made using Tukey's honestly significantly different (HSD) test ($p < 0.05$). Mortality data of *C. includens* fed nano-ST leaf tissue was arcsine-square root transformed to achieve normality and analyzed using a one-way ANOVA, with year treated as a random effect in the model. Flower abortion and pod data were natural logarithm transformed to achieve normality and analyzed using a repeated-measures ANOVA comparing flowers to treatment over time. Hundred-seed weights and

TABLE 2 Analysis of variance results for soybean seed germination and root growth.

Effect	*df*	*F* value	*p* value
Soybean seed germination			
Treatment	2/45	0.276	0.760
Concentration	4/45	0.753	0.561
Treatment × concentration	8/45	0.196	0.990
Soybean seed growth rate			
Treatment	2/45	0.46	0.633
Concentration	4/45	0.20	0.936
Treatment × concentration	8/45	0.18	0.992

Abbreviation: *df*, degrees of freedom.

DI and reduction of seed weights were analyzed using a one-way ANOVA, evaluating the interactions between treatment groups, with year as a random effect. Comparisons between treatments were made using Tukey's HSD test ($p < 0.05$).

3 | RESULTS

3.1 | Soybean seed germination and root growth

Differences were not detected in the treatments and cumulative germination rates, or with the interaction between treatments and concentrations and cumulative germination rates nor between treatments and the growth rates of soybean roots (Table 2). When comparing growth rates of soybean roots, differences also were not detected between treatments or between nanoparticle concentrations, nor were differences found in the interaction between treatments and concentrations with respect to the growth rates of soybean roots (Table 2). The cumulative germination rate and cumulative root growth rates for soybean grown in nanoparticle solutions are listed in Tables 3 and 4, respectively.

3.2 | Impacts of biopolymer nanocarriers applied as seed treatments on plant growth and yield

Stand counts were taken after 14 days to determine field germination of nano-ST soybeans. Differences were not detected between treatments in a specific year (Figures 1 and 2). However, when taking year into account, significant differences were observed (Table 5). Height differences of soybeans with nano-STs were not observed when evaluating between treatments (Figures 3 and 4). As expected, differences in heights were noted between DAP of 14, 21, and 28 days (Table 5, Figures 1 and 2); however, differences were not detected in the interaction between the treatments and DAP. Differences were also observed in soybean heights with respect to year (Table 5, Figures 3 and 4).

TABLE 3 Cumulative germination rate (standard error) of soybean seeds reared in concentrations of nanoparticle and methoxyfenozide solutions.

Treatment	Concentration (zein | methoxyfenozide)(ppm)	Cumulative germination rate (seeds day^{-1})
(+)ZNP	0 | 0	13.6 (0.3)a
	10 | 0	10.5 (0.3)a
	100 | 0	13.7 (0.4)a
	1000 | 0	12.4 (0.4)a
	2000 | 0	11.8 (0.5)a
(+)ZNP(MFZ)		
	0 | 0	13.6 (0.3)a
	10 | 0.62	11.7 (0.4)a
	100 | 6.2	12.0 (0.2)a
	1000 | 62	11.5 (0.6)a
	2000 | 124	10.6 (0.4)a
Intrepid 2F		
	0 | 0	13.6 (0.3)a
	10 | 0.62	12.8 (0.5)a
	100 | 6.2	12.3 (0.5)a
	1000 | 62	13.4 (0.5)a
	2000 | 124	11.3 (0.2)a

Note: Values followed by the same letters within a column do not differ significantly at $\alpha = 0.05$.
Abbreviations: (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(MFZ), positively charged zein nanoparticles loaded with methoxyfenozide.

The growth of soybean (cm day^{-1}) was not different between the treatments, but it appeared that planted soybeans grew at a rate of 1.28 cm a day in 2021 and 0.95 cm a day in 2022. Soybean grown in 2022 was found to be shortest, being an average 20.5% shorter than soybean grown in 2021. Soybeans from 2021 ranged from 10 to 14 cm at 14 DAP, 16 to 20 cm at 21 DAP, and 28 to 32 cm at 28 DAP. This is contrasted by those nano-ST soybeans planted in 2022 that ranged from 8 to10 cm at 14 DAP, 17 to 18 cm at 21 DAP, and 22 to 23 cm at 28 DAP. Control soybean plots decreased 26.4%, Dynasty soybean decreased 22.3%, (+)ZNP decreased 24.7%, (+)ZNP(AZO) decreased 6.2%, LNP decreased 21.9%, and LNP(AZO) decreased 21.8%. However, when accounting for changes between years, differences were not observed. Due to this, soybean in 2022 matured slower, being 1–2 stages behind their 2021 counterparts. These differences in stage were not

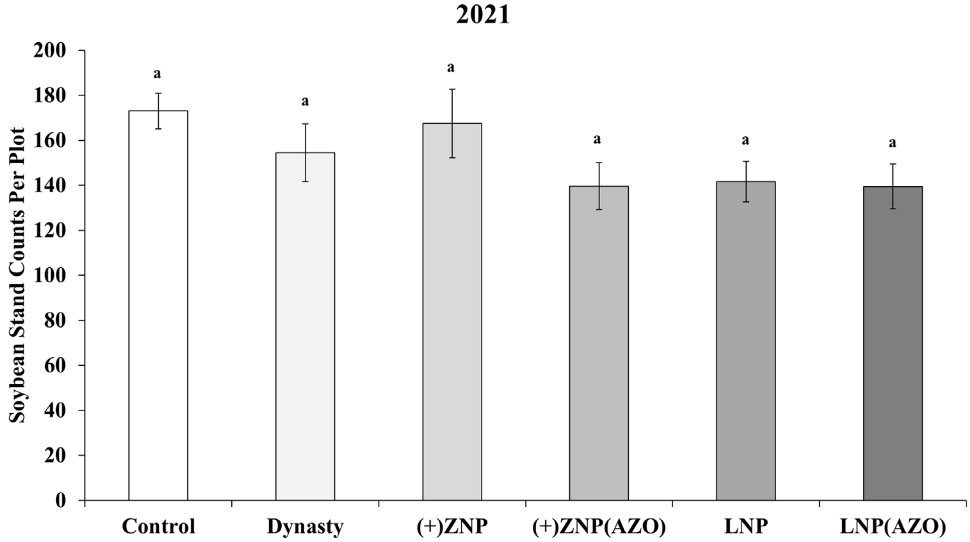

FIGURE 1 Mean stand counts of soybean plants per plot between nano-ST for 2021. Error bars show the standard error of the mean. The same letters atop bars indicate that treatments are not significantly different at $\alpha = 0.05$. LNP, lignin biopolymer nanoparticles; LNP(AZO), lignin biopolymer nanoparticles loaded with azoxystrobin; nano-ST, biopolymer nanoparticle seed treatments; ST, seed treatment; (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(AZO), positively charged zein nanoparticles loaded with azoxystrobin.

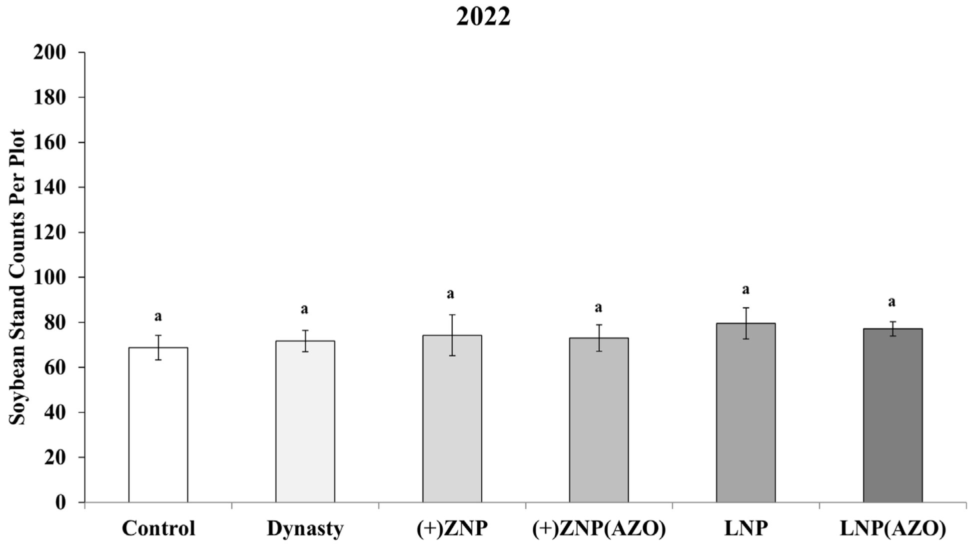

FIGURE 2 Mean stand counts of soybean plants per plot between nano-ST for 2022. Error bars show the standard error of the mean. The same letters atop bars indicate that treatments are not significantly different at $\alpha = 0.05$. LNP, lignin biopolymer nanoparticles; LNP(AZO), lignin biopolymer nanoparticles loaded with azoxystrobin; nano-ST, biopolymer nanoparticle seed treatments; ST, seed treatment; (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(AZO), positively charged zein nanoparticles loaded with azoxystrobin.

noted between treatments on sampling days within a specific year.

The calculated hundred-seed weight was found not to be different between treatments in sampled years. However, when comparing hundred-seed weight between years, differences were detected. The average hundred-seed weight for all treatments was between 11.02 and 11.71 mg in 2021, and between 9.15 and 11.77 mg in 2022 (Table 6).

3.3 | Impacts of biopolymer nanocarriers applied as seed treatments on herbivore susceptibility

Significant differences in larval weights were noted between the years of 2021 and 2022, but the differences in years were treated as a random effect in modeling (Table 7). Taking year into account, there were no differences found between the

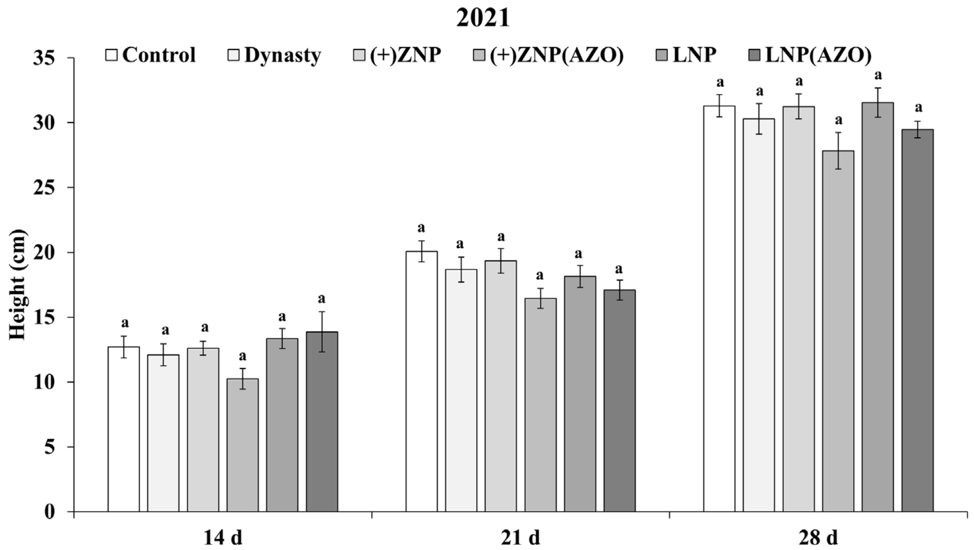

FIGURE 3 Mean height of nano-ST soybeans 14, 21, and 28 days after planting for 2021. Within each day, heights were not found different. Treatments within a day match order of the legend from left to right. Error bars show the standard error of the mean. The same letters atop bars indicate that treatments are not significantly different at $\alpha = 0.05$. LNP, lignin biopolymer nanoparticles; LNP(AZO), lignin biopolymer nanoparticles loaded with azoxystrobin; nano-ST, biopolymer nanoparticle seed treatments; ST, seed treatment; (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(AZO), positively charged zein nanoparticles loaded with azoxystrobin.

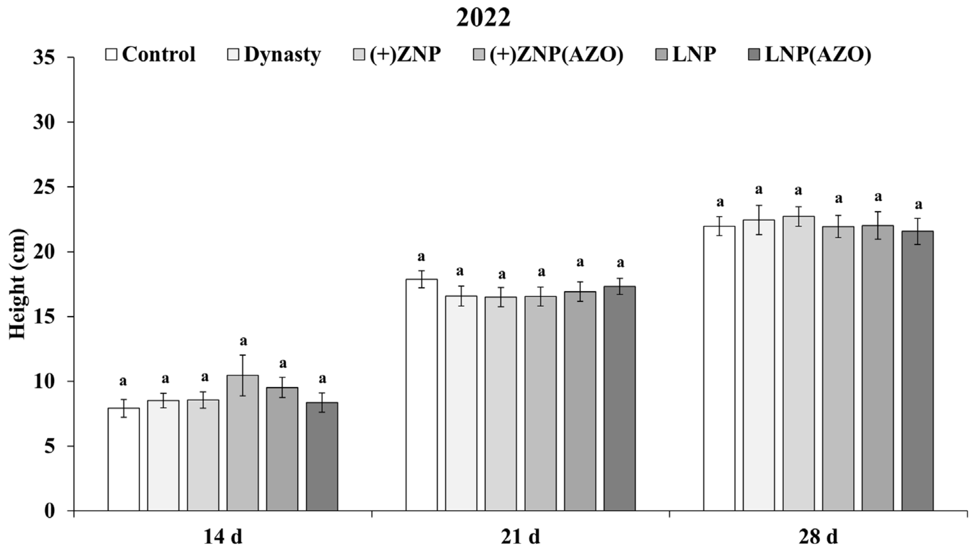

FIGURE 4 Mean height of nano-ST soybeans 14, 21, and 28 days after planting for 2022. Within each day, heights were not found different. Treatments within a day match order of the legend from left to right. Error bars show the standard error of the mean. The same letters atop bars indicate that treatments are not significantly different at $\alpha = 0.05$. LNP, lignin biopolymer nanoparticles; LNP(AZO), lignin biopolymer nanoparticles loaded with azoxystrobin; nano-ST, biopolymer nanoparticle seed treatments; ST, seed treatment; (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(AZO), positively charged zein nanoparticles loaded with azoxystrobin.

weights or mortalities of neonate *C. includens* that fed on nano-ST soybean leaf tissue (Table 8). Differences between years were likely due to quality of leaf tissue due to the poor growing conditions in 2022. Soybean DI in Terral 48A76 soybeans that were treated with nano-STs were not found to be different based upon treatment (Table 6); however, when evaluating DI in soybean seed between years, differences were detected. Despite differences noted between 2021 and 2022, nano-STs did not decrease the plant's ability to protect itself from insect attack within a respective year.

TABLE 4 Cumulative root growth rate (standard error) of soybean seeds reared in nanoparticle solutions and methoxyfenozide solutions.

Treatment	Concentration (zein | methoxyfenozide) (ppm)	Cumulative growth rate (mm day^{-1})
(+)ZNP	0 | 0	15.7 (1.6)a
	10 | 0	13.6 (1.9)a
	100 | 0	17.7 (1.9)a
	1000 | 0	14.9 (1.4)a
	2000 | 0	16.6 (1.6)a
(+)ZNP(MFZ)		
	0 | 0	15.7 (1.6)a
	10 | 0.62	15.9 (1.8)a
	100 | 6.2	16.0 (1.8)a
	1000 | 62	12.5 (1.2)a
	2000 | 124	16.3 (0.9)a
Intrepid 2F		
	0 | 0	15.7 (1.6)a
	10 | 0.62	14.8 (1.2)a
	100 | 6.2	15.1 (1.6)a
	1000 | 62	21.1 (1.8)a
	2000 | 124	14.5 (1.3)a

Note: Values followed by the same letters within a column do not differ significantly at $\alpha = 0.05$.
Abbreviations: (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(MFZ), positively charged zein nanoparticles loaded with methoxyfenozide.

TABLE 5 Analysis of variance results for the impacts of biopolymer nanocarriers applied as seed treatments on plant growth and yield.

Effect	*df*	*F* value	*p* value
Soybean stand counts			
Treatment	5/89	0.89	0.489
Year	1/89	211.19	<0.001*
Soybean plant heights			
Treatment	5/18	0.31	0.901
Year	1/18	51.55	<0.001*
Days after planting	2/18	136.22	<0.001*
Treatment × days after planting	10/18	0.34	0.958
Soybean hundred-seed weight			
Treatment	5/40	0.54	0.748
Year	1/40	4.70	0.038*

Abbreviation: *df*, degrees of freedom (numerator/denominator).
*Significant differences were detected at $\alpha = 0.05$.

3.4 | Impacts of foliarly applied biopolymer nanocarriers on flower abortion and pod set

The repeated-measures ANOVA found no statistical differences in the number of flowers between the treatments at any given point in time, nor were any differences found in the interaction between days sampled and treatment (Table 9). When compared to the control, no differences in flowers were noted between the treatments at any given sampling period following application of nanoparticle spray (Table 10). As expected, there were differences noted between the days after sampling and the number of flowers in soybeans. One-way ANOVA found no differences between the treatments with respect to pods present. There was no observed phytotoxicity in soybean plants following foliar application. The average number of pods found on soybean plants was 20 per plant when sampled at R5, which was 58 DAP (Table 10). The indeterminate variety used in this experiment AG3555 was found to have increased flowers at an estimated rate of one per day.

4 | DISCUSSION

This study was a multiyear field experiment that evaluated the agronomic effects of several biopolymer nano-STs on soybean. As nanoparticles become utilized in agriculture for fertilizers, pesticides, and pesticides-carriers, applied research is imperative. This research presents the next stage in nanotechnology development, which shifts the focus of nanotechnology to its application phase (Roco, 2018; Roco et al., 2011). However, environmental and health concerns exist from using metal/metal oxide nanoparticles. A viable alternative is the use of biopolymer nanoparticles due to their degradability and biocompatibility (Banu et al., 2019; Patel et al., 2012).

The experiments conducted were proof of concept, and due to limited funding, not all of the same biopolymer nanoparticle formulations were used in each of the conducted studies. The biopolymer nanoparticles used in the field study were applied as STs or as foliar sprays. Zein, one of the biopolymers used, is advantageous due to its characteristic ability to create films, which could be useful in creating a polymeric matrix for seed coatings (Arvanitoyannis & Dionispoulou, 2010). Additionally, zein biopolymer nanoparticles synthesized with DDAB as a surfactant were positively charged, aiding in their attachment to the plant tissue. The results suggested that (+)ZNP-STs had no impact on the plant, as demonstrated by the lack of immediate deleterious effects on soybean when measured by stand counts and heights 14 DAP. The sampled leaf tissue from 28 DAP also failed to decrease larval weights or mortality, suggesting that these

TABLE 6 Average damage index and hundred-seed weight (standard error) of harvested soybean by treatment and year.

	Average damage index		Average hundred-seed weight (g)	
Treatment	**2021**	**2022**	**2021**	**2022**
Control	1.4 (0.2)a	2.6 (0.1)a	11.11 (0.49)a	11.77 (0.30)a
Dynasty	1.5 (0.1)a	2.7 (0.1)a	11.61 (0.15)a	10.74 (0.50)a
(+)ZNP	1.5 (0.1)a	2.7 (0.1)a	11.60 (0.25)a	10.14 (0.46)a
(+)ZNP(AZO)	1.2 (0.2)a	2.7 (0.1)a	11.42 (0.13)a	10.84 (0.58)a
LNP	1.5 (0.1)a	2.7 (0.1)a	11.02 (0.31)a	11.52 (0.50)a
LNP(AZO)	1.4 (0.0)a	2.9 (0.1)a	11.71 (0.24)a	9.15 (1.41)a

Note: Values with the same letters within each column do not differ significantly at $\alpha = 0.05$.
Abbreviations: LNP, lignin biopolymer nanoparticles; LNP(AZO), lignin biopolymer nanoparticles loaded with azoxystrobin; (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(AZO), positively charged zein nanoparticles loaded with azoxystrobin.

TABLE 7 Analysis of variance results for the impacts of biopolymer nanocarriers applied as seed treatments on herbivore susceptibility.

Effect	*df*	*F* value	*p* value
***C. includens* larval weights**			
Treatment	5/41	0.73	0.603
Year	1/41	13.84	<0.001*
Soybean damage index			
Treatment	5/42	1.22	0.319
Year	1/42	393.80	<0.001*

Abbreviation: *df*, degrees of freedom (numerator/denominator).
*Significant differences were detected at $\alpha = 0.05$.

biopolymer nanoparticles do not induce systemic toxicity in the plant. Foliar (+)ZNP also failed to reduce flower abortion and pod formation compared to the controls, indicating that these biopolymer nanoparticles do not impart a deleterious effect on soybean plants.

Lignin, the other biopolymer used, is the second most abundant plant material on the planet and is a complex hydrocarbon polymer that gives rigidity to plants by toughening plant stems and leaf tissue, giving added durability and protection to the plant in the early stages of its development (Khan et al., 2019; Patel & Parsania, 2018). Research has indicated that the leaf hardness and water retention ability of plants increases as they mature and lignify (Gao et al., 2019; Song et al., 2021; Wang et al., 2010). However, research conducted here does not suggest that exogenous lignin in nano-ST form imparted any of the aforementioned effects. In addition, results from this study do not indicate any differences in larval weights of mortalities between the control and lignin-ST treatments when leaves were gathered 28 DAP. Moreover, no differences were found in plants grown from ZNP- and LNP-treated seeds, suggesting that these biopolymer nano-STs do not negatively impact the growth of the plant.

As nanocarriers, biopolymer nanoparticles offer advantages to conventional chemical formulations. These systems provide targeted drug delivery, increase specificity and bioavailability, sustain or control the release of active ingredients, protect the active ingredients from degradation, and provide improved adhesion (Ulbrich & Lamprecht, 2010; Dutta et al., 2022; Gupta et al., 2013; Machado et al., 2022). For example, the use of chitosan biopolymer nanoparticles loaded with paraquat preserved effectiveness after encapsulation, but soil reduced sorption and caused less chromosome damage in *A. cepa* compared to the free herbicide (Grillo et al., 2014). Seed germination studies with lignin biopolymer nanoparticles have shown that they cross the seed tegument and penetrate seedling tissues with no toxicity, which provides an opportunity for the movement of active ingredients to increase crop production while decreasing environmental toxicity (Falsini et al., 2019).

The presented study evaluated methoxyfenozide and azoxystrobin as model active ingredients to be encapsulated by zein and lignin biopolymer nanoparticles as STs. Methoxyfenozide is a diacylhydrazine insecticide that acts as an ecdysone agonist on 20-hydroxyecdysone receptors in Lepidoptera (Doucet et al., 2009; Palli & Retnakaran, 2001; Wing et al., 1988). The fungicide azoxystrobin is a strobilurin compound, which acts by blocking the electron transport in the mitochondrial respiratory chain in fungi (Bertelsen et al., 2001). Both have low acute and chronic toxicity to non-target species (USEPA, 2015, 2020). These active ingredients were selected as models due to their biorationality and low environmental impact, which provide safe alternatives to when used in conjunction with biodegradable nanoparticles, such as zein and lignin.

The results of this study agree with previous research that evaluated the effects of nano-STs soybeans grown in greenhouse conditions on *C. includens* (Kacsó et al., 2022), which found that nano-STs had no effect on feeding or mortality. Plant growth did not appear to be negatively impacted by the presence of nano-STs, as demonstrated by equivalent growth rates across all treatments. Entrapment of active ingredients in biopolymer nanoparticles also demonstrated no deleterious effects on seedlings. These results suggest that nano-STs are no more harmful to soybean seeds than the formulated

TABLE 8 Effect of soybean leaves grown from nanoparticle seed treatments on mean (standard error) 7-day larval weights and mortality of *C. includens*.

	Larval weights (mg)		Larval mortality (%)	
Treatment	**2021**	**2022**	**2021**	**2022**
Control	16.18 (2.33)a	15.99 (1.11)a	0.02 (0.05)a	0.05 (0.08)a
Dynasty	15.97 (3.29)a	13.08 (1.97)a	0.04 (0.08)a	0.04 (0.09)a
(+)ZNP	18.22 (2.60)a	11.93 (1.90)a	0.02 (0.04)a	0.06 (0.07)a
(+)ZNP(AZO)	16.81 (0.67)a	9.75 (1.79)a	0.01 (0.02)a	0.05 (0.05)a
LNP	17.06 (2.60)a	12.43 (3.26)a	0.05 (0.03)a	0.06 (0.08)a
LNP(AZO)	22.34 (3.76)a	12.66 (1.25)a	0.02 (0.03)a	0.04 (0.08)a

Note: Means with the same letters within a column do not differ significantly at $\alpha = 0.05$.
Abbreviations: LNP, lignin biopolymer nanoparticles; LNP(AZO), lignin biopolymer nanoparticles loaded with azoxystrobin; (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(AZO), positively charged zein nanoparticles loaded with azoxystrobin.

TABLE 9 Analysis of variance results for the impacts of foliarly applied biopolymer nanocarriers on flower abortion and pod set.

Effect	*df*	*F* value	*p* value
Soybean flower abortion			
Treatment	3/36	1.03	0.393
Days after treatment	2/36	45.53	<0.001*
Treatment × days after treatment	6/36	0.15	0.989
Soybean pod set			
Treatment	3/12	0.174	0.911

Abbreviation: *df*, degrees of freedom (numerator/denominator).
*Significant differences were detected at $\alpha = 0.05$.

TABLE 10 Effects of foliar applied nanoparticles formulations on mean (standard error) flowers and pods of soybean plants. All values were rounded to the nearest whole number.

	Flowers			
Treatment	**Pre-spray**	**3-DAT**	**7-DAT**	**Pods**
Control	3 (0)a[a]	6 (0)a	11 (0)a	21 (1)a
(+)ZNP	3 (0)a	6 (0)a	11 (1)a	18 (1)a
(+)ZNP(MFZ)	3 (0)a	6 (0)a	12 (1)a	22 (1)a
Intrepid 2F	3 (0)a	5 (0)a	9 (0)a	20 (1)a

Note: Values followed by the same letter within a column are not significantly different at $\alpha = 0.05$.
Abbreviations: DAT, days after treatment; (+)ZNP, empty positively charged zein nanoparticles; (+)ZNP(MFZ), positively charged zein nanoparticles loaded with methoxyfenozide.

product, demonstrating the safety of these particles to soybean plants.

Of note were the differences in nano-STs between the 2021 and 2022, specifically the decreased plant heights, reduced soybean seed weights, and increased damage to seed. Decreases in height and stage between years were noted and likely due to the drought-like conditions during and after planting. The significant differences in nanoparticle seed treatments found between the years could be a response to the high soil temperatures and lack of precipitation at the Doyle Chambers Central Research Station in 2022. Air temperature during planting was 31.8˚C and 33.6˚C for 2021 and 2022, respectively. Planting soil temperature was 27.9˚C and 28.9˚C for 2021 and 2022, respectively, with 0.78 and 0.09 cm of rainfall 7 DAP. The optimum air temperature for soybean is 29˚C and 1.8 cm of water in the vegetative stage per week (Matchem & Conley, 2022; Moseley, 2022). Temperatures above 29˚C cause soybean plants to suffer heat stress, which will lead to a decrease in photosynthesis, thereby limiting growth and development of the crop (Moseley, 2022). According to a USDA-NASS report for the week in which the soybean was planted, topsoil moisture fell 33% adequate and 53% short, while subsoil moisture supplies were 36% adequate and 54% short and (USDA-NASS, 2022). This is compared to the planting dates of 2021, which had a 65% adequate and a 1% short topsoil moisture supply, a 66% adequate and 1% short subsoil moisture supply, with light to moderate precipitation reported in 2022 throughout Louisiana (USDA-NASS, 2021). When evaluating the temperature with the amount of rainfall and available moisture, it appears that the higher average soil temperatures was the likely culprit of the decrease in soybean germination in 2022.

The findings presented in this paper demonstrate that positively charged zein biopolymer nanoparticles and lignin-PLGA nanoparticles as STs have no obvious deleterious effects on soybeans plants as a seed treatment. Evidence of this is supported by similarities in pods found in the flower termination study among treatments, and the similar heights and development found in nano-ST treatments. Use of nano-STs is in its infancy, and this research shows that their use did not negatively affect plant growth compared to non-treated seeds.

Ristroph et al. (2017) found that when put into a hydroponic solution, zein biopolymer nanoparticles would visibly adhere to the root and marginally translocate to leaf tissue. However, no observations were recorded with regards to plant quality or health following the experiment's 10 days hydroponic exposure to the biopolymer nanoparticles. When evaluating soybean STs of both (+)ZNP and LNP, Kacsó et al. (2022) found that seed germination and plant metrics were not adversely affected by the presence of the particles. The soybean plants in our study were grown to the V4 stage (Fehr & Caviness, 1977) and no tests evaluated yield. The aforementioned studies highlight a deficit in scientific literature that emphasizes plant health following exposure to biopolymer nanoparticles. The objective of this paper was to fill that deficit; our findings demonstrate that both (+)ZNP and LNP did not negatively impact growth of soybean plants under field conditions and that this nano-enabled strategy of agrochemical delivery may be a highly useful tool in future precision of agricultural practices.

AUTHOR CONTRIBUTIONS

C. A. R. Bonser: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing–original draft, writing–review and editing. **J. Borgatta**: Data curation, formal analysis, funding acquisition, investigation, methodology, validation, writing–review and editing. **J. C. White**: Project administration, writing–review and editing. **C. E. Astete**: Data curation, formal analysis, methodology, validation, writing–review and editing. **C. M. Sabliov**: Funding acquisition, writing–review and editing. **J. A. Davis**: Conceptualization, funding acquisition, methodology, project administration, validation, visualization, writing–original draft, writing–review and editing.

ACKNOWLEDGMENTS

This work was supported by the USDA National Institute of Food and Agriculture AFRI project # 2019-67021-29449, the USDA-NIFA Hatch Project #1008750, the USDA-NIFA Hatch Project Accession #1021546, and the Louisiana Soybean and Grain Research and Promotion Board. This article was approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript No. 2022-234-38309.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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How to cite this article: Bonser, C. A. R., Borgatta, J., White, J. C., Astete, C. E., Sabliov, C. M., & Davis, J. A. (2023). Impact of zein and lignin-PLGA biopolymer nanoparticles used as pesticide nanocarriers on soybean growth and yield under field conditions. *Agrosystems, Geosciences & Environment*, *6*, e20350. https://doi.org/10.1002/agg2.20350