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Metalloid and metal oxide nanoparticles suppress Sudden Death Syndrome of soybean

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18 ▪ **ABSTRACT**

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20 Soybean (*Glycine max*) (V3 stage) were sprayed once with nanoparticles (NPs) of AgO, B, CeO,
21 CuO, MnO, MoO_3, SiO, TiO, or ZnO and exposed to *Fusarium virguliforme,* the cause of sudden
22 death syndrome. Up to 80% root rot was observed in greenhouse experiments. However, NP CuO,
23 B, MoO_3, or ZnO reduced root rot severity by 17-25%. Infected roots and shoots had significant
24 changes in B, Mg, P, S, Si, and Zn but NP treatment restored levels to that of healthy control. For
25 example, the increased root Mg and Mn content induced by disease was reversed by NP B and Mn
26 amendment. I*n vitro* assays found the NPs did not inhibit the pathogen. This, along with the
27 restoration of altered nutrient levels in the plant tissue, suggests that modulated plant nutrition
28 increased disease defense. Treatment of seedlings with nanoscale micronutrients may be a new
29 tool in promoting soybean health.

30 Key words: nanoparticles; soybean; *Fusarium virguliforme*; Foliar application, Disease
31 suppression.

32

▪ INTRODUCTION

Current estimates predict that global food production must increase by 60-70% by 2050 to maintain food security.[1] However, the consensus in the literature is that current agricultural practices are unsustainable. For example, year-over-year increases for most crops have decreased over the last 30-40 years; additional challenges posed by a changing climate and decreases in arable soil have further confounded efforts to systematically increase food production.[2] Consequently, dramatic changes are needed as part of an "Agri-Tech" revolution.[1, 3] One area of particular concern in the current inefficiency in agrichemical delivery, with 70-90% of applied fertilizers and pesticides not reaching the intended target.[2] Nanoparticle (NP) forms of metalloid and metallic oxides of essential micronutrients have been shown to have important applications in plant protection and nutrition.[2] More rapid particle dissolution and greater activity of NP forms leads to improved growth and metabolic function.[4-5] The rate at which these elements can activate defensive physiological and biochemical processes can often control the level of host resistance and eventual consequence of disease.[6] Given that the role of micronutrients in plant metabolism and host defense directly affects the production of important secondary metabolites such as phenolics, lignin, quinones, tannins, and flavonoids, as well as membrane and cell wall stability,[6-7] continued efforts to further tune and enhance micronutrient availability and function in nanoscale form are warranted.

Although the role of micronutrients in the suppression of crop disease is well documented,[8] significant obstacles exist in delivering and distributing these elements to the infected tissues. For soil application to be an effective route of micronutrient delivery, the rates must be inordinately high due to element precipitation as insoluble oxides in slightly acid to neutral soils.[9] Conversely, most micronutrients are poorly translocated to the roots following foliar application,[10] which is

particularly problematic for root diseases since nutrition[11] in that tissue is critical to the balance between health and disease. Notably, foliar "feeding" to enhance plant health is an established practice,[12] but incorporating the use of nanoscale forms of micronutrients is a more novel approach.[13] Work from our group has demonstrated that applying NPs of copper oxide (CuO), copper phosphate nanosheets ($Cu_3(PO_4)_2$), and zinc oxides (ZnO) to seedlings grown in fungal-infested media resulted in improved nutrient uptake, translocation, and function when compared to the larger bulk equivalent or salt forms.[14-17] These efforts have focused almost exclusively on vegetable species infected by fungal root pathogens, such as *Fusarium* and *Verticillium*. Alternatively, nonessential elements, such as silver (Ag) and cerium (Ce), have also been shown to enhance plant growth under certain conditions when applied in nanoscale form.[18-21] In a recent review of the limited literature on NPs and plant disease, Ag, CuO, and ZnO were the materials shown to most consistently suppress crop disease;[5] notably, the mode of action for many of these materials is likely different and in some cases, unknown. For example, a 2006 study by Park et al. demonstrated that NP Ag could suppress powdery mildew of pumpkin,[22] likely due to the direct antibacterial activity of the treatment. Similarly, Graham et al. (2016) demonstrated that foliar application of NP ZnO on citrus reduced citrus canker after *Xanthomonas citri* subsp. *citri* was injected into the leaf intercellular space.[23] Notably, the antibacterial activity of zinc directly against the bacteria seems responsible for the reduced disease and this work has led to a commercially available nanoscale Zn formulation (Zinkicide®). Alternatively, work from our group has focused on foliar application of different nanoscale forms of Cu as a means to modulate plant nutrition in the root and stimulate plant defense against disease. Specifically, in both greenhouse and field studies, foliar treatment of vegetable species (eggplant, tomatoes, and watermelon) with Cu NPs in different forms and concentrations was shown to suppress Fusarium and Verticillium wilt to

varying degrees (Borgatta et al. 2018; Elmer and White 2016; Elmer et al. 2018; Ma et al. 2019).[14-17]

Sudden death syndrome (SDS) of soybean (*Glycine max* (L.) Meer) caused by *Fusarium virguliforme* has increased in distribution and economic importance in the Midwestern United States. Since 2014, between $200 to 700 million are estimated to have been lost due to SDS in the United States alone.[24-25] *F. virguliforme* (*Fv*) is the causal agent of SDS in North America, but the species complex differs in the southern hemisphere.[26] Early symptoms include poor root development and root rot that may progress into foliar symptoms later in the life cycle, including interveinal chlorosis and necrosis, defoliation, and early death.[27] Foliar symptoms can be quite variable and often manifest aggressively (i.e., sudden plant death) at anthesis.[28] The management of SDS has been difficult, although some advances have been made.[29] Selecting for host-plant resistance has been successful in identifying some cultivars with modest tolerance, but screening for resistance is difficult because disease onset and expression are strongly dependent on environmental factors.[30] Fungicides as seed treatments have some value,[31-32] although extensive use can lead to negative environmental consequences and potentially residues in the crop. Alternatively, crop rotation can provide suppression in some fields if other crops are available for growth.[29, 33] Cultural management of field parameters, such as improving soil drainage and reducing soil compaction, can reduce the severity of SDS,[29, 34] but no management strategy has consistently suppressed disease across a range of conditions. Additionally, host nutrition is another factor that can affect SDS severity.[35-36] The role of micronutrients in nanoscale metal oxide form as a foliar treatment strategy for soybean diseases has never been evaluated. Given the above-described successes with vegetable species and the strong need for novel management strategies

to achieve sustainable agriculture, investigations with disease systems such as SDS is highly warranted.

In the current study, our objectives were: 1) Determine the appropriate *F. virguliforme* inoculum concentration for three separate soybean cultivars to promote consistent levels of root rot, 2) Determine the *in vitro* antifungal activity of nanoscale B, CuO, Mn_2O_3, and ZnO NPs against *F. virguliforme* and 3) Determine the efficacy of foliar applications of the nanoscale essential micronutrients B, CuO, Mn_2O_3, MoO_3, and ZnO and the nonessential metals Ag, CeO_2, SiO_2 and TiO_2 at suppressing SDS in soybean in a series of asymmetric soil-based greenhouse studies. The measured endpoints included plant growth and root rot severity, as well as the elemental composition of roots and stems.

▪ MATERIALS AND METHODS

Nanoparticles, plants, and inoculum. NPs of Ag (20 nm, 99.99 % pure); B (100 nm, 99.9% pure); CuO (40 nm, 99.00% pure); CeO_2 (25 nm, 99.97% pure); Mn_2O_3 (30 nm, 99.20% pure); MoO_3 (13-80 nm, 99.94% pure); SiO_2 (60-70 nm, 98.00% pure), TiO_2 (rutile, 10-25 nm, 99.50% pure); and ZnO (10-30 nm, 99.00% pure) were obtained from US Research Nanomaterials Inc. (Houston, TX). Bulk oxide equivalents were obtained from Fisher Scientific (New Jersey, USA). Depending on the experiment, suspensions of NPs were prepared at 500 or 1,000 µg/ml distilled water amended with a nonionic surfactant (1 ml/liter) (Regulaid®, Kalo Inc., Overland Park, KS). Suspensions were sonicated for 2 min in a probe sonicator (Fisher Scientific, FB505) at 50% amplitude immediately before application to achieve a stable dispersion. Particle zeta potential and hydrodynamic size were characterized in 500 mg/L solutions (prepared as above) by dynamic light

scattering (DLS) on a zetasizer (Malvern Zetasizer, Nanoseries ZS90). The particles were also characterized by transmission electron microscopy (TEM) (Hitachi HT7800).

Soybean cultivar 'Seedranch' (Seedranch, Odessa, FL) belong to Maturity Group I and its susceptibility to SDS was not known. Cultivar 'Sloan' (provided by Dr. Glen Hartman, University of IL) belongs to maturity groups II and has moderate resistant to some foliar diseases, but is highly susceptible to SDS.[63] Culitvar 'Spencer' (provided by Dr. Martin Chilvers, Michigan State University) belong to Maturity group IV and is highly susceptible to SDS.[64] Seeds were germinated in 36-celled (5.66 × 4.93 × 5.66 cm) plastic liners (1 plant/cell) filled with soilless potting mix (ProMix BX, Premier Hort Tech, Quakertown, PA, USA). The potting mix characteristics were as follows: pH = 6.5, NO_3-N = 3 μg/g, NH_4-N = 12 μg/g, P = 100 μg/g, K = 180 μg/g, Ca = 1,66 μg/g, Mg = 125 μg/g, and soluble salts = 0.3 ms/cm as determined by Morgan Test (Lunt et al. 1950). The seedlings were fertilized once after three weeks with 40 ml of Peters' soluble 20-10-20 (N-P-K) fertilizer (R.J. Peters, Inc., Allentown, PA). Greenhouse temperatures averaged 17 to 22 °C night and 19 to 25 °C day. Seedlings in the V3 leaf stage were used to initiate all studies described below.[37]

The pathogen inoculum was prepared on Japanese millet that had been autoclaved with distilled water (1:1 wt/vol) for 1 hour on two consecutive days.[38] The millet was seeded with three agar plugs colonized by *F. virguliforme* (Isolate Mont-1).[39] The culture was allowed to grow for 2 weeks at 22-25 °C and the millet was air-dried, and ground in a coffee mill for 30 sec. The millet inoculum was thoroughly mixed by hand into potting mix (ProMix BX, without mycorrhizae, Premier Hort. Tech, Quakertown, PA, USA). Imidacloprid was applied (0.3 g/pot) once as a granular amendment to suppress fungus gnats.

Cultivar sensitivity to infection. The sensitivity of each of the three soybean cultivars to *F. virguliforme* infection was determined so as to guide design of the nanoscale amendment experiments. Cultivars 'Seedranch', 'Sloan' and 'Spencer' were transplanted at the V3 stage into 1-liter plastic pots filled 0.8 liters of potting mix and were infested with 0, 0.5, 1.0, 2.0 or 3.0 g millet inoculum/liter. The inoculum was enumerated by serially diluting potting mix onto Peptone PCNB agar plates,[40] followed by incubation for 5 days and subsequent pathogen colony counting. The colony forming units (CFU) of *F. virguliforme* /g potting mix was then calculated. There were two soil samples per inoculum concentration and three plates per dilution at 10^{-2} or 10^{-3} ml/g soil (oven dry weight equivalent) were prepared. Replicate seedlings of each cultivar were transplanted into a pot filled with each inoculum density/concentration and were set on greenhouse bench in a 3 (soybean varieties) × 5 (inoculum concentrations) randomized complete block design with six replicates per treatment. Each pot received 50 ml of a complete fertilizer solution (20-20-20, N-P-K) once per month. The experiment was repeated eight months later with three replicates. After 5 weeks of growth, the experiments were terminated and the plants were removed from pots, washed in tap water to remove all potting mix, and weighed. The root systems were visually rated for the percentage root rot as the percent root area with reddish-brown discoloration. The root systems and above ground tissue were weighed separately, dried to a constant weight at 50 °C, and then re-weighed.

NP toxicity against *F. virguliforme*. The *in vitro* toxicity of select nanoscale micronutrients against *F. virguliforme* was determined by a shake culture method. Fifty ml of sterile potato dextrose broth (Difco Laboratories, Livonia, MI) was added to 125-ml Erlenmeyer flasks that were subsequently amended with 0, 100, and 1,000 µg/ml of NP B, CuO, Mn_2O_3, or ZnO.

169 Flasks were seeded with a colonized agar plug of *F. virguliforme* and were set on a platform
170 shaker at 125 rpm for 5 days at 22 °C. Mycelial mats were harvested under vacuum onto pre-
171 weighed Whatman® #1 filter paper that had been dried at 50 °C for 18 hr. The mycelia-
172 containing filter papers were re-dried at 50 °C for at least 18 hours and weighed again. The dried
173 mycelial mass was calculated after subtracting the weight of NP treatment that was added to the
174 flask. There were three replicate flasks per NP type and concentration. The experiment was
175 repeated to confirm the findings.
176
177 **Greenhouse experiments.** With the above information on cultivar-specific pathogen inoculum
178 size, a series of asymmetric greenhouse experiments to investigate the effect of foliar applications
179 of nanoscale forms of essential (B, CuO, Mn_2O_3, MoO_3, and ZnO) and non-essential (Ag, CeO_2,
180 SiO_2, TiO_2) metal/metal oxides at 500 or 1000 μg/ml distilled water. Two separate experiments
181 were conducted with the soybean cultivar 'Sloan'. In the first experiment, the effect of foliarly
182 applied NPs B, CuO, Mn_2O_3, and ZnO (each at 500 or 1000 μg/ml) was investigated. Rates were
183 based on past studies where positive growth benefits were observed at these rates. [14-17]
184 Polyvinylidene chloride film (Saran™ wrap) was securely fitted around the stem of each plant to
185 cover the soil and prevent NP contamination of the growth media. Seedlings were sprayed using
186 plastic spray atomizers until the leaves were visibly wet (1-2 ml/plant; 0.5-1.0 mg NP/plant); the
187 plants were allowed to dry and the film was removed. Control plants were sprayed with sonicated
188 distilled water. The seedlings of each treatment were immediately transplanted into non-infested
189 potting mix or to potting mix infested with 2 g/liter of millet inoculum. After transplanting, the
190 plants were individually irrigated to avoid wetting the leaves. For this and other greenhouse
191 experiments (unless otherwise noted), temperatures averaged 17 to 22 °C night and 19 to 25 °C

day. Three days after transplanting, one half of the plants in each treatment/infestation received 100 ml of either a high fertilization regime of 100 µg N/ml (as NH_4NO_3) or low fertilization of 50 µg N/ml regime. Each pot subsequently received 50 ml of a complete fertilizer solution (20–20–20 N-P-K) every 2 wks. The experiment was arranged on a greenhouse bench as a randomized block design with five NPs treatments (untreated control, B, CuO, Mn_2O_3, ZnO) x two inoculum levels (infested with *F. virguliforme* or not infested) x two fertilization regimes (high or low). Imidacloprid was applied (0.3 g/pot) once as a granular amendment to suppress fungus gnats. After 5 weeks, plants were harvested and fresh and dry weights were measured as described above. The roots were washed free of potting mix, weighed, and the percent root rot was visually determined. Samples of the feeder roots were surface-disinfested in 4% household bleach for 4 min, rinsed in distilled water, and placed on two petri dishes containing Peptone PCNB medium. Dishes were placed over a 13 mm grid and the total length of the root pieces were estimated by the line intercept method.[41] After 5-7 days, *Fusarium* colonies were counted and expressed as colonies per cm root. Dried root and above ground tissues were analyzed for elemental composition described below. The data collected included plant wet and dry mass, SDS severity (percent root rot), and elemental composition of roots and above ground tissues (described below).

A second experiment with 'Sloan' was established that excluded NP B, included six replicates per treatment (instead of three), and directly compared the efficacy of 500 µg/ml CuO, Mn_2O_3, and ZnO NPs to their larger bulk equivalents on SDS disease progression. The particles were prepared and applied as above; the replicate seedlings were arranged on a greenhouse bench as a randomized block design with three NPs (CuO, Mn_2O_3, ZnO) x two metal forms (NP versus bulk forms) x two inoculum levels (infested with *F. virguliforme* or not infested). Untreated infested and non-infested plants were included as controls. Plants were grown as described above and the

experiment was terminated after 5 weeks. At harvest, plant mass, SDS severity, and elemental composition were determined.

With the soybean cultivar 'Spencer,' the impact of foliar application of 500 µg/ml NPs B, CuO, Mn_2O_3, MoO_3, or ZnO, as well as the nonessential elements Ag, CeO_2, TiO_2, or SiO_2, on soybean growth, SDS, and elemental composition of stems and roots was evaluated. These non-essential elements were chosen because of known antimicrobial, antioxidative, photoactive, or secondary metabolic activity. The experimental design was similar to above; there were six replicates per treatment that were arranged on greenhouse benches as a 10 NP (Untreated control, Ag, B, CeO_2, CuO, Mn_2O_3, MoO_3, SiO_2, TiO_2, and ZnO) × 2 inoculum levels (infested with *F. virguliforme* or not infested) factorial randomized complete block design. Based on the pathogen inoculation data, we used 3 g of millet/liter inoculum as 'Spencer' is significantly more tolerant to SDS. Growth conditions in the greenhouse were warmer than other experiments (20-24 °C night, 26 to 30 °C day). After 5 weeks, plants were harvested and biomass, disease severity in the roots and tissue elemental content was determined.

With the soybean cultivar 'Seedranch,' the impact of foliar application of 500 µg/ml NPs B, CuO, or ZnO was directly compared to similar treatment with the nonessential elements Ag and CeO_2. However, here the particle amendment was not by foliar spray application; instead, the plants were exposed to the NP treatment by inverting the seedling and then immersing the leaves into the NP suspensions for 3-5 seconds, followed by hanging the seedlings upside down until dry. This application technique likely provides more complete foliar coverage than spraying the shoots. Plants were then transplanted into infested or non-infested potting mix as described above. In this trial, there were nine replicates and plants were arranged on greenhouse benches as a six NP

(Untreated control, Ag, B, CeO_2, CuO, and ZnO) x two inoculum levels (infested with *F. virguliforme* or not infested) in a factorial randomized complete block design.

Elemental analysis. Root and foliar tissues from the greenhouse experiments were analyzed for the elemental composition. Tissues were dried in an oven at 50 °C, ground in a Wiley mill, and passed through a 1 mm sieve. Acid digestion of ground samples (0.5 g) was done in 50 ml polypropylene digestion tubes with 5 ml of concentrated nitric acid at 115 °C for 45 min using a hot block (DigiPREP System; SCP Science, Champlain, NY). The elements Ag, B, Ca, Ce, Cu, Fe, K, Mg, Mn, Mo, P, S, Si, Ti and Zn were quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES) on an iCAP 6500 (Thermo Fisher Scientific, Waltham, MA). Elemental content was expressed as µg/g (dry plant weight). In the 'Spencer' trial, tissue from replicates 1 and 2, 3 and 4, and 5 and 6 were composited, yielding three replicates per treatment. In the 'Seedranch' trial, tissue from replicates 1, 2 and 3; 4, 5, and 6; and 7, 8, and 9 were composited, yielding three replicates per treatment. Tissue from the other studies were not composited.

Statistical analyses. Data sets of biomass and elemental composition were subjected to Shapiro-Wilk's Test for equality of variance. Normally distributed data with equal variance were analyzed for treatment effects (NPs and *F. virguliforme* infestation) using ANOVA for a mixed model factorial blocked design. Treatment effects (NP and Fusarium inoculation) were tested as fixed variables with block and replication as random effects. Means separated using Tukey's Honestly Significant Difference Test at $P < 0.05$. Disease severity values (percent root rot) were analyzed non-parametrically using Wilcoxon Signed Fisher's Test at ($P = 0.05$) (Conover and Iman 1981).

Regression analysis was used to analyze the inoculum concentration on SDS and recovery of *F. virguliforme* from potting mix. All statistical analyses were performed using SYSTAT V.10 (Cranes Software International Limited, Bangalore, Karnataka, INDIA)

▪ RESULTS

Particle characterization. The hydrodynamic size and zeta potential of the particles used in the various experiments are shown in Table S1. Not surprisingly, significant particle aggregation occurred in solution prior to DLS analysis; particle sizes ranged from 96.8 nm (TiO_2) to 1449 nm (MoO_3). The zeta potentials of all particles were negative, ranging from -5.82 mV (CuO) to -59.5 (Mn_2O_3). Images from TEM analysis can also be found in the SI.

Cultivar sensitivity to infection. An experiment was conducted to determine the appropriate pathogen inoculum for each of the soybean cultivars; the millet inoculum was added to potting mix at 0, 0.5, 1.0, 2.0 and 3.0 g of per liter and disease severity was monitored. A curvilinear increase was evident in the recovered CFU of *F. virguliforme* per g of oven dried potting mix (Figure 1 lower panel). The data were best fit by the polynomial equation $CFU = 184.8x^2 – 155.5x$ ($R^2 = 0.98$, $P = 0.001$) where Y = CFU and X equal millet inoculum/g mix (dry weight equivalent). Disease severity as assessed by root rot ratings for soybean cultivars ‘Seedranch’ and ‘Sloan’ reached 50 to 75 % at 2.0 g inoculum/liter soil. In spite of an increase from 300 to 1,200 CFU/ g potting mix (Figure 1) at 3.0 g *F. virguliforme* inoculum/liter, the disease severity remained constant. Conversely, for ‘Spencer’ a maximum disease severity of approximately 25% occurred at 1.0 g inoculum/liter soil and root rot was unaffected by higher levels of pathogen inoculum. Thus, it appears that ‘Seedranch’ and ‘Sloan’ are more susceptible to the pathogen than is

283 'Spencer'. Given these findings, we used an inoculum size 2 g per liter soil for 'Seedranch' and
284 'Sloan,' and for 'Spencer,' 3 g was used.
285
286 **NP toxicity against *F. virguliforme*.** An *in vitro* toxicity assay of 0, 100 and 1000 μg/ml NP B,
287 CuO, Mn_2O_3, and ZnO against *F. virguliforme* growth of was conducted in two separate
288 experiments (Figure S1). No interaction was detected between the separate experiments and as
289 such, the data sets were combined. At 100 μg/ml, there was no significant decreases in fungal
290 growth for any of the NPs. At 1000 μg/ml, NPs ZnO were the most toxic to *F. virguliforme*,
291 completely inhibiting fungal growth at the, followed by CuO which caused more than 50%
292 reduction in mycelial biomass. Nanoscale B exerted no fungal toxicity at 1000 μg/ml, whereas NP
293 Mn_2O_3 unexpectedly enhanced *F. virguliforme* growth.
294
295 **Greenhouse experiments.** In the first greenhouse experiment with 'Sloan,' the fresh and dry
296 weight data yielded the same results so only the fresh weight data are presented (Figure 2). Here,
297 inoculation with *F. virguliforme* reduced total plant biomass by approximately 50% in the controls
298 (Figure 2a) and percent root rot was nearly 90%, regardless of N fertilization rate. Significant
299 interactions between the NP treatment and infestation with *F. virguliforme* were detected ($P <$
300 0.001) and were evident by the strong effect of the NP treatment on the mass of non-infested plants.
301 The two main treatment effects were also significant; NP treatment at $P < 0.001$ and infestation
302 with *F. virguliforme* at $P < 0.001$. For healthy plants at the lower N fertilization rate, nanoparticle
303 amendment had no impact on plant biomass, regardless of NP concentration (Figure 2a). However,
304 at the higher N fertilization level (Figure 2b), NP CuO, B, ZnO at 500 mg/L significantly increased
305 plant biomass (55-102%) in the healthy plants; a similar trend was evident at the 1000 mg/L level

306 for these three micronutrients, although the magnitude of increase was less. The trend for Mn_2O_3
307 was also for increased biomass, although neither amendment level resulted in statistically
308 significant increases. Interestingly, in the diseased plants the increases in biomass with NP
309 amendment were no longer evident; all treatments were statistically equivalent to the controls.
310 Given that this pathogen manifests most overtly during plant flowering, this lack of impact on
311 biomass is not surprising; as such, the more sensitive and valuable endpoint will be disease severity
312 in the root system.

313 The root rot severity ratings are shown in Figure 3a; an example of root rot is shown in Figure
314 4. For uninfected soils, plant root rot severity was obviously low and not impacted by treatment;
315 for infected control plants, percentage rot was unaffected by N fertilization rate and ranged from
316 86-89%. At the 500 mg/L NP treatment level, fertilization rate significantly impacted disease
317 progress. Specifically, at low N fertilization, foliar application of nanoscale CuO, ZnO, and Mn_2O_3
318 significantly decreased root rot by 18.9, 24.7 and 17.1%, respectively. However, at the higher N
319 fertilization rate, none of the treatments significantly impacted root disease. At the 1000 mg/L NP
320 treatment level, fertilization rate had no impact on plant response and as such, the low and high N
321 data were pooled for analysis (Figure 3b). Here, foliar amendment with CuO and ZnO significantly
322 reduced diseased roots by 10.7 and 10.0%, respectively. Treatment with NP B or Mn_2O_3 had no
323 impact on disease onset.

324 Cultivar 'Sloan' root and shoot tissues were acid digested and analyzed for a range of elements
325 (Table S2). The main effects of NP treatment, infestation, and tissue type were statistically
326 significant for B and Cu. For the unamended control plants, tissue element content was not
327 significantly impacted by N fertilization rate. At the 500 mg/L NP rate and lower N fertilization
328 rate, the presence of disease significantly increased the root content of K, Mg, P, S, Si, and Zn.

329 Interestingly, at the 1000 mg/L NP rate, although the trends for nutrient increases were still evident,
330 none of these increases were statistically significant, except Mg. Similarly, at the higher N
331 fertilization level, disease significantly increased the root content of K, Mg, Mn, P, S, Si, and Zn
332 at the 500 mg/L NP rate but many of these increases were lost at the higher rate. Specifically, at
333 the 1000 mg/L NP amendment rate, only K, Mg, P and Zn levels were significantly increased in
334 plant roots as a function of disease. The presence of disease in this experiment also impact shoot
335 nutrient content; at the 500 mg/L level with low N fertilization, disease decreased the shoot content
336 of B, Mg, Na, P, S, and increased the content of Si, and Ti. Unlike the roots, effects were similarly
337 robust at the higher 1000 mg/L rate; shoot content was decreased for B, Cu, Mg, P, and Zn and
338 was increased for Si, and Ti. At the higher N fertilization level, disease altered the shoot content
339 of a large number of nutrients in the 500 mg/L NP treatment, including decreased Cu, Fe, Mg, Mn,
340 Na, and Zn, as well as increased K, P, Si and Ti. Interestingly, at the 1000 mg/L NP level, the
341 changes were far more modest, with Si and Ti being significantly increased. In terms of the
342 amended elements in healthy plants, the Cu treatment was the only applied nanoscale nutrient that
343 was present at significantly greater amounts in plant shoots; none of the foliar applied elements
344 were present at significantly greater concentrations in the roots than found unamended controls.
345 For the diseased plants, only B amendment at the 500 mg/L low N fertilization level and Mn_2O_3
346 at the 1000 mg/L high fertilization level resulted in significantly greater shoot B and Mn content,
347 respectively. In the roots of diseased plants, none of the amended elements were present at
348 significantly greater levels in the roots of treated plants.

349 A number of the micronutrient changes induced by disease were reversed or restored to control
350 levels upon nanoscale amendment, although this “return to control” was not observed in all cases.
351 For example, in the roots of the 500 mg/L low N fertilization plants, the increased root content of

Mg induced by disease was restored to control levels by foliar B and Mn_2O_3 amendment. Foliar B also restored root S levels to that of the controls. In the roots of the 1000 mg/L low N treatment, nanoscale B also restored Mg levels that had been elevated by disease. In the roots of 500 mg/L NP low N fertilization treatment, NP ZnO restored K content; NP B, Mn_2O_3, and ZnO restored Mg content; and NP B, CuO, and Mn_2O_3 restored the Mn content. Last, at the 1000 mg/L high N level, the elevated root content of P induced by disease was restored to control levels upon foliar NP B treatment. In the shoots of diseased plants in the 500 mg/L low N treatment, foliar amendment with NP B and Mn increased the shoot B, P, and S to the level of uninfected control plants; similarly, in the 500 mg/L high N treatment, NP B and Mn reduced to shoot P content to that of the controls as well. Last, for the 1000 mg/L low N treatment, NP B increased the shoot B content to control values and both NP B and Mn restored P levels to that of the controls.

For the second 'Sloan' experiment, the B treatment was excluded and replicate numbers for the CuO, Mn_2O_3, and ZnO NP treatments were increased. Similar to the first 'Sloan' experiment, the fresh and dry weight data yielded the same findings and as such, only the fresh weight data are presented. Infestation with *F. virguliforme* was significant (P = 0.001) and reduced the overall average fresh mass by 26%. Unlike the first 'Sloan' experiment, treatment with NP CuO, Mn_2O_3, and ZnO had no impact on the biomass of healthy plants. Similarly, bulk forms of the metal oxides also had no effect on plant biomass in the un-infested controls. For the diseased plants, none of the nanoscale or bulk treatments increased plant mass of the infected plants relative to the diseased controls and percent reductions from the respective amended controls were approximately 20-36%. As noted above, this pathogen typically manifests during the reproductive stage and as such, impacts on biomass under the current experimental design are not necessarily anticipated. The percent root rot values of plants are shown in Figure 5. Disease was not detected in the healthy

plants; however, infestation in the control plants increased root rot severity to nearly 60%. Foliar treatment with bulk forms Mn_2O_3 and CuO had no impact on disease severity; similarly, NP Mn_2O_3 had no impact as well. However, foliar application of bulk and NP ZnO and of NP CuO reduced disease severity by 17, 30, and 28% respectively. The nanoscale-specific nature of the response to these two elements is notable and is discussed below.

With regard to element content, there were interactions between the NP treatments x plant tissue, likely due to the elevated foliar levels verses the roots (Table S3). Significant main effects were also detected for NP treatment for Cu ($P< 0.001$), Mn ($P < 0.001$), and Zn ($P = 0.005$). For the untreated controls, the presence of disease significantly decreased root Na content. In addition, there were trends for decreased Mg, as well as increased Ca, P, and Z, as a function of disease but because of significant replicate variability, these differences were not statistically significant. In the shoots, disease resulted in significantly greater P content, with a statistically insignificant trend for increased Na content. Regardless of disease presence, foliar treatment with NP or bulk CuO, Mn_2O_3, and ZnO resulted in higher above ground tissue concentrations of these respective elements. For a given treatment element, there was no difference in shoot content as a function of particle size. These increased shoot levels did not correspond to increased levels of any of the elements in the roots, regardless of disease. Similarly, for the other measured elements, there were no other changes of significance across the different treatments.

For the experiment with the soybean cultivar 'Spencer', the efficacy of nanoscale micronutrients (B, CuO, ZnO, Mn_2O_3, MoO_3) at suppressing disease was directly compared with non-essential elements of interest to nano-enabled agriculture; NP Ag, SiO_2, TiO_2 and CeO_2. Similar to the two 'Sloan' experiments, fresh and dry biomass data were equivalent and as such, fresh weight results are discussed. The main effects of NP treatment ($P < 0.001$) and infestation with *F. virguliforme*

($P < 0.001$) were significant, but there was no interaction ($P = 0.898$). Infestation with *F. virguliforme* decreased soybean biomass by approximately 8% in the control plants, although the decrease was not statistically significant. With regard to treatments for the healthy plants, foliar amendment with all particles resulted in average biomass values that were greater than the controls, although variability was high and only CeO_2, Si and MoO_3 were of statistical significance at $P < 0.10$. Similarly, for the diseased plants, all treatments yielded trends of greater biomass, although variability was again rather high and none of the increases were of statistical significance. The infested plants had a percent root rot value of 81% (Figure 6); plants receiving foliar treatments of NP CeO_2, Mn_2O_3, MoO_3, B, and SiO_2 had severity ratings that were not significantly different from the diseased controls and ranged from 77-85%. Conversely, NP CuO and ZnO had statistically significant reductions in disease severity by 34 and 18%, respectively. NP Ag and TiO_2 foliar treatment resulted in disease reductions of 13 and 14%, respectively but these values were only different from the diseased controls at $p<0.10$. With regard to element content, the presence of disease did not significantly impact the element content of the shoots or roots in this experiment, although trends for many of the elements were similar to previous experiments (Table S4). Not surprisingly, nearly all elements that were added as part of treatments were indeed detected at significantly greater levels in the shoots than present in the unamended controls; the exceptions were Si and Ti, which were not increased. However, the amounts of these added elements did not differ as a function of disease presence. Interestingly, the concentrations Cu in the roots was significantly greater when plants were foliar treated with nanoscale CuO. Specifically, levels of root Cu in the control and CuO-treated plants were 7.4 and 15.5 mg/kg, respectively ($p<0.05$). There were no other changes of note in the element content of soybean across the various treatments.

421 The design for the soybean cultivar 'Seedranch' was similar to the previous 'Spencer'
422 experiment, although with a decreased number of particles; the efficacy of NP micronutrients
423 (CuO, B, ZnO) at suppressing disease was directly compared with NP Ag and CeO_2. However,
424 instead of a foliar application, the materials were applied as a "dip" treatment. The presence of
425 disease reduced soybean significantly biomass by 31% in the unamended control plants. Foliar
426 application of nanoscale Ag, B, CeO_2, CuO, and ZnO had no impact on the biomass of either
427 healthy or diseased plants. The diseased controls had a percent root rot of 50%; NP amendment
428 with Ag, B, and CuO resulted in root rot values of 45, 38, and 57%, respectively; these values are
429 not significantly different from the diseased controls. NP CeO_2 and ZnO foliar treatment resulted
430 in root rot values of 31 and 36% respectively; by one-way ANOVA with the full data set, these
431 values are not significantly different from the controls. However, a *t*-test with each treatment
432 against the control (an admittedly weaker test) shows that both CeO_2 and ZnO values are
433 significantly reduced from the diseased controls. With regard to element content, the presence of
434 disease significantly altered the root and shoot element content for this cultivar (Table S5).
435 Specifically, infestation with F. *virguliforme* significant decreased both Mg and Mn in the root
436 tissue relative to disease-free controls; there were non-significant trends for decreased S and Zn as
437 well, and for increased Ca content. Similarly, the shoots of infested plants contained significantly
438 lower amounts of Ca and Mn, as well as significantly greater amounts of P and Si, as compared to
439 healthy controls. There was also a trend for reduced Mg in the shoots as a function of disease but
440 significant replicate variability confounded statistical significance. Similar to previous
441 experiments, the concentration of most amended elements was significantly elevated in the shoots
442 of plants receiving those specific treatments; the exception being Zn in the ZnO amended infested
443 plants. However, none of the amended elements were present at significantly increased levels in

444 the roots of treated plants. Interestingly, shoot amendment with nanoscale CeO_2 restored the levels
445 of Mg and Mn in the infected root tissue to that of control levels; similarly, NP CuO shoot
446 amendment restored root Mn levels to the non-disease condition. In addition, NP CeO_2 treatment
447 of the diseased plants restored shoot Mn and Si to non-disease levels; NP Cu had a similar
448 restorative effect for shoot Mn content, as did NP Ag and B for the Si content of diseased shoots.
449 There were no other notable changes in element content of the plants as a function of treatment.
450

451 ▪ **DISCUSSION**

452 Management strategies for SDS have included the use of moderately resistant cultivars,
453 fungicides, and cultural rotation strategies, but none of these approaches have proven to be
454 consistently.[29, 31-34] The current study explored the use of foliar application of metalloid and metal
455 oxide NP to determine efficacy in suppressing SDS. After determining the optimal inoculum size
456 (2-3 g/millet) to produce consistent root rot symptoms across three separate cultivars, we
457 conducted four asymmetric greenhouse experiments that demonstrated the effects of nanoscale
458 micronutrients and nonessential elements on SDS of soybean. Estimates of percent root rot were
459 consistently the most sensitive endpoint to assess the efficacy of NP treatments; biomass was far
460 more variable, both as a function of disease and treatment. As noted earlier, this lack of biomass
461 effects is not entirely surprising given that the pathogen exerts the most severe symptoms at
462 flowering stage, which our experimental design did not allow. Although not all NPs were evaluated
463 in each experiment, we observed disease suppression of root rot across all four experimental trials,
464 although significant cultivar-specific and particle-specific results were observed. Effective
465 nanoscale amendments within specific experiments included NP B, CuO, ZnO, Mn_2O_3, and CeO_2,

with reduced root rot values ranging from 17-36%. Once again, the nano-scale produce was more effective than the large bulk equivalents which aligns with a number of past studies. [14,16,42]

The finding that NP CuO and ZnO suppress SDS follows a number of other reports where foliarly applied NPs of these nutrients suppressed plant disease. NP of CuO suppressed Fusarium and Verticillium diseases in tomato, eggplant, and watermelon (Borgatta et al. 2018; Elmer et al. 2018; Elmer and White 2016; Ma et al. 2019).[15, 17] In most cases, the disease suppression was associated with increased yield. In the current study, root tissue analysis revealed higher levels of Cu in select but not all experiments, which agrees with previous studies (Elmer et al. 2018; Elmer and White 2016; Ma et al. 2019). Variable root element content results may be a function of experimental design and growth dilution, since plants are grown for a number of weeks (5 in the current study) after NP foliar application. Hong et al (2016) noted that CuO NPs applied at lower concentrations (50 – 200 μg/ml) to cucumber (*Cucumis sativus*) plants did not significantly change the amount of Cu in the roots, suggesting a threshold may reached and that lower rates may not be as useful.[42] Alternatively, particle properties may be tuned to yield enhanced transport and activity (Borgatta et al. 2018 ; Ma et al. 2019). Wang et al. (2012) suggested that the shoot-to-root transport of CuO NPs takes place via phloem, although the mechanism is still largely unexplored. There is limited information on the interactions of plants with Cu-based NPs applied foliarly as compared to what has been published focusing on root exposure studies.[43] In roots, the uptake of Cu can occur as intact NPs or as ions released from Cu-based NPs.[44] In Bt-transgenic cotton, NP CuO applied to leaves were accumulated by endocytosis, while the NPs are retained in the cell wall of conventional cotton.[45] In the current study, it is not known whether the NPs are remaining on the leaf surface and slowly dissolving and releasing ions into the leaf through stomatal openings with subsequent ion transport to the roots, or if the NPs themselves are being accumulated and

transferred through the plant. In watermelon, the increased Cu root levels following foliar treatment with NP CuO were associated with strong up-regulation of polyphenol oxidase and PR1 genes in the roots but only when NP CuO and *F. oxysporum* f. sp. *niveum* were both present.[46] Ma et al. (2019) reported similar gene expression changes with Fusarium-infested tomato that had been foliar treated with different forms of nanoscale Cu.[17] Interestingly, in that study nanomaterial morphology (amorphous, nanosheet) and composition (phosphate vs no-phosphate) significantly impacted biomass, disease progress, and the expression of defense-related genes. A similar mechanism may be occurring with soybean. Cu availability was shown to be a strong driver of polyphenol oxidase activity in soybean.[47] Copper serves as a cofactor for plastocyanins, peroxidases, and multi-Cu oxidases (Evans et al. 2007); all of which serve as key components of host defense. Interestingly, it appears that nanoscale Cu-induced defense reactions are somewhat non-specific with regard to pathogen and may serve as a highly useful management option in a range of disease systems.[5] More complex and tunable Cu composites may further enhance defense reaction by allowing targeted release of Cu ions.[48]

Zn nutrition has long been associated with disease suppression and the nutrient functions as a cofactor in superoxide dismutase (SOD) enzymes that quench free radicals.[49] Delivering Zn to plant in the nanoscale form has been shown to enhance host resistance in citrus, rose, and sugarbeets,[23, 50-51] but information on its uptake or accumulation in nanoscale versus ionic form is generally lacking. We also observed that NP ZnO had a significant positive effect on soybean resistance to SDS, significantly reducing root rot in two of the three cultivars. Similar to NP CuO, particle aggregation likely occurred in spite of probe sonication of the suspension for 2 min before application; this aggregation is known to reduce NP dissolution and highlights the need to tune particle properties such as charge and morphology through the use of coatings or by specific

formulation components. For example, researchers in Florida have begun to address this obstacle by formulating ZnO with various coatings.[23]

There is a history of Mn nutrition being associated with suppression disease.[52] Mn is an activator of Phenylalanine ammonia lyase and phenol synthesis.[52] The association between Mn and root health has been demonstrated in asparagus, beets, eggplant, strawberries, and wheat.[8, 52] However, the potential benefits of nanoscale Mn to suppress SDS are unclear based on the current findings; there was only significant disease reduction with one cultivar (Spencer). Notably, *F. virguliforme* growth was stimulated *in vitro* by NP Mn_2O_3 suggest, raising concerns over the potential of NP Mn_2O_3 for SDS management.

The role of B in crop disease was reviewed by Stangoulis and Graham (2007);[53] the authors noted that in 20 reports where B was studied, 18 (90%) were associated with disease suppression. In those reports, disease was incited by foliar and root infecting fungi, bacteria, and viruses, suggesting B nutrition may mediate a wide array of defense mechanisms. Bellaloui et al. (2012) subsequently reported that soybean plants with enhanced B nutrition were more tolerant to the charcoal rot disease caused by *Macrophomina phaseolina* and had higher levels of phenolics, seed coat lignin, isoflavones, and sugars.[54] As such, although our work appears to be one of the few studies looking specifically at nanoscale B, the finding that foliar NP application of this nutrient suppressed SDS is not entirely surprising. In addition, an earlier study from our group examined the effect of NP B on watermelon in a field trial in B-deficient soil to determine effects on growth, yield, and Fusarium wilt disease progress.[46] When compared to untreated controls, a reduction in disease rankings was observed, although no effect on yield was detected. Given that soybeans are responsive to B application even in the absence of disease, NPs of B show promise as nanoenabled fertilizer to promote crop health.[55]

Nanoscale Ag was investigated in experiments involved two cultivars (Spencer, Seedranch) and in both cases, there was a trend for reduced root rot, although values were only statistically significant at $p<10$. The antimicrobial properties of NPs Ag are well known,[56] and it was among the first NP to be used for plant disease.[22] There are a number of reports using NP Ag as part of various platforms. For example, Ocsoy et al. (2013) used NP Ag to functionalize graphene oxide, which was then foliar sprayed to suppress *Xanthomonas perforans* on tomatoes.[57] In the current report, the mechanism of potential disease suppression with NP Ag is not known. Direct toxicity to the pathogen seems unlikely given the temporal and spatial separation of the particle and pathogen. However, low levels of Ag were detected in the roots of one foliar-treated soybean cultivar, making it impossible to rule out direct effects. Alternatively, there could be an induced resistance stimulated by Ag amendment, although the physiological basis for this effect is not known. Specific root physiological and transcriptomic analyses are needed to determine if NP Ag can induce host resistance.

Ce is a nonessential element that has recently received attention as health promoting element in plants when applied in bulk and nanoscale form.[21, 58] The current study shows that NP CeO_2 decreased root rot severity in one of two trials. Adisa et al. (2018) demonstrated that NP CeO_2 was suppressive to Fusarium wilt of tomato, and also increased the content of chlorophyll, lycopene, catalase, peroxidase, polyphenol oxidase, fruit production, and total biomass when compared to untreated plants or to those amended with Ce acetate.[21] Although the mechanisms of disease suppressive effects are unclear, nanoscale Ce is known to quench ROS in plants.[59] It is unclear if NP CeO_2 will have a role in disease management platforms, but additional research is certainly warranted.

In the *in vitro* assay, we observed that the dried mycelial biomass of *F. virguliforme* was relatively unaffected by 100 and 1,000 µg/ml NP B, slightly inhibited by NP CuO at the high dose, and was actually stimulated by NP Mn_2O_3. The greatest inhibition was observed with NP ZnO at the highest level where no fungal growth occurred. Others have also found that NP ZnO were inhibitory to *F. graminearum*,[60] as well as to *Botrytis cinerea* and *Penicillium expansum*.[61] Elmer and White (2016) incorporated NP CuO, Mn_2O_3 or ZnO into 25% potato dextrose agar and also found that only Zn was inhibitory to the radial expansion of *F. oxysporum* f. sp. *lycopersici*. In both that study and the current one, NP Mn_2O_3 were stimulatory to *Fusarium* species.[14] Given that NP CuO were generally non-toxic up to 1,000 µg/ml in the *in vitro* assay, it is unlikely there was a direct fungicidal effect on the pathogen in the plant-based assay. Indirect positive effects through increased host defense is a much more likely mechanism of action.

Fertilization and plant nutrition are often overlooked as components of disease suppression.[8] This oversight may result from reports where the addition of micronutrients in the absence of disease do not increase yield.[62] As a result, the use of nutrition to influence plant disease is significantly underutilized as a disease management strategy. Considering the findings herein and the supportive existing literature, the positive effects of nanoscale micronutrients/elements B, CuO, CeO_2, and ZnO hold promise in the suppression of soybean SDS; NP Ag, Mn_2O_3, MoO_3 need more study, but may also have potential. Ongoing studies are currently exploring more tunable forms and shapes of many different nano-elements. Field studies are planned to determine the role of these NPs on enhancing yield and grain quality. Although formulation and delivery of NPs will require considerable interest from the chemical industry prior to wide-scale acceptance by soybean growers, it has become increasingly clear that the role of NPs in plant health has great potential as a new tool for growers as foliarly applied nanofertilizers.[2] We

recognize that potential environmental risks need to be recognized and addressed, but the low dose applications of required or non-essential nutrients in nanoscale form to young seedlings could offer significant benefit with much lower environmental and economic impact.[5]

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▪ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: …

▪ AUTHOR INFORMATION

The authors declare no competing financial interest.

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771 Figure 1.

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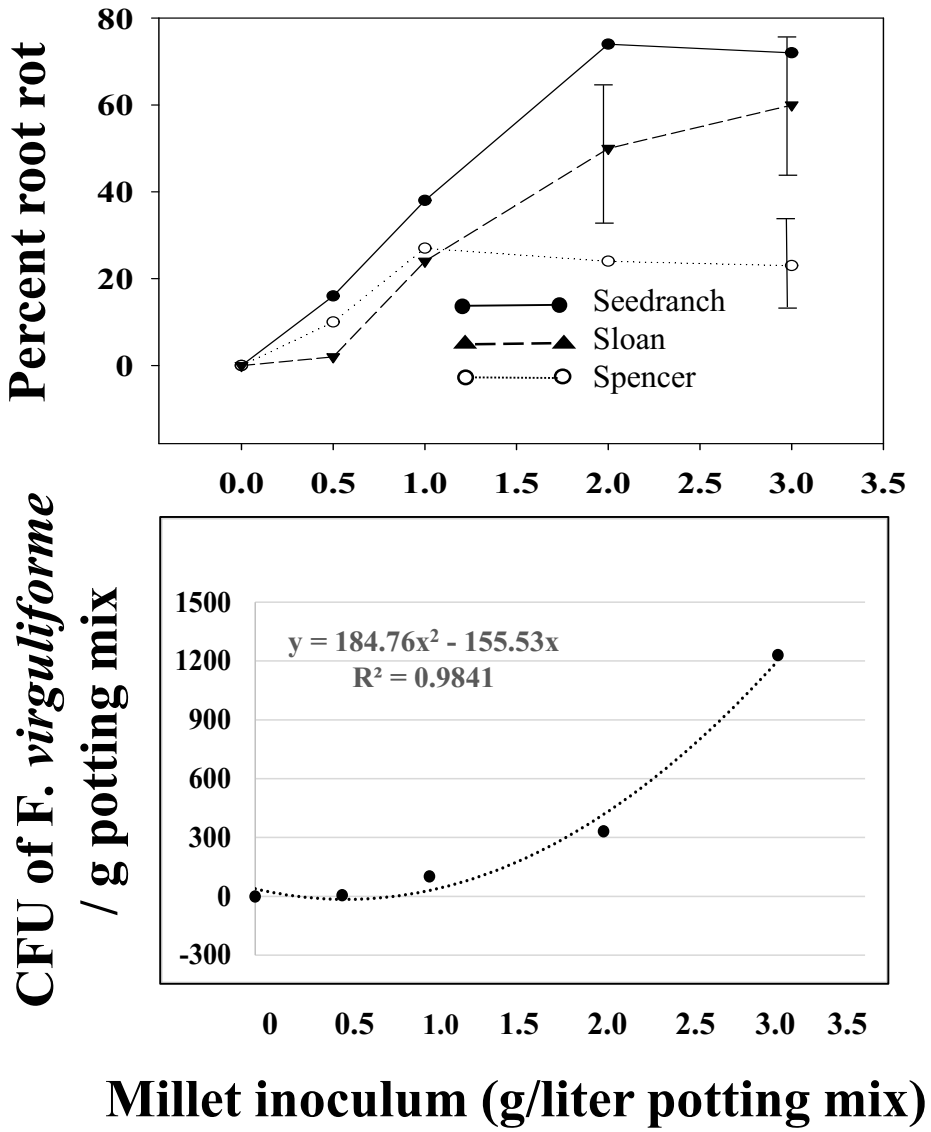

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774 Figure 1. (Upper panel) The effect of increasing rates of millet inoculum of *Fusarium*

775 *virguliforme* on the percent root of three soybean cultivars, Seedranch, Sloan, and Spencer;

776 values represent the mean of six replicates error bars represent the standard error of the mean;

777 (Lower panel) the effect of increasing rates of millet inoculum of the recovery *Fusarium*
778 *virguliforme* following serial dilutions on agar; values represent the mean of four replicates.
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Figure 2A. Wet biomass of healthy soybean grown in un-infested media for 5 weeks at low N fertlization (100 ml of 50 mg N/ml). The disease control is included for comparison. Select seedlings were foliar treated with 1-2 ml of 500 (solid bars) or 1000 (hatched bars) mg/L NP CuO, ZnO, MnO, or B prior to transplanting. Bars with different letters are significantly different (one way ANOVA with Student Newman Keuls MCT).

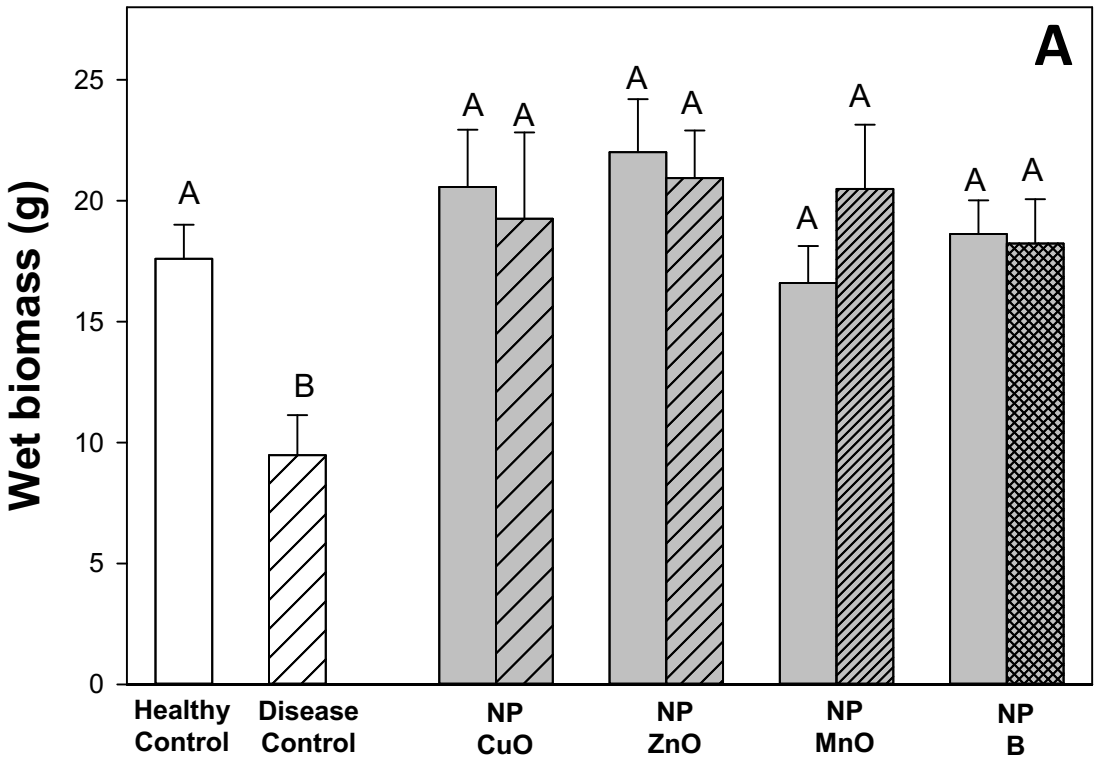

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Figure 2B. Wet biomass of healthy soybean grown in un-infested media for 5 weeks at high N fertlization (100 ml of 100 mg N/ml). The disease control is included for comparison. Select seedlings were foliar treated with 1-2 ml of 500 (solid bars) or 1000 (hatched bars) mg/L NP CuO, ZnO, MnO, or B prior to transplanting. Bars with different letters are significantly different (one way ANOVA with Student Newman Keuls MCT). * indicates statistical significance at $p<0.10$.

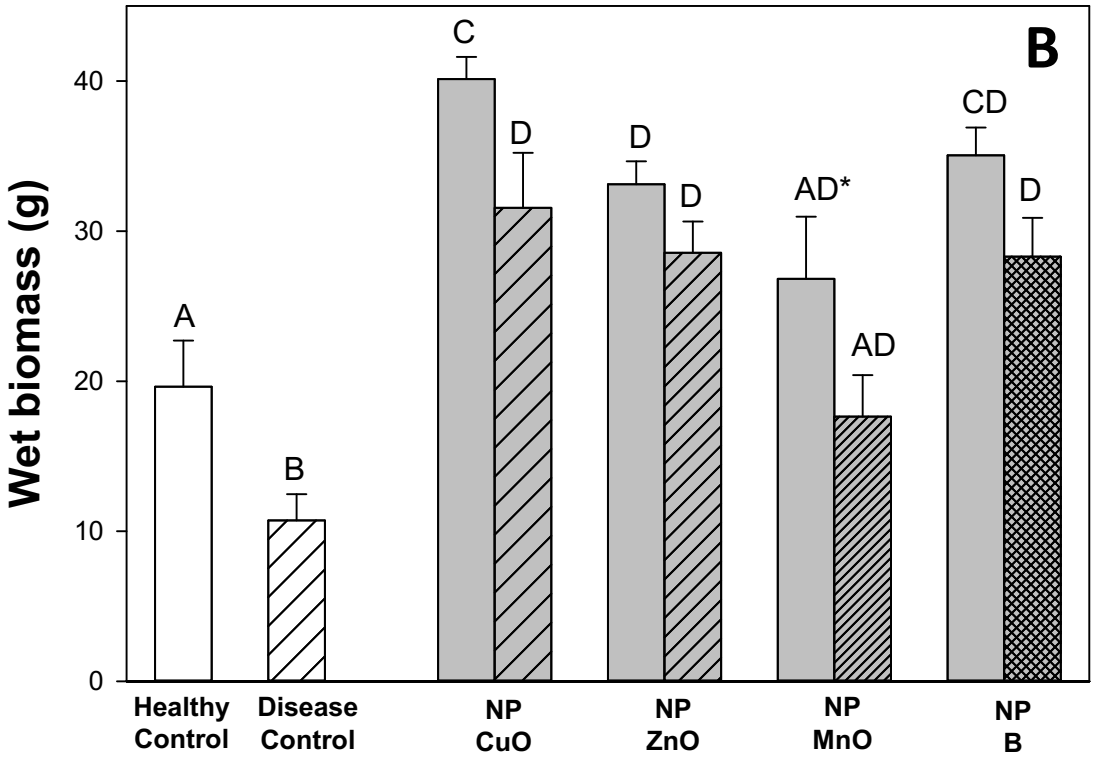

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Figure 3A. Percent root rot in soybean grown in media infested with *Fusarium virguliforme* for 5 weeks under low N fertilization (100 ml of 50 mg N/ml). Select seedlings were foliar treated with 1-2 mL of 500 mg/L NP CuO, ZnO, MnO, or B prior to transplaning into infested media. Bars with different letters are significantly different (one way ANOVA with Student Newman Keuls MCT)

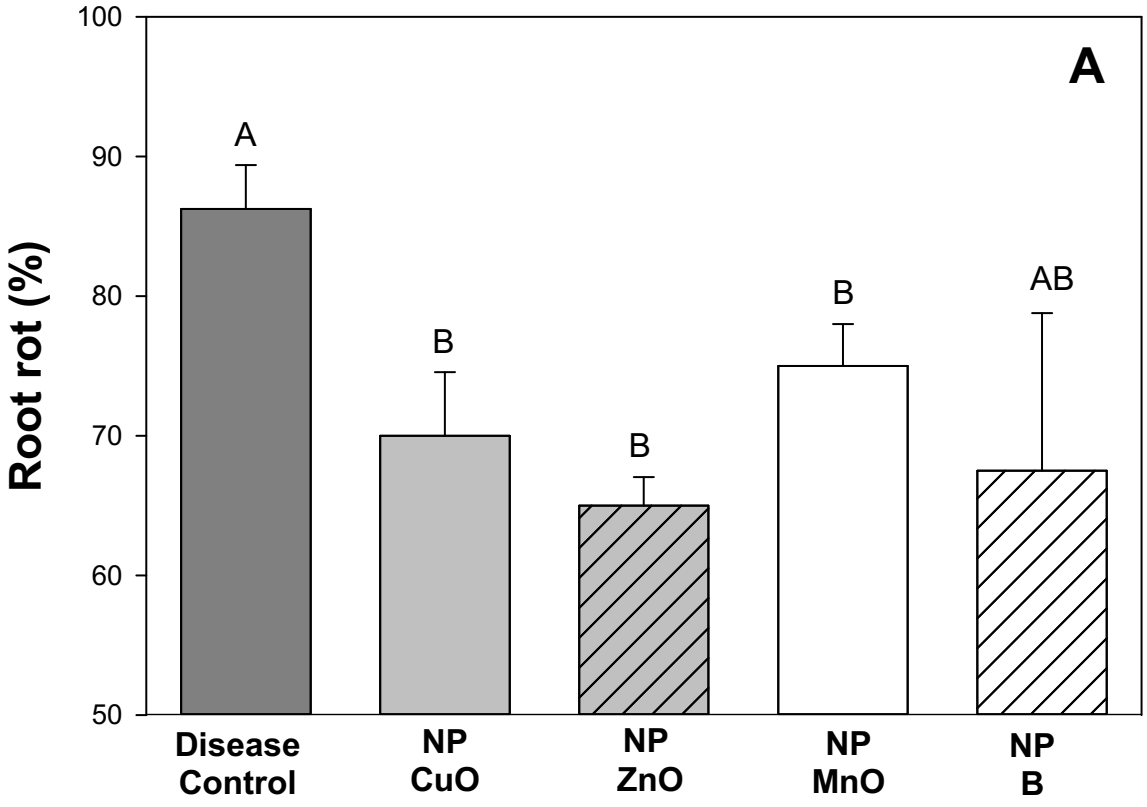

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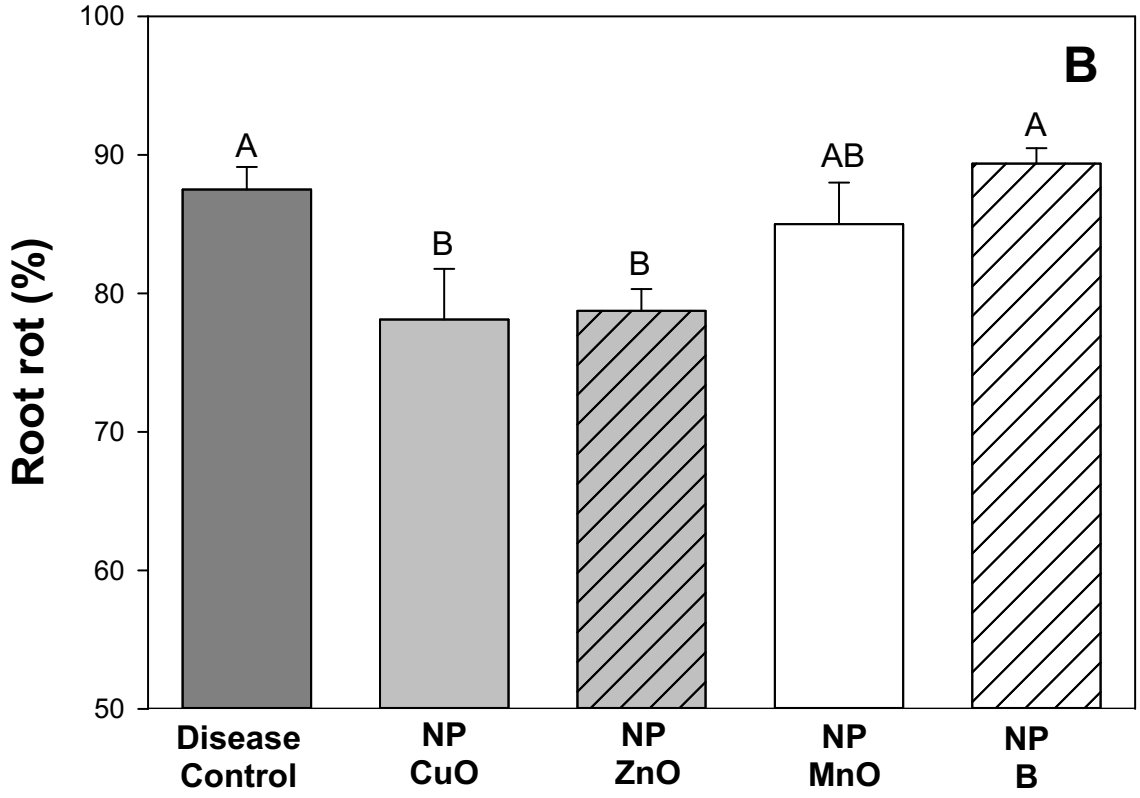
Figure 3B. Percent root rot in soybean grown in media infested with *Fusarium virguliforme* for 5 weeks. Select seedlings were foliar treated with 1-2 mL of 1000 mg/L NP CuO, ZnO, MnO, or B prior to transplaning into infested media. Percent root rot was unaffected by N fertilization rate; as such, data for low and high fertilization rates were pooled.Bars with different letters are significantly different (one way ANOVA with Student Newman Keuls MCT)



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796 Figure 4. Images of healthy (left) and Fusarium-infected (right) soybean roots. Dark coloration
797 or root rot is evident.

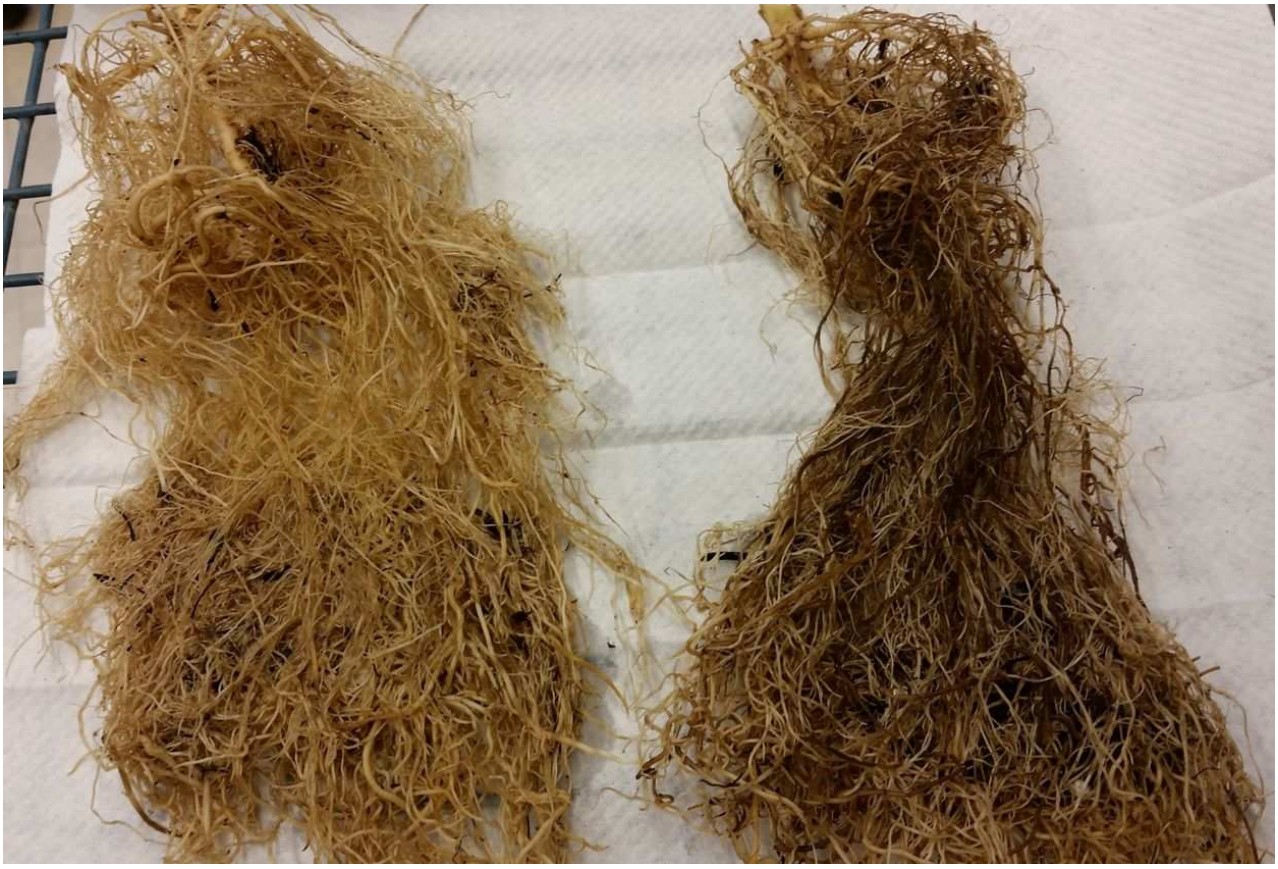

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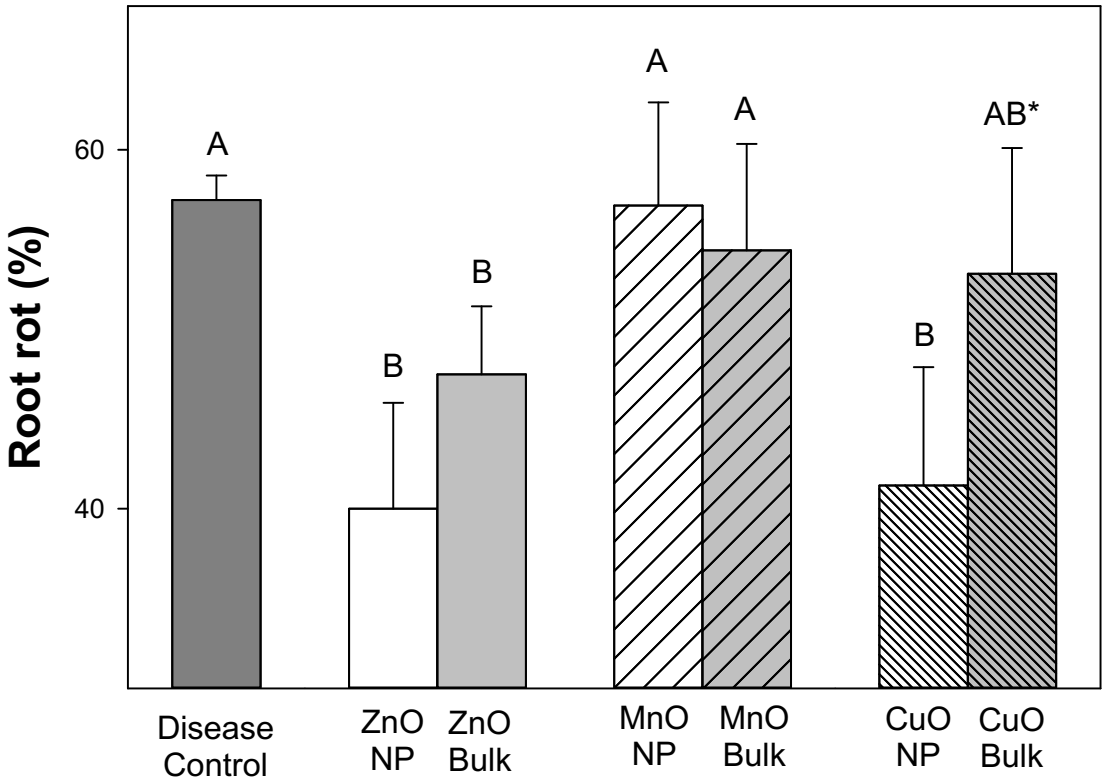
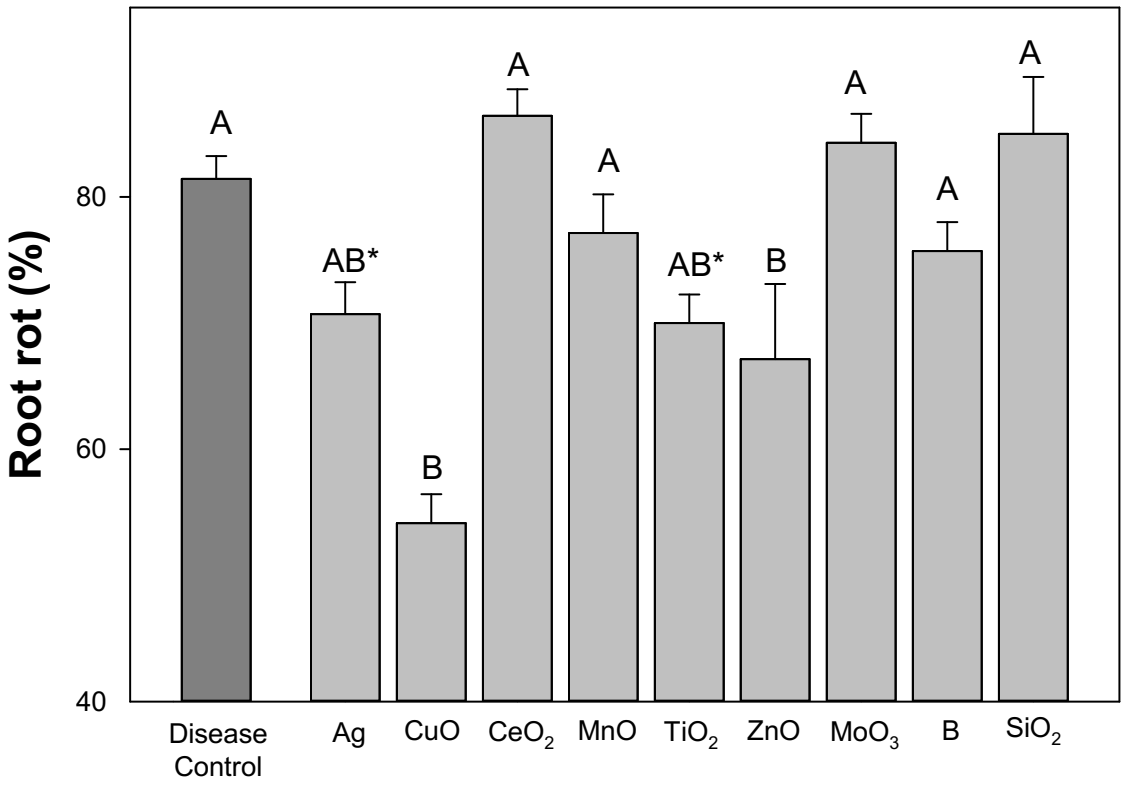
Figure 5. Percent rot root in soybean (Sloan) grown in infested media with *Fusarium virguliforme* for 5 weeks. Select seedlings were foliar treated with 1-2 ml of either NP or bulk ZnO, Mn_2O_3 or CuO prior to transplanting into infested media. The statistical anlaysis was one way ANOVA was done on controls, bulk, and NP form of an element. Bars with different letters are significantly different within an element type ($p<0.05$). * indicates significant difference at $p<0.10$



811 Figure 6. Percent rot root in soybean (Spencer) grown in infested media with *Fusarium*
812 *virguliforme* for 5 weeks. Select seedlings were foliar treated with 1-2 ml of 500 ug/ml NP Ag,
813 CuO, CeO_2, Mn_2O_3, TiO_2, ZnO, MoO_3, B, or Si prior to transplanting into infested media. Bars
814 with different letters are significantly different (One way ANOVA with SNK MCT $p<0.05$). *
815 indicates significant difference at $p<0.10$



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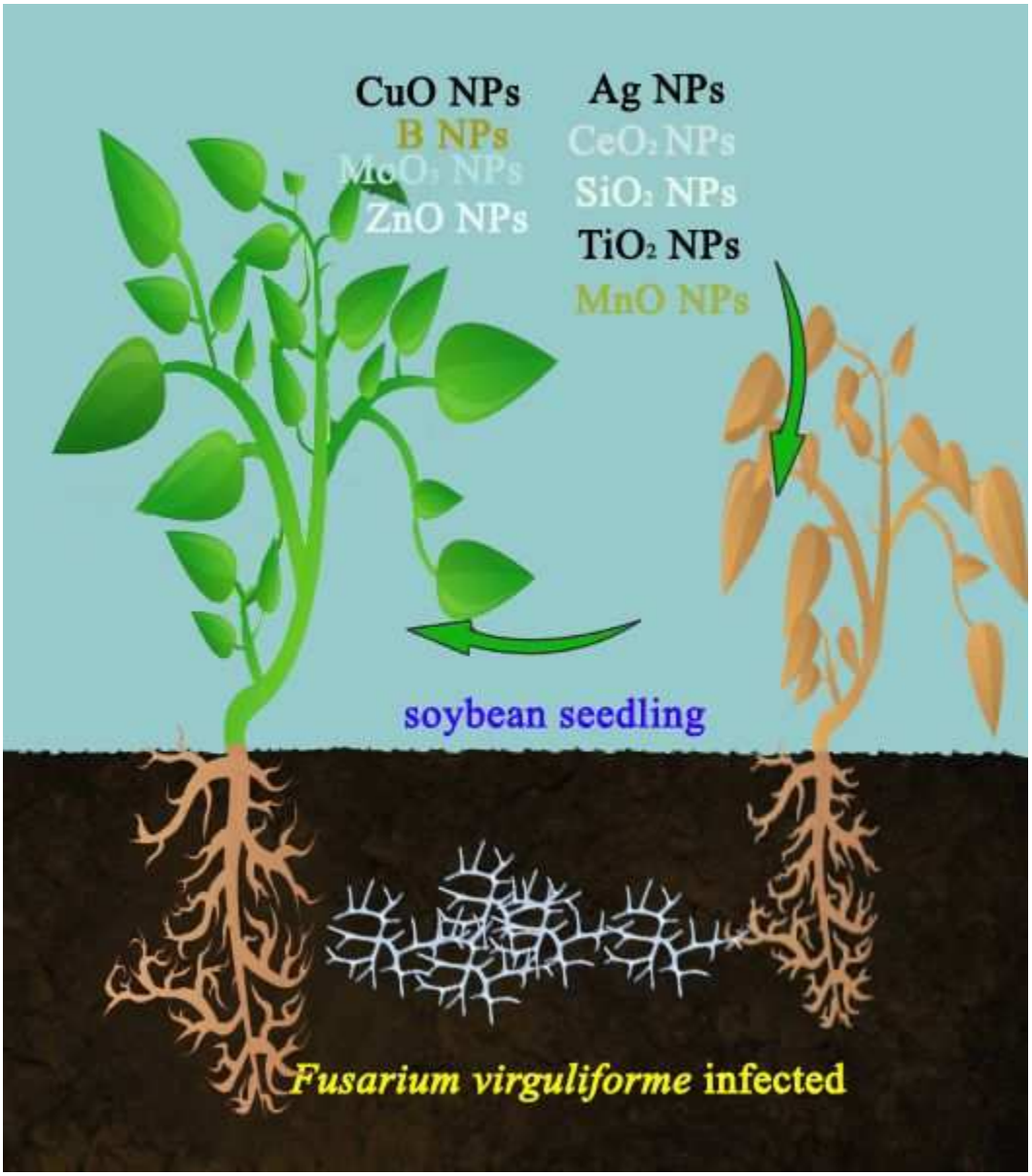

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