Effect of metalloid and metal oxide nanoparticles on Fusarium wilt of watermelon.

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Abstract This study explored the use of foliar sprays with nanoparticles (NP) of B, CuO, MnO, SiO, TiO, and ZnO to protect watermelon against Fusarium wilt. Leaves of young watermelon plants were sprayed (1 to 2 ml per plant) with NP suspensions (500 to 1,000 µg/ml) and were planted in potting mix infested with *Fusarium oxysporum* f. sp. *niveum*. In five out of eight greenhouses experiments, CuO NPs suppressed disease, and in six out of eight experiments, CuO NPs increased biomass or yield more than in untreated controls or other tested NPs. More root Cu was detected in CuO NP-treated plants than other treatments ($P = 0.015$). In Griswold, Connecticut (CT), plants treated with CuO NPs yielded 39% more fruit than untreated controls. In Hamden, CT, treatment with CuO NPs produced 53% more fruit when compared to controls ($P = 0.02$) and was superior to other Cu fungicides. Gene expression in watermelon roots revealed strong up-regulation of polyphenol oxidase (PPO) and PR1 genes when CuO NPs

and *F. oxysporum* f. sp. *niveum* were both present. Enzymatic assays for PPO supported the gene expression results. CuO NPs may serve as a highly effective delivery agent for this micronutrient to suppress disease.

Introduction

Engineered nanoparticles (NPs) (<100 nm) of metalloid and metal oxides have great potential in agriculture as a means to deliver micronutrients to plants (Khan and Rizvi 2014; Servin et al, 2015). It is well documented that the micronutrients B, Cu, Mn, and Zn can play pivotal roles in plant health by activating important enzyme systems such as those of phenol metabolism (Römheld and Marschner 1991). Since micronutrients, in general, have poor basipetal and intraplant mobility (Bukovac and Wittwer 1957) and are less available in neutral soils (Leeper 1952), compromised nutrition of the plant roots often results in increased susceptibility to wilts and root rots.

Past research from our groups demonstrated that CuO NPs and other metal oxide micronutrients possess the potential to fertilize roots when applied foliarly, presumably due to nano-enabled basipetal translocation. Both Wang et al. (2013) and Elmer and White (2016) provided evidence in support of basipetal translocation of CuO NPs in eggplant, maize, and tomatoes. Using maize grown in split root pots, Wang et al (2013) detected increased Cu in unexposed roots following application of CuO NPs to one side. Tomato and eggplant roots had more Cu when the leaves were exposed to CuO NPs and had less disease when these plants were grown in soil with *Fusarium oxysporum* f. sp. *lycopersici* or *Verticillium dahliae,* respectively. The role of NP in delivering nutrition for disease management is otherwise largely unexplored.

The underlying mechanisms of CuO NPs on plants are not clear. The antifungal and anti-oomycete activities of CuO NPs is known (Kanhed et al. 2014; Zabrieski et al. 2015), and recent discoveries with new engineered composites of Cu NP have been shown to be effective against Cu-tolerant bacterial pathogens of tomatoes (Strayer-Scherer et al. 2017). However, the role of the nutritional effects of CuO NPs on root disease suppression is still not clear (Servin et al.

2015). Copper is a cofactor for three important proteins: plastocyanins, peroxidases, and multi-Cu oxidases (Evans et al. 2007), many of which serve in the creation of host defense barriers (Chmielowsk et al. 2010). One major group of enzymes are polyphenol oxidases (PPO) that show increased activity in the presence of Cu ions when attacked by pathogens (Evans et al. 2007; Mayer and Harel 1979). It is not known how CuO NPs might influence the activity of these enzymes.

Fusarium wilt of watermelon (*Citrullus lanatus* var. *lanatus* (Thunb.) (Matsum. & Nakai), caused by *Fusarium oxysporum* f. sp. *niveum,(F. o. niveum),* appears wherever watermelon is grown, including North America, Asia, Australia, Europe, and the Middle East (Everts and Himmelstein 2015). The incidence and severity of Fusarium wilt on watermelon has increased due to several factors. The loss of methyl bromide application (due to concerns over ecotoxicity), shorter rotations, and the advent of highly susceptible triploid seedless watermelons have resulted in widespread outbreaks in many areas where the disease had previously not been a problem (Egel and Hoke 2010; Everts and Himmelstein 2015; Wu et al. 2013). Recent improvements in grafting, cover cropping, and biological control have lessened damage from Fusarium wilt to some extent, but additional management strategies are still needed (Everts and Himmelstein 2015; Ren et al. 2008)).

The effect of metalloid or metal oxide NPs on Fusarium wilt of watermelon are unknown. In this work, our objectives were first to examine the effect of several NP metalloid (B) and metallic oxides (Cu, Mn, Si, Ti, and Zn) on plant growth and elemental root composition of watermelon plants, to evaluate the ability of the NP's to suppress Fusarium wilt and affect plant growth and yield. Secondly, we examined the effect of increasing rates of CuO NPs on plant growth and Fusarium wilt. Our third objective was to compare CuO NPs with two commercial

forms of Cu fungicides for effect on yield and disease control. Our fourth objective was to explore the effect of CuO NPs on the expression and activity of PPO using transcriptomics and enzymatic assays.

Material and Methods

Greenhouse Experiments.

The first two greenhouse experiments were conducted to address Objective 1 and screen several NP for their ability to affect plant growth and Fusarium wilt. For greenhouse experiment 1, foliar sprays of NP suspensions of CuO (30 nm), MnO (40 nm), SiO (20-30 nm), TiO_2 (30 nm, rutile) and ZnO (10–30 nm) were applied to watermelon and compared to untreated watermelon plants for effects on biomass and Fusarium wilt. Watermelon seeds (cv. Sugar Baby, Harris Seed Co., Rochester, NY) were germinated on March 19th in 36 cell (5.66 x 4.93 x 5.66 cm) plastic liners filled with soilless potting mix (ProMix BX, Premier Hort Tech, Quakertown, PA, USA) and fertilized three weeks later with 40 ml of Peter's soluble 20–10–20 (N–P–K) fertilizer (R. J. Peters Inc., Allentown, PA). When plants reached the 3- to 4-leaf stage, medium size plants were selected and polyvinylidene chloride film (Saran™ wrap) was securely fitted around the stem to cover the soil and prevent soil exposure from the NP spray. Healthy 3 to 4 wk-old seedlings (10 replicates) were sprayed with NP suspensions of CuO (30 nm), MnO (40 nm), SiO (20-30 nm), TiO_2 (30 nm, rutile) and ZnO (10–30 nm) (US Research Nanomaterials, Houston, TX) at 1000 µg/ml. NP suspensions were sonicated for at least 10 min in a FS20H Ultrasonic cleaner (Fisher Scientific Inc., Pittsburgh, PA) prior to application; a stable dispersion was evident in each case. Plants were sprayed using plastic spray atomizers until leaves were wet (1 to 2 ml per plant), allowed to dry, and the film was removed. Plants were sub-irrigated to avoid wetting the leaves.

Control plants were sprayed with sonicated distilled water. Greenhouse temperatures averaged 17 to 22 C° night and 19 to 25 C° day.

One week later, 10 treated seedlings were then transplanted into 10 cm pot filled with non-infested potting mix (one plant/pot). An additional 10 replicates of each treatment were transplanted into pots filled with potting mix infested with dried ground millet that had been colonized by inoculum *F. o. niveum* (one plant/pot). Inoculum was prepared on Japanese millet autoclaved with distilled water (1:1, wt/wt) for 1 hour on two consecutive days, then seeded with three agar plugs colonized by a *F. o. niveum* isolate and allowed to grow for 2 weeks at 22-25 °C. Millet was air-dried, ground in a mill, and passed through a 0.5-mm sieve. The millet inoculum was incorporated into potting mix at 1 g inoculum/liter potting mix. The isolate of *F. o. nivuem* was isolated from infested watermelon seeds; its race was not determined. One week later, 1 g of infested potting mix and non-infested potting mix from greenhouse pots was serially diluted onto Komada's selected agar (Komada 1977); the inoculum stock was found to contain approximately 1×10^5 CFU of *F. oxysporum* per g of potting mix, whereas non-infested soil contain 0.9×10^2 CFU of *F. oxysporum* per g of potting mix producing an inoculum load of 9.9×10^4 CFU/g soil. No effort was made at this time to distinguish *F. oxysporum* from the morphologically identical *F. oxysporum* f. sp. *niveum*.

The pots were placed on greenhouse benches in a 2 (Infestation) X 5 (NP treatments) in a randomized complete block design with 10 replicates/treatment. Each pot received 50 ml of a complete fertilizer solution (20–20–20 N-P-K) once per month. As symptoms of disease developed, plants were rated for severity approximately twice per week, for a total of seven times, on a scale of 1 to 5 where 1 = no disease, 2 = slightly stunted, 3 = stunted and or partially wilted, 4 = completely wilted, and 5 = dead. The disease ratings were rank-transformed as

discussed below. The pathogen was re-isolated from wilted stem tissue to confirm its association with the disease. No *Fusarium* spp. were isolated from healthy stems. After 5 weeks, the experiment was terminated and the plant tops and roots were weighed for fresh and dry weights. Root tissue was later ground for elemental analysis described below.

Greenhouse experiment 2 was a repeat of experiment 1, but differed from the first experiment in that five additional treatments were added. The larger bulk equivalents of CuO, MnO, SiO, TiO_2, and ZnO (Fisher Scientific, New Jersey, US) were compared to the NP forms used in experiment 1 to assess the effect of NP size on growth and disease. In addition, NP suspensions were sonicated using a probe sonicator (Fisher Scientific, FB505) at 50% amplitude for 2 min to aid in dispersing particles prior to treatment. On April 10th, seeds were germinated 36 cell liners (1 plant/cell) filled with potting mix and fertilized three weeks later with 40 ml of Peter's soluble 20–10–20 (N–P–K) fertilizer. Plants were treated with NP or bulk equivalents (1000 µg/ml) on May 8th. Therefore, a 2 (infestation) X 10 (NP or bulk treatments) factorial randomized complete block design, with ten replicate plants per treatment, was used. Growth conditions in the greenhouse were warmer (17 to 22 C° night and 20 to 27 C° day) for greenhouse experiment 2 than those in first experiment, so experiment 2 was terminated after 5 weeks because the disease appeared sooner. Plants were rated five times beginning three weeks after transplanting, and the rank sum transformation was calculated as discussed below. At the end of the experiment, plants were harvested for fresh and dry weights; the dry root tissue was digested for elemental analysis as described below.

Greenhouse experiment 3 was established to assess the effect of increasing rates of CuO NPs on watermelon growth and Fusarium wilt. The experiment was conducted three times with five, six, and eight replicates, respectively. Seeds were germinated on November 5th, June 16th,

and August 21st. Healthy 3 to 4-wk old seedlings were sprayed with 0, 250, 500, and 1,000 µg/ml of CuO NPs prepared as described above. Plants were transplanted into soil infested with *F. o. niveum* or into non-infested soil, and rated once after five weeks, as described above. Fresh and dry weights were determined.

Field experiments. Field experiment 1 examined the effect of foliar sprays of NP of B, CuO, MnO, ZnO on watermelon yield and Fusarium wilt when compared to an untreated control. Fertilizer (10-10-10, NPK) was broadcasted over a 0.9 m wide rows at 112 kg/ha. The rows were set 6 m apart, covered in black plastic mulch and lined with irrigation drip tape. Rows were partitioned into 30 microplots (5.6 m^2). Seeds were germinated on May 26th in 36 cell liners (1 plant/cell) filled with potting mix and fertilized three weeks later with 40 ml of Peter's soluble 20–10–20 (N–P–K) fertilizer. Four wk-old transplants were sprayed on June 23rd in the greenhouse with 1 to 2 ml of one of five treatments: NP of B (2 nm), CuO, MnO, or ZnO applied at 500 µg/ml, or with 1 to 2 ml of distilled water for the untreated control. Metalloid NPs of B were included in this experiment since soil tests at this site indicated very low to no B in the soil and it was of interest to know if foliar sprays could benefit growth and/or disease suppression. One week later, (July 1st), two transplants were set 30 cm apart in the center of each microplot. There were six replicate microplots/treatment. Planting holes were each infested with approximately 2 g of millet inoculum and hand mixed into the soil immediately before transplanting. In addition, another 30 microplots were planted and prepared the same way, but were not infested with the millet inoculum. Plants were sprayed again on July 7th with 1 to 2 ml of NP solutions one week after planting. Effort was made to direct the spray onto foliage and minimize any contact with the black plastic or soil. Plants were rated for disease three times on

July 7th, July 21, and August 5th using the same scale described above. The experiment was conducted only once in Griswold, CT.

Field experiment 2 was designed to compare CuO NPs to the bulk form of CuO along with two commercial Cu fungicides, Kocide 2000 (Certis USA, Columbia, MD) and Copper Fungicide (Cu octanoate, Bonide Product Inc, Oriskany, NY), for their effect on yield and Fusarium wilt of watermelons. Seeds were germinated on May 26th in 36 cell liners (1 plant/cell) filled with potting mix and fertilized three weeks later with 40 ml of Peter's soluble 20–10–20 (N–P–K) fertilizer. Four weeks later (June 17th) healthy plants were sprayed with one of five treatments. Since the CuO NPs treatment (500 µg/ml) provides 400 µg/ml of elemental Cu, the amount of product in the other treatments was adjusted to deliver the same amount (400 µg/ml) of elemental Cu. Five treatments were applied: No treatment (control); CuO NPs 500 µg/ml), a corresponding bulk equivalent form of CuO, (500 µg/ml); Kocide 2000 (1142 µg/ml; 35% metallic copper); and Copper Fungicide (0.22 ml/ml, 1.8% metallic Cu).

The experimental plots were prepared in Hamden, CT on a Cheshire fine sandy loam (Typic Dystrocrept) (pH 6.1). The field was prepared by broadcasting 10-10-10 NPK fertilizer at the 112 kg/ Ha and rototilling. Raised beds were prepared under 4 mil black plastic along with drip irrigation tape (Berry Plastics Holding Corp., Evansville, IN). Root systems of treated transplants were removed from plastic cell linings and dipped in spore suspensions of *F. o. niveum* before planting on May 23rd. Inoculum was increased on 25% Potato Dextrose Agar by seedling cultures for 2.5 weeks at 23-28 °C, rinsing the spores and mycelium off with distilled water, and adjusting to (5×10^4 conidia/ml) with a hemocytometer. A one-way randomized blocked design with six replicates was used. After harvest, the field was planted with winter rye, and plowed again in May 2016. The experiment was repeated in 2016. Seed were germinated on

June 1st, treated on June 23rd and planted on June 30th. Plots were artificially infested with 2 g of millet inoculum which was hand mixed into the soil before planting. Plots were randomly re-assigned to the different treatments and planted on Jun 23th. Disease ratings were conducted three times in 2015 on July 29th, August 15th, and August 30th and three times in 2016 on August 3rd, August 16th, and August 31st.

Elemental analysis. Root tissues from greenhouse experiment 1 and 2, and fruit tissues from field experiments 1 (2015) and 2 (2015 and 2016), were assayed for Cu levels. The edible fruit tissue (minus seeds) was sampled from one medium size fruit from each plot in late August. Tissue was dried in an oven at 50°C, ground in a Wiley mill, and passed through a 1 mm sieve. Digests on ground samples (0.5 g) were 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 Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn content was quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES) on an iCAP 6500 (Thermo Fisher Scientific, Waltham, MA), and element content is expressed as µg g−1 (dry weight) plant tissue.

Gene-Expression Analysis. For transcriptomic analysis, two separate greenhouse experiments were performed on watermelon transplants. On Aug 25th, seeds were germinated in 36 cell liners (1 plant/cell) filled with potting mix and fertilized three weeks later with 40 ml of Peter's soluble 20–10–20 (N–P–K) fertilizer. Plants were treated on September 25th with 1 to 2 ml of CuO NPs (500 µg/ml) or with distilled water and then grown for three weeks in soil infested with millet inoculum of *F. o. niveum* or in non-infested potting mix. Symptoms became evident in the inoculated controls 3 weeks after planting. Plants were then harvested and roots were washed clean of soil and stored at -80 C. Roots were bulked and total RNA from 0.1 g of fresh roots was

extracted using a Sigma-Aldrich Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO). Total RNA sample quality and quantity was assessed by a Thermo Scientific Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and gel electrophoresis. Two-step reverse transcription was performed on 1 µg of the total RNA extracted using the Qiagen QuantiTect Reverse Transcription kit (Qiagen, Velno, The Netherlands). Reverse- transcription real-time PCR (RT-qPCR) was carried out using the Bio-Rad SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) in an optical 96 well plate with the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Based on previous work with *Arabidopsis thaliana,* ortholog gene coding sequences (CDS) were obtained through the BLAST tool of Cucurbigene database resource (http://cucurbigene.net/) for *C. lanatus* (Pagano et al. 2016). A 1·e−20 (E-value) threshold with the query sequence (of *A. thaliana*) was used to identify the orthologous coding sequences in watermelon: a total of 9 orthologs were identified in both of the species (**Table 1**). Specific primers for each selected gene transcript were designed using the Primer3 software (http://primer3.ut.ee/); the thermal profile for RT-qPCR amplifications was: 95 °C for 10′, 95 °C for 15″, and 60 °C for 60″ for 40 cycles. Confirmation of the single amplicon in each reaction was performed by a dissociation-curve step. Relative expression was estimated through ΔΔCt method using β-actin of *C. lanatus* as the housekeeping gene. Gene expression was expressed relative to control plants that were not inoculated or treated with *F. oxysporum* f. sp. *niveum.*

Polyphenol oxidase and protein analysis. For enzyme activity analysis, watermelon that had been sprayed in the greenhouse with 1 to 2 ml of CuO NPs, MnO and ZnO at 500 µg/ml were grown in soil infested with *F. oxysporum* f. sp. *niveum* in the greenhouse, along with an untreated control and non-inoculated untreated control. Plants were harvested 6 weeks later.

Roots were frozen at -80^{o}C. Frozen roots were ground in liquid nitrogen using mortar and pestle, and a 10% homogenate was prepared in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) PVP and 0.5% Triton X-100 at 4 °C. The samples were centrifuged at 10,000 x g for 20 min at 4°C, and the supernatant was collected and stored at -80 °C. PPO activity was measured in a 96-well microplate reader, each well containing 150 ul of 50 mM catechol as substrate and 50 ul of enzyme extract; each sample was repeated twice in different wells. The absorbance of the reaction mixture was measured at a wavelength of 490 nm at ambient temperature using SpectraMax® M5 (PN 0112-0115, from Molecular Devices Corp., Sunnyvale, CA) at 0 min and after 60 min incubation at 37°C. The PPO activity was expressed as U min^{-1} (mg protein)$^{-1}$ (Soffan et al. 2014). The total soluble protein content in the tissues was examined according to Bradford using bovine serum albumin standard (Bradford 1976).

Statistical analyses Data for fresh weight and yield from experimental repetitions in the greenhouse and field were analyzed using the SYSTAT V.10 (Cranes Software International Limited, Bangalore, Karnataka, INDIA) procedure for mixed model ANOVA with year (or experimental repetition) and replication as random effects and NP treatments and inoculations as main effects. Field data from plots with NP treatments in artificially infested plots or in non-infested plot were analyzed separately as one way ANOVA. Data from Greenhouse experiment 3 and Field experiment 2 sets were combined when experimental repetition x treatment interactions were not significant. Yield data (kg) were transformed to square root of yield when it was necessary to satisfy requirements for homogeneity of variance. Means were separated using Tukey's Honesty Significant Difference Test at $P < 0.05$. Since disease severity values were categorical and ordinal, they were subjected to a rank transformation where disease ratings

were totaled over the course of the experiment and analyzed using the nonparametric Wilcoxon Signed Fisher's Test at (P = 0.05) (Conover and Iman 1981).

Results

Greenhouse experiments. Watermelon plants typically showed wilt symptoms 14 to 21 days after inoculation. Discolored stem tissue consistently gave rise to *F. oxysporum* when placed on selective agar (Komada 1979) where the fungus was absent from healthy stems.

In Greenhouse experiment 1, fresh weights were harvested after 5 weeks. Plants treated with CuO NPs were significantly (21%) larger than the untreated plants and were approximately 33% larger than plants amended with NP MnO, SiO, TiO_2, or ZnO (**Fig. 1**). Seven disease ratings were rank-transformed and analyzed by Wilcoxon Rank-Signed Test. Plants treated with CuO NPs, and ZnO had rank sums of disease rating values that were significantly less than untreated controls (**Fig. 1**). Root Cu levels from CuO NPs treatment (44.0 µg Cu /g root) was 31 % more than the untreated control roots (30.5 µg Cu /g root) (P = 0. 012). No difference in root Zn levels was observed between the control (203.6 µg Zn/g root) and the NP ZnO treatment (198.7 µg/ g root). Roots from the other treatments were not assayed in this experiment since they had no marked effect on growth or disease.

In the repetition (greenhouse experiment 2), CuO NPs, MnO, SiO, TiO_2, or ZnO were again examined along with five additional treatments of the bulk forms of the metal oxides on watermelon plants in soil infested with *F. o. niveum*. Neither the NP nor the bulk forms of the metal oxides affected plant fresh weights when compared to untreated controls, but the CuO NPs treatment produced the largest fresh weights which were significantly larger than the bulk form of CuO (P = 0.048) and TiO (P = 0.035) and the NP forms of MnO (P =0.035), Ti) (P = 0.035) and ZnO (P 0.038) (**Fig. 2**). Symptoms of disease appeared after two weeks. Five disease

evaluations were then made over a three week period and rank-transformed. Compared to the control, none of the bulk metal oxides were effective in reducing disease (**Fig. 3**). However, CuO NPs significantly reduced the rank sum of disease ratings by approximately 35% compared to the untreated control ($P = 0.012$). When acid-digested root tissues from plants were assayed by ICP-OES for element content, a 133% increase in Cu levels were observed in plants treated with CuO NPs when compared to untreated controls; notably, Cu content in the NP treatment was double that in roots treated with corresponding bulk CuO (**Fig. 4**). Root levels of other treatment elements did not statistically differ from controls (data not shown).

Given that CuO NPs perform better that other metallic oxides, greenhouse experiment 3 was conducted three times to examined the effect of increasing CuO rates on plant growth and disease severity (**Fig. 5**). CuO NPs applied at 500 µg/ml and 1.000 µg/ml increased plant growth, in infested potting soil, but only the 500 µg rate increased growth in non-infested potting soil. Similarly, the final disease ratings were lowest at 250 and 500 µg/ml when compared to controls; the highest rate was statistically insignificant (**Fig. 6**). Given these results, subsequent studies used 500 µg/ml as the chosen application rate. Assuming 1 to 2 ml was being sprayed per plant, we estimate that only 0.5 to 1.0 mg of CuO (0.4 to 0.8 mg metallic Cu) was being applied per plant.

Field plot experiments. Field experiment 1 was designed to test the effect of NP B, CuO, MnO and ZnO on yield and disease of watermelons grown in soil infested with *F. o. niveum* or left non-infested. Plots that were artificially infested had 19% less yield than healthy control plots. The total yield among the infested plots was highest for plants treated with CuO NPs; yields were increased by 35% relative to untreated controls **(Fig. 7**). Disease appeared in infested plots approximate 3 wks after transplanting Plots treated with NP of B, MnO and ZnO did not differ

from the untreated control or plots treated with CuO. All NP treatments (B, CuO, MnO and ZnO) significantly reduced the rank sum of the disease ratings relative to the control, but plants treated with CuO NPs had rank sums significantly lower than the other NP treatments **(Fig. 8**). No disease appeared on plants grown in non-infested soil. Fruit number was not affected indicating fruit size was being affected by the treatments (data not shown). Compared to untreated controls, the NP treatments had no effect on the edible fruit concentration of B (range 3.5-31.6 µg/g, mean 17.0 µg/g); Cu (range 1.3 – 5.8 µg/g, mean 3.7 µg/g); Mn (range 1.7 – 8.7 µg/g mean, 4.4 µg/g); or Zn (range 5.9 – 17.5 µg/g, mean 10.5 µg/g). In addition, fruit concentrations of Ca, Fe, K, Mg, Mo, Na, P, or S were unaffected by the NP treatments (data not shown).

Field experiment 2 was designed to compare CuO NPs to the corresponding bulk equivalent forms of CuO, along with a conventional Cu hydroxide fungicide (Kocide 2000) and an organic Cu fungicide soap (Cu octanoate). In 2015, all transplants were inoculated with a root drench of conidia of *F. o. niveum*, and disease severity was extremely low. In 2016, transplants were planted into soil infested with millet inoculum and the disease severity was more evident, and appeared 4 weeks after planting. Although the plants in 2016 that were treated with CuO NPs had rank sums of disease ratings 25% lower than the control, these values were not significantly different from other treatments (data not shown). The yield was significantly lower in 2016 than in 2015 ($P < 0.001$). The average yield per plot in 2015 was 45.1 kg, whereas in 2016 yields averaged 16.0 kg. Even though the years differed, there were no significant interactions observed in the yield data between the years and the treatments ($P = 0.97$) ; as such, the five treatments for both data sets were combined and analyzed for yield using the square root transformation (**Fig. 9**). The combined data revealed the yield (kg) was highest in plants treated with CuO NPs and was the lowest in the untreated control plots ($P = 0.020$). CuO NPs treatment

resulted in an averaged 53% increase in yield over the untreated control; Other than CuO NPs, no other treatments resulted in statistically significant differences from the untreated control or from each other. Acid digests of watermelon flesh were analyzed by ICP-OES in each year and found no significant differences in the Cu levels present in the fruit across all treatments (**Fig. 10**).

Gene expression and protein analysis. Gene-expression analysis was conducted to identify up- and down-regulated genes for plants treated with CuO NPs and *F. o. niveum.* Plants were harvested in two experiments for fresh weights when symptoms became evident in plants grown in infested soils. Healthy plants exposed to CuO NPs were 26% larger in fresh weight while inoculated plants were 21% larger when they were treated with CuO NPs (**Table 2**). In this experiment, inoculation did not significantly reduce plants weights. Root digests revealed a significant reduction in Cu following inoculation with *F. o. niveum.*

The genes of interest, along with their ID number from *C. lantus* gene bank are presented (**Table 1)**. Genetic analyses performed with RT-qPCR are expressed relative to the untreated non-inoculated control (**Fig. 11**). Of the nine genes examined, three (PPO, PR1, and PAO) showed significant up regulation. In both repetitions of the experiment, PPO expression was strongly upregulated when CuO NPs was combined with *F. oxysporum* f. sp. *niveum* inoculation (Experiment 1 = 9 times higher, Experiment 2 = 29 times higher), when compared to the untreated healthy control. Gene expression was unchanged in other treatments. The root expression of the gene encoding PR1 protein was also upregulated compared to the untreated healthy control (Experiment 1 = 6 times higher, Experiment 2 =119 times higher) in plants treated with both the CuO NPs and the pathogen. The expression of the PR1 gene was also increased in plants treated with CuO NPs alone, but not in plants inoculated with *F. oxysporum* f.

sp. *niveum* alone. Polyamine oxidase 1 (PAO) activity was only increased in one of the experimental repetitions. Across all genes and treatments, the greatest upregulation was consistently observed upon treatment with both CuO NPs and *F. oxysporum* f. sp. *niveum.*

Polyphenol oxidase activity in the roots of watermelons treated with CuO NPs and *F. oxysporum f. sp. niveum* increased with CuO and pathogen exposure, although the effects were statistically insignificant (**Fig. 12**). PPO activity was unaffected by NP MnO or ZnO. Similarly, total protein amount was not significantly affected by treatment.

Discussion

The ability of metal oxides NPs via foliar application to affect disease resistance is a relatively new and unexplored concept. Most studies on NP in plant pathology have examined direct antifungal activity against the pathogen of concern (Dimkpa et al. 2013a; Jo et al. 2009; Kaned et al. 2014; Kim et al. 2009; 2012; Ocsoy et al. 2013; Saharan et al. 2015; Strayer-Scherer et al. 2017; Wani and Shah 2012; Zabrieske et al. 2015). The current study showed that foliar application of NP of the metalloid B and the metal oxides could positively affect growth and inhibit disease development, presumably through enhanced mineral nutrition and host defense. While NP performance varied across our studies, we observed that CuO NPs was more consistently associated with increases in growth and yield of plants, regardless of disease status. Our findings are in agreement with a past study from our laboratory where CuO NPs were superior to six other NP metallic oxides (AlO, FeO, MnO, NiO TiO, or ZnO) in their ability to improve growth of eggplant and tomato grown in soil infested with *Verticillium dahliae* and *F. oxysporum* f. sp. *lycopersici,* respectively (Elmer and White 2016). In that study, as in the current one, CuO NPs had a disease-suppressing influence on the host. In the 2015 and 2016 field studies, only CuO NPs produced statistically greater yields when compared to untreated

controls ($P = 0.02$). Both commercial Cu fungicides/bactericides, Kocide 2000 and a Cu octanoate soap, as well as the corresponding bulk CuO equivalent, had no effect on disease or yield. The active ingredient in commercial Cu products is the Cu ion, but it is unclear how CuO NPs function in plants. The unique size of the NP certainly influences interaction with the infected plants; however, it is not known if CuO NPs are allowing entry into the plant leaf or if the NP remains in the cuticle and epidermal tissue and serves as a reservoir for slow release of Cu ions.

Copper is an essential plant micronutrient that plays a pivotal role in growth as well as defense (Evans et al. 2007; Römheld and H. Marschner 1991; Yruela, 2009). Copper is a cofactor for three important proteins: plastocyanins, peroxidases, and multi-Cu oxidases (Evans et al. 2007). Many of these proteins serve as defense products synthesized in response to pathogenic infection. For example, polyphenol oxidase (PPO) activity in plants is increased many fold in the presence of Cu ions when attacked by pathogens (Evans et al. 2007; Mayer and Harel 1979). These defense reactions are non-specific and protect plants against a wide array of pathogens. Evans et al. (2007) summarized 70 different disease systems on 30 different crops and found Cu suppressed disease in 65 of these instances (93%). Although it was not clear if the Cu effect was as a fungicide/bactericide or by enhanced host resistance, it was noted that in 30 cases the disease system involved a soil borne pathogen, which suggests a likely role for mineral nutrition.

Deficiency symptoms of Cu (stem dieback, chlorosis of leaves, stunted growth) are very rarely observed on watermelon, so it is assumed that Cu availability is adequate for normal plant growth in unstressed conditions. Thus, it is important to question why CuO NPs suppress disease. One hypothesis is that when roots are under pathogenic attack, the level of Cu needed to

activate host defense enzymes may rapidly become limiting. Evidence of this phenomenon was observed in the present study (**Table 2**). This hypothesis is further supported by the findings of Chmielowska et al (2009), who found that augmenting soil with high levels of CuSO4 induced PPO, PR1, phenolics, perioxidases, and glucanases in pepper and subsequently increased resistance to *V. dahliae*. The benefits of Cu fertilization on watermelons in the absence of disease or deficiency symptoms have been documented (Everett et al. 1966). The current work and our previous study (Elmer and White 2016) demonstrate that greater Cu is present in the roots of plants treated (foliarly) with CuO NPs when compared to a corresponding bulk equivalent or to untreated plants. Although Wang et al. (2012), Dimkpa et al. (2013b) and Pagano et al. (2016) all reported on the presence of Cu NPs within exposed plant tissues (upon root exposure), in this study it is unclear whether the NPs or dissolved ions from the NPs applied to the leaves are actually transported to the root. Cu is classified as an immobile element (Bukovac and Wittwer 1957), suggesting that the unique size of NPs may allow for better transport and/or dissolution of Cu ions into the symplast. Additional studies are underway to answer this question.

In the current gene expression experiments, root PPO was strongly upregulated upon exposure to the pathogen and CuO NPs. PPO is strongly associated with enhanced resistance to plant disease (Constabel and Barbehenn 2008; Thipyapong et al. 2004); the enzyme is activated by Cu, (Marziah and Lam 1987), Si (Suriyaprabha, et al. 2014) and by both pathogenic and nonpathogenic bacteria (Chen et al 2000). It is reasonable to assume the CuO NPs are suppressing disease by enabling the production of phenolic defense barriers through up regulation of this enzyme. Additional confirmation is needed to validate the role of PPO. Future studies should include the extraction of phenolic products and the demonstration that a level

418 sufficient to inhibit *F. oxysporum* f. sp. *niveum* exists. Although the PPO enzymatic assay found
419 no statistical difference in its activity among the plants treated with CuO, MnO and ZnO NPs,
420 collectively, the finding suggests that CuO NPs may offer a novel, safe and sustainable treatment
421 platform for fungal diseases of important food crops.

422 Upregulation of the PR1 protein genes was not unexpected, given that these genes are
423 known to be upregulated following pathogen invasion or other stressors. However, it was
424 interesting that the levels were highest in plants treated with both CuO NPs and *F. oxysporum* f.
425 sp. *niveum.* Conversely, polyamine oxidase (PAO) was strongly upregulated in plants treated
426 with both CuO NPs and/or *F. oxysporum* f. sp. *niveum*. This enzyme catalyzes polyamines; these
427 are metabolites known to accumulate during incompatible interactions between plants and
428 pathogens (Walters 2003). There are several possible roles for polyamines in plant disease,
429 including the hypersensitive response and as an inducer of PR proteins. We do note that NPs of
430 other elements have also been reported to increase host defense metabolites. Suriyaprabha et al.
431 (2014) conducted a similar study and found that NP Si were effective at inducing phenols,
432 phenylalanine ammonia lyase, peroxidase ,and PPO in maize, all of which was associated with
433 increasing resistance to *Aspergillus* spp. The authors also reported particle size dependence,
434 with significantly higher resistance in maize treated with the NP form versus the corresponding
435 bulk equivalent.

436 One surprising discovery in the current study is that a single or double application of
437 CuO NPs to young seedlings, often amounting to a total treatment level of less than 1.0-2.0
438 mg/seedling, is associated with increased root Cu content, season long pathogen suppression, and
439 yield enhancement. This finding is similar to that reported in our previous study on eggplant and
440 tomato (Elmer and White 2016). One possible explanation for the long lasting benefits could be

441 that Fusarium root infection occurs on young plants early in the season (Hart and Endo 1979),
442 highlighting the importance of a disease/treatment window. If plant roots have sufficient Cu
443 availability, host defenses may prevent or minimize infection and delay the onset of symptoms to
444 the extent that disease does not significantly take hold. Clearly, additional research on the role of
445 Cu in host defense is warranted, as are efforts to optimize disease suppression through alternative
446 treatment regimens and with more well designed, tunable forms of the nanoparticle.
447

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452 LITERATURE CITED

453 Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities
454 of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
455 Bukovac, M. J., and Wittwer, S. H. 1957. Absorption and mobility of foliar applied nutrients,
456 Plant Physiol. 32:428–434.
457 Chmielowska, J., Veloso, J., Gutiérrez, J., Silvar, C., and Díaz, J., 2010. Cross-protection of
458 pepper plants stressed by copper against a vascular pathogen is accompanied by the
459 induction of a defense response. Plant Sci. 178:176-182.
460 Conover, W.J., and Iman, R.L. 1981. Rank transformations as a bridge between parametric and
461 nonparametric statistics. The American Statistician, 35:124-129.

462 Constabel, C.P. and Barbehenn, R., 2008. Defensive roles of polyphenol oxidase in plants. Pages
463 253-270 in: Induced Plant Resistance to Herbivory, A. Schaller, ed., Springer Science
464 New York.
465 Dimkpa, C.O., McLean, J.E., Britt, D.W., and Anderson, A.J. 2013a. Antifungal activity of ZnO
466 nanoparticles and their interactive effect with a biocontrol bacterium on growth
467 antagonism of the plant pathogen, *Fusarium graminearum*. BioMetals 26:913-924.
468 Dimkpa, C.O., Latta, D.E., McLean, J.E., Britt, D.W., Boyanov, M.I., and Anderson, A.J. 2013b.
469 Fate of CuO and ZnO nano and micro particles in the plant environment. Environ. Sci.
470 Technol 47:4734-4742.
471 Egel, D. S., and Hoke, S. 2010. Evaluation of triploid watermelon for resistance to Fusarium
472 wilt, 2010. Plant Dis. Management. Rept. 4, V135.
473 Elmer, W. H. and White, J. 2016. Nanoparticles of CuO improves growth of eggplant and
474 tomato in disease infested soils. Royal Chemical Society, Environ. Sci.: Nano 3:1072-
475 1079.
476 Evans, I., Solberg, E. and Huber, D.M., 2007. Copper and plant disease. Pages 177-188, in:
477 Mineral Nutrition and Plant Disease, L. E. Datnoff, W. H. Elmer, and D. M. Huber, eds.,
478 APS Press, St. Paul, MN.
479 Everett, P.H., Locascio, S.J., and Fiskell, J.G.A., 1966. Phosphorus and copper effects on growth
480 and yield of watermelons. Vine 3:20-3.
481 Everts, K.L., and Himmelstein, J.C. 2015. Fusarium wilt of watermelon: Towards sustainable
482 management of a re-emerging plant disease. Crop Protection. 73:93-99.

Hart, L.P., and Endo, R.M., 1981. The effect of time of exposure to inoculum, plant age, root development, and root wounding on Fusarium yellows of celery. Phytopathology 71:77-79.

Jo, Y.K., Kim, B.H., and Jung, G., 2009. Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi. Plant Dis. 93:1037-1043.

Kanhed, P., Birla, S., Gaikwad, S., Gade, A., Seabra, A.B., Rubilar, O., Duran, N., and Rai, M., 2014. In vitro antifungal efficacy of copper nanoparticles against selected crop pathogenic fungi. Materials Letters 115:13-17.

Khan, M. R., and Rizvi, T.F., 2014. Nanotechnology: scope and application in plant disease management. Plant Pathol J. 13:214-231.

Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev. Plant. Prot. Res. 8:114-124.

Leeper, G.W. 1952. Factors affecting availability of inorganic nutrients in soils with special reference to micronutrient metals. Ann. Rev. Plant Physiol. 3:1-16.

Marziah, M., and Lam, C. H. 1987. Micronutrients: Polyphenol oxidase from soybeans (*Glycine max* V. Palmetto) and its response to copper and other micronutrients, J. Plant Nutr.10:2089–2094.

Mayer, A.M., and Harel, E., 1979. Polyphenol oxidases in plants. Phytochemistry 18:193-215

Pagano, L., Servin, A.D., De La Torre-Roche, R., Mukherjee, A., Majumdar, S., Hawthorne, J., Marmiroli, M., Maestri, E., Marra, R., Parkash Dhankher, O., Isch, S.M., White, J.C., and Marmiroli, N. 2016. Molecular response of crop plants to engineered nanomaterial exposure. Environ. Sci. Technol. 50:7198–7207.

505 Ren, L., Su, S., Yang, X., Xu, Y., Huang, Q., and Shen, Q., 2008. Intercropping with aerobic rice
506 suppressed Fusarium wilt in watermelon. Soil Biol. Biochem. 40:.834-844.
507 Römheld, V. and Marschner, H. 1991. Function of micronutrients in plants, Pages 297-328 In:
508 Micronutrients in Agriculture, J. J. Mortvedt, F. R. Cox, L. M. Shuman and R. M. Welch,
509 eds., Soil Science Society of America, Madison, Wisconsin, USA, 1991, pp. 297-328.
510 Saharan, V., Sharma, G., Yadav, M., Choudhary, M. K., Sharma, S. S., Pal, A., Raliya, R., and
511 Biswas, P. 2015. Synthesis and in vitro antifungal efficacy of Cu–chitosan nanoparticles
512 against pathogenic fungi of tomato. Int J. Biologic Macromolecules *75:*346-353.
513 Servin, A., Elmer, W., Mukherjee, A., De la Torre-Roche, R., Hamdi, H., White, J.C.,
514 Bindraban, P. and Dimkpa, C. 2015. A review of the use of engineered nanomaterials to
515 suppress plant disease and enhance crop yield. J Nanoparticle Res 17:92.
516 Soffan, A., Alghamdi, S.S. and Aldawood, A.S., 2014. Peroxidase and polyphenol oxidase
517 activity in moderate resistant and susceptible *Vicia faba* induced by *Aphis craccivora*
518 (Hemiptera: aphididae) infestation. J. of Insect Sci, 14:285.
519 Strayer-Scherer, A.L., Liao, Y.Y., Young, M., Ritchie, L., Vallad, G.E., Santa, S., Clark, D.,
520 Freeman, J.H., Jones, J.B. and Paret, M.L., 2017. Advanced copper composites against
521 copper-tolerant *Xanthomonas perforans* and tomato bacterial spot. Phytopathology
522 https://doi.org/10.1094/PHYTO-06-17-0221-R.
523 Suriyaprabha, R., Karunakaran, G., Kavitha, K., Yuvakkumar, R., Rajendran, V. and Kannan,
524 N., 2014. Application of silica nanoparticles in maize to enhance fungal resistance. IET
525 nanobiotechnolog, 8:133-137.
526 Thipyapong, P., Hunt, M.D. and Steffens, J.C., 2004. Antisense downregulation of polyphenol
527 oxidase results in enhanced disease susceptibility. Planta, 220:105-117.

528 Walters, D.R., 2003. Polyamines and plant disease. Phytochemistry 64:97-107.

529 Wang, Z.; Xie X.; Zhao, J.; Li, X.; Feng, W.; White, J.C.; Xing, B. 2012. Xylem- and phloem-
530 based transport of CuO nanoparticles in maize (*Zea mays* L). Environ. Sci. Technol. 46,
531 4434-4441.

532 Wani, A.H. and Shah, M.A., 2012. A unique and profound effect of MgO and ZnO nanoparticles
533 on some plant pathogenic fungi. J. Appl. Pharmaceutical Sci.2:4.

534 Wu, H.-S., Gao, Z.-Q., Zhou, X.-D., Shi, X., Wang, M.-Y., Shang, X.-X., Liu, Y.-D., Gu, D.-L.,
535 and Wang, W.-Z., 2013. Microbial dynamics and natural remediation patterns of
536 Fusarium-infested watermelon soil under 3-yr. of continuous fallow condition. Soil Use
537 Manage. 29: 220-229.

538 Yruela, I., 2009. Copper in plants: acquisition, transport and interactions. Functional Plant
539 Biology, 36:409-430.

540 Zabrieske, Z., Morrell, E., Hortin, J., Dimkpa, C., McLean, J., Britt, D., and Anderson, A. 2015.
541 Pesticidal activity of metal oxide nanoparticles on plant pathogenic isolates of *Pythium.*
542 Ecotoxicology 24:1305-1314.

543 Ze, Y., Liu, C., Wang, L., Hong, M., and Hong, F. 2011. The regulation of TiO2 nanoparticles
544 on the expression of light-harvesting complex II and photosynthesis of chloroplasts of
545 *Arabidopsis thaliana.* Biological Trace Element Res. 143:1131-1141.

Table 1 Nine orthologs from *Arabidopsis thaliana* also found in *Citrullus lanatus* var. *lanatus*

Ref[x]	Gene ID *C. lanatus*	Gene Function
CCH	Cla020497	Copper chaperone, cch
COX11	Cla002392	Cytochrome c oxidase assembly protein ctag / Cox11 family
HMA1	Cla006819	Heavy metal atpase 1, hma1
HMA5	Cla011458	Heavy metal atpase 5, hma5
RAN1	Cla009875	Heavy metal atpase 7, hma7, ran1
CSD1	Cla011299	Copper/zinc superoxide dismutase 1, csd1, sod1
PAO	Cla015262	Polyamine oxidase 1, pao1
PPO	Cla019486	Polyphenol oxidase chloroplastic-like
PR1	Cla001623	Pathogenesis-related gene 1

[x] BLAST tool of Cucurbigene database resource (http://cucurbigene.net/) for *C. lanatus.*

Table 2. Effect of nanoparticles (NP) of CuO and *Fusarium oxysporum* f. sp. *niveun* on the fresh weights and root concentrations of copper.

Treatment[x]	Fresh weight (g)	Cu levels (µg/g root tissue)[y]
NonInfested potting mix - Untreated Control	9.3 ab	84 b
NonInfested potting mix - NP of CuO	11.7 b	107 c
Infested with *F. o. niveum* - Untreated control	8.8 a	33 a
Infested with *F. o. niveum* - NP of CuO	10.7 ab	44 ab

[x] Treatments - Plants were infested with dried ground millet inoculum described in the text; plants were sprayed with 2-4 ml of NP of CuO (500 µg/ml).

[y] Cu was determined by ICP OES following acid digests

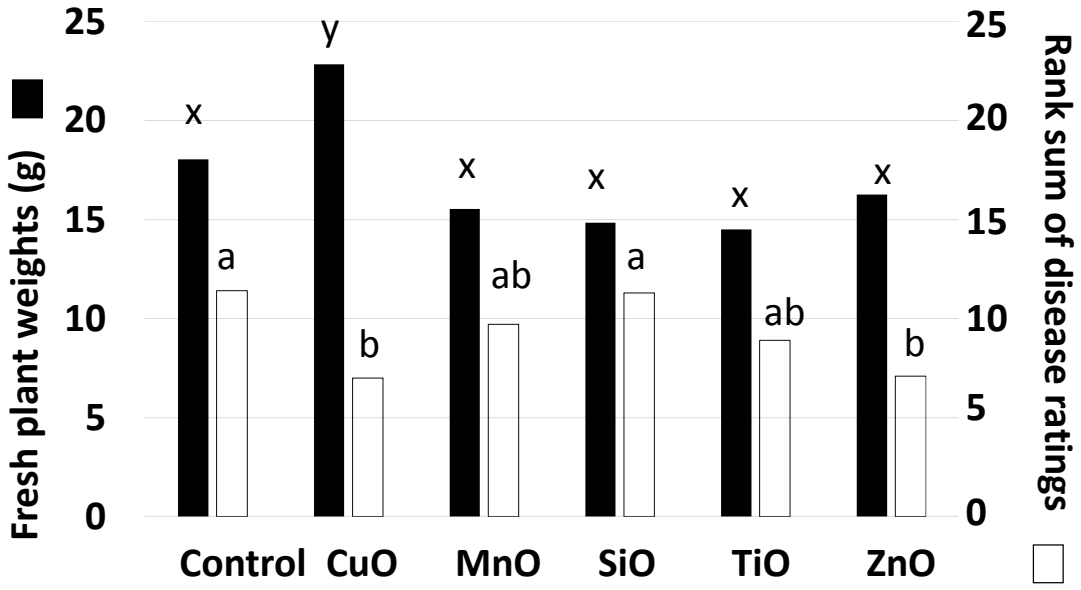


Fig. 1. Effect of foliar applications of nanoparticles (NPs) of metal oxides on fresh weights and the rank sums of disease ratings of watermelon plants grown in soil infested with *Fusarium oxysporum* f. sp. *niveum* in Greenhouse experiment 1. Plant weight data designated with differing letters (x, y) are significant different according to Tukey's Honest Significant Test at $P = 0.05$. Rank sums of disease ratings ($n = 10$) represent sums of seven disease ratings (1 – 5 scale) taken over 16 days; values with differing letters (a,b) are significantly different according to Wilcoxon Signed-Rank Test ($P = 0.05$).

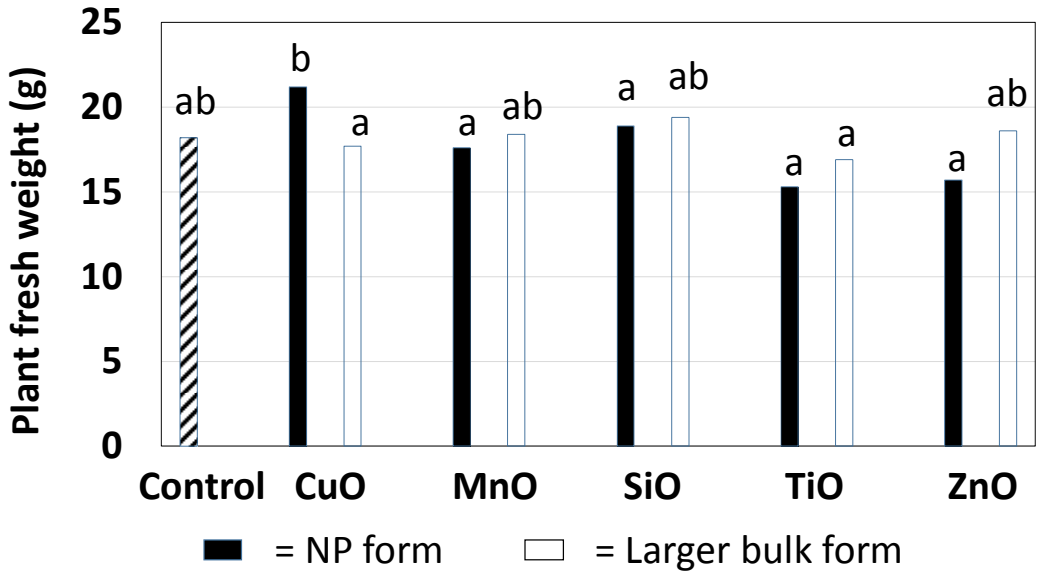


Fig. 2. Effect of foliarly sprayed nanoparticles (NPs) of metallic oxides and their larger bulked oxide equivalents on fresh weights of watermelons grown in soil infested with *Fusarium oxysporum* f. sp. *niveum* in Greenhouse experiment 2. Plant weight data designated with differing letters are significant different according to Tukey's Honest Significant Test at $P = 0.05$.

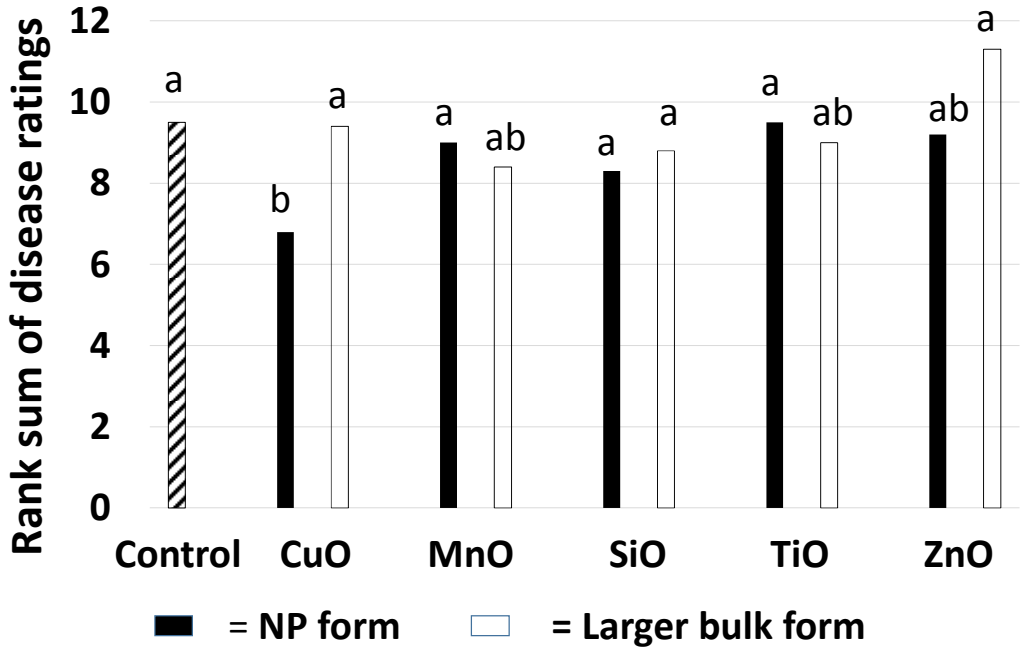


Fig. 3. Effect of foliarly sprayed nanoparticles (NPs) of metal oxides and the larger bulk oxide equivalents on rank sums of disease ratings of watermelon plants grown in soil infested with *Fusarium oxysporum* f. sp. *niveum* in Greenhouse experiment 2. Values (n = 10) represent the rank sums of five disease ratings (1 – 5 scale) taken over 19 days; values with differing letters are significantly different according to Wilcoxon Signed-Rank Test (P = 0.05).

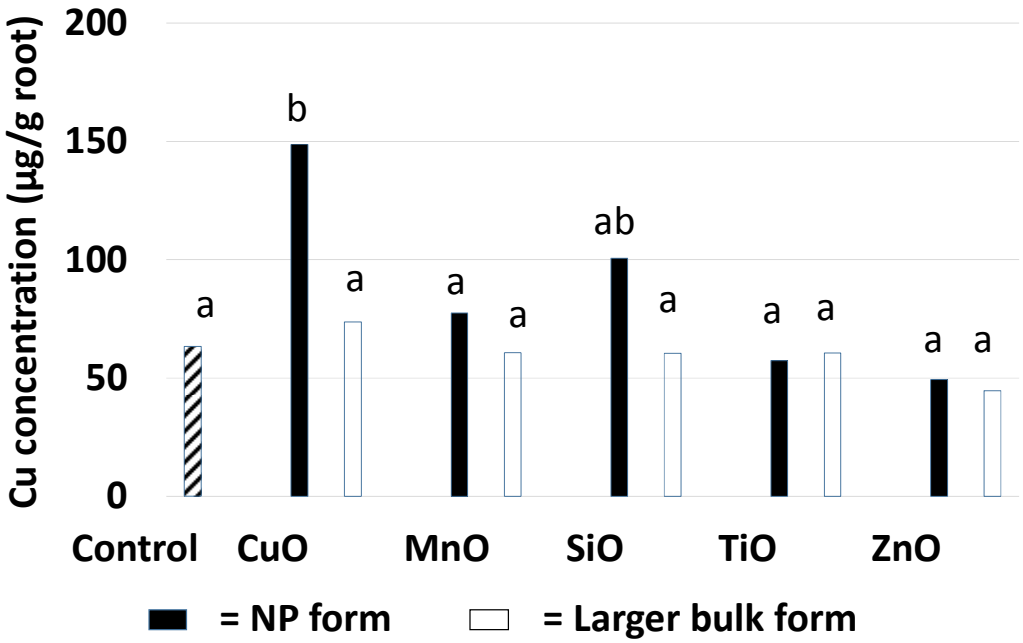


Fig. 4. Effect of foliarly sprayed nanoparticles (NPs) of metal oxides and the larger bulk oxide equivalents on the concentration of Cu in roots of watermelons grown in soil in Greenhouse experiment 2. Mean Cu concentrations designated with differing letters are significant different according to Tukey's Honest Significant Test at $P = 0.05$.

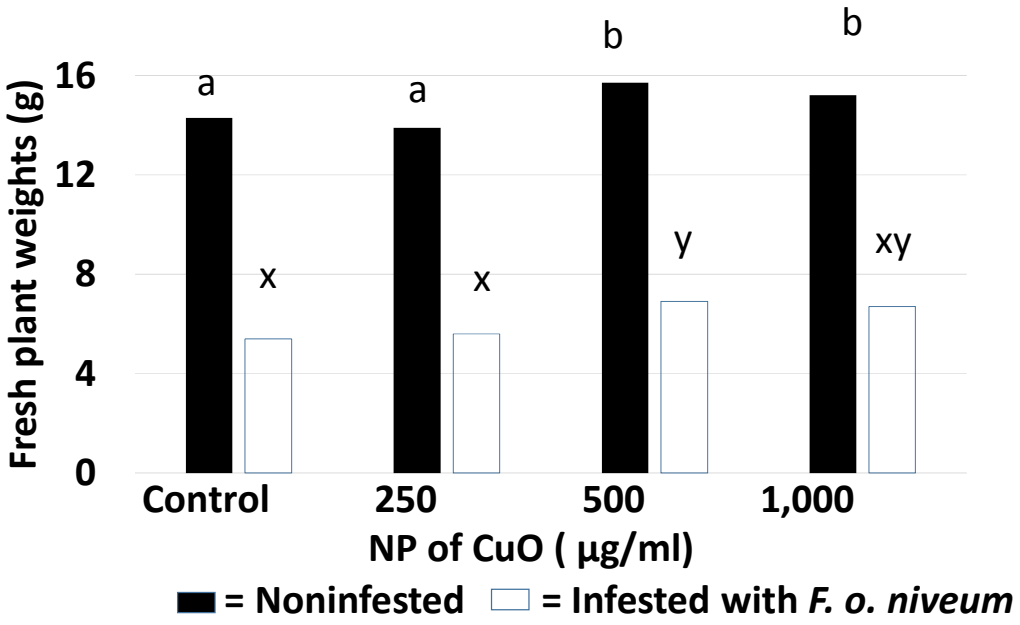


Fig. 5. Effect of the rate of foliarly sprayed nanoparticles (NPs) of CuO on fresh weights of watermelons grown in soil infested with *Fusarium oxysporum* f. sp. *niveum* or in noninfested soil in Greenhouse experiment 3; means designated with differing letters (Noninfested refers to letters a, b) or (Infested refers to letter x, y) are significant different according to Tukey's Honest Significant Test at $P = 0.05$.

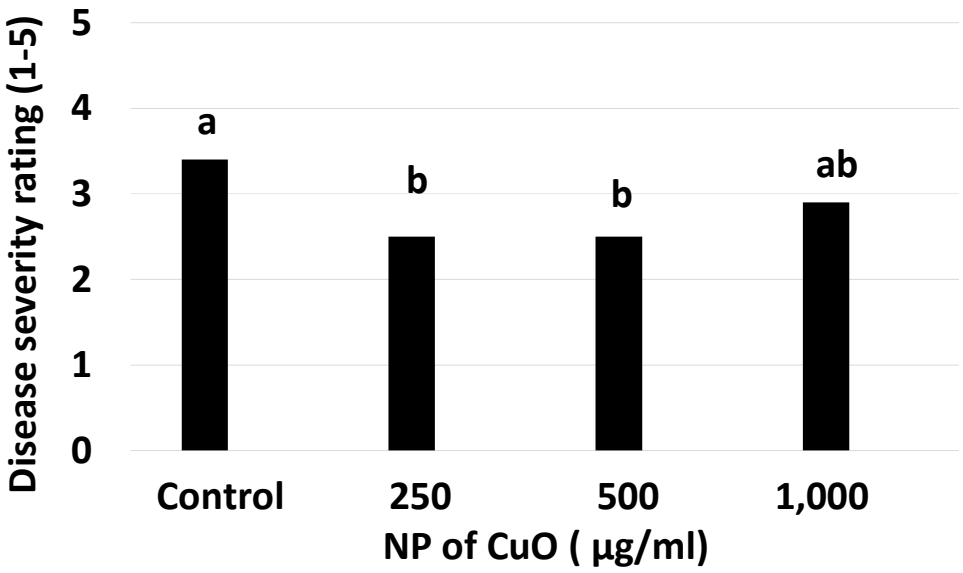


Fig. 6. Effect of the rate of foliarly sprayed nanoparticles (NPs) of CuO on the final disease severity rating (1-5) of watermelons grown in soil infested with *Fusarium oxysporum* f. sp. *niveum* in Greenhouse experiment 3. Values represent mean disease severity ratings from three trials (n = 19); values with differing letters are significantly different according to Wilcoxon Signed-Rank Test (P = 0.05).

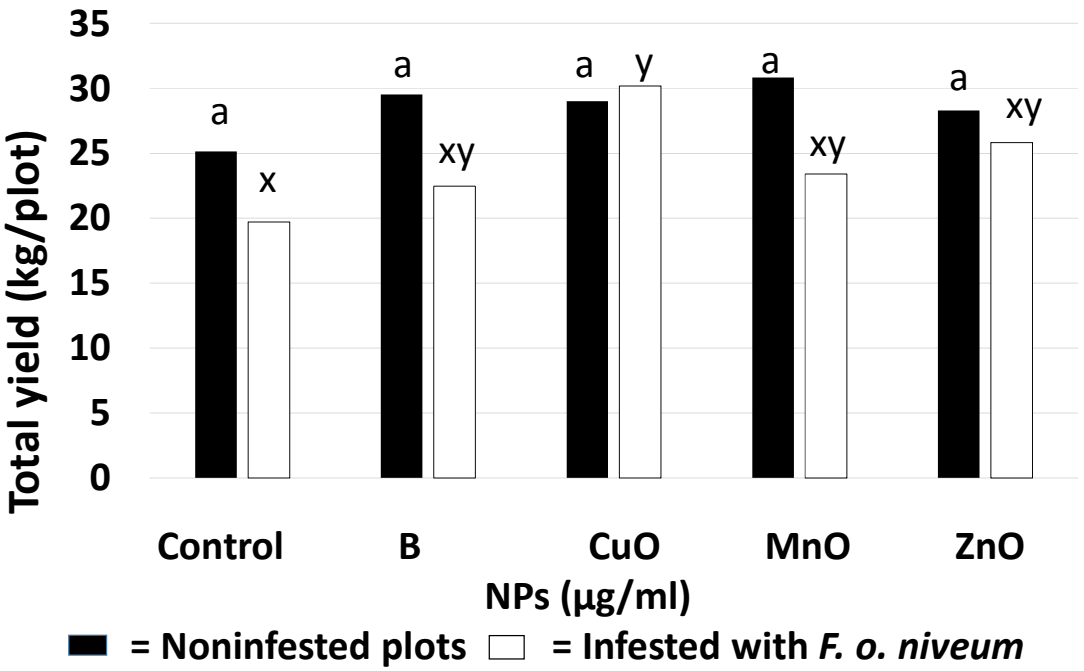


Fig. 7. Mean watermelon yield from plants treated with foliarly sprayed nanoparticles (NPs) of B, CuO, MnO, or ZnO in noninfested soil or in soils artificially infested of watermelons infested with *Fusarium oxysporum* f. sp. *niveum* in Field experiment 1. Differing letters represent significant differences by Tukey Honest Significant difference Test.

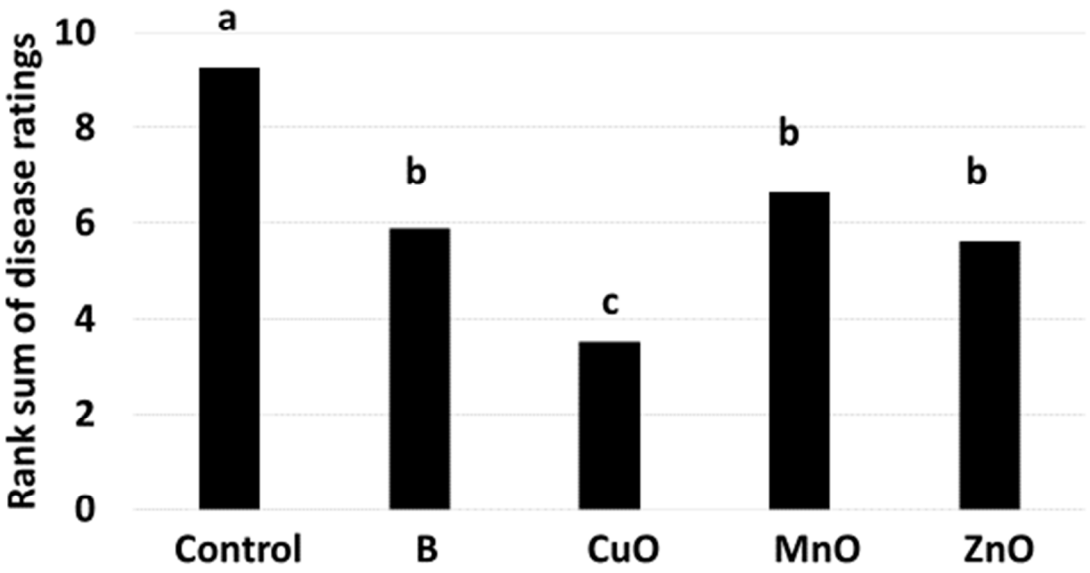


Fig. 8. Means of rank sums of three disease ratings (1 – 5 scale) taken over 30 days of watermelon plants grown in experimental field plots in soil infested with *Fusarium oxysporum* f. sp. *niveum* and treated with nanoparticles of B, CuO MnO or ZnO in Field experiment 1. Values with differing letters are significantly different according to Wilcoxon Signed-Rank Test ($P = 0.05$).

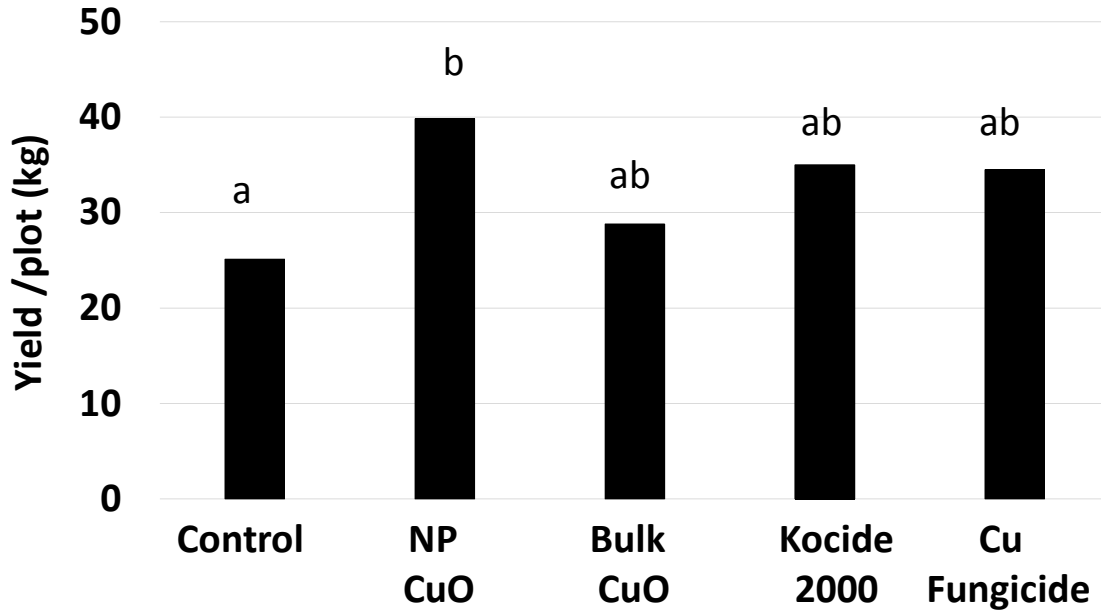


Fig. 9. Mean watermelon yield from plants treated with foliarly sprayed CuO nanoparticles (NPs), the larger bulk oxide equivalent, or Cu fungicides (Kocide 2000 or Copper fungicide (Cu octanoate) Hamden, Connecticut in Field experiment 2. Mean yield values designated with differing letters are significant different according to Tukey's Honest Significant Test at $P = 0.05$.

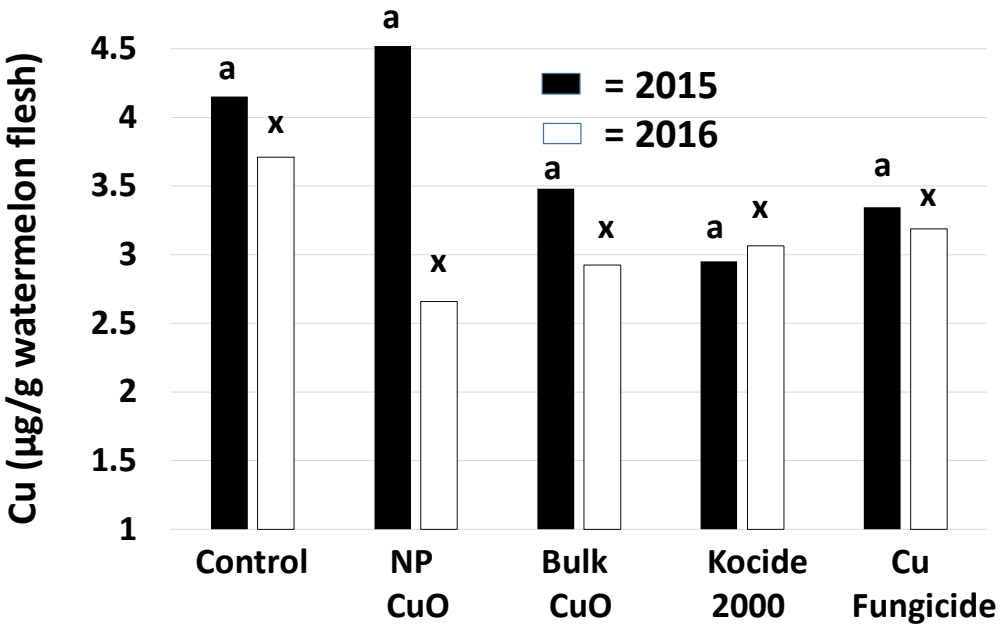


Fig 10. Mean Cu concentrations in edible fruits of watermelon treated with foliarly sprayed CuO nanoparticles (NPs), the larger bulk oxide equivalent, or Cu fungicides (Kocide 2000 or Copper fungicide (Cu octanoate) in Hamden, Connecticut in Field experiment 2. Cu concentrations were not significantly different according to Tukey's Honest Significant Test at $P = 0.05$ in either year (2015 refers to letter, a; and 2016 refers to the letter, x).

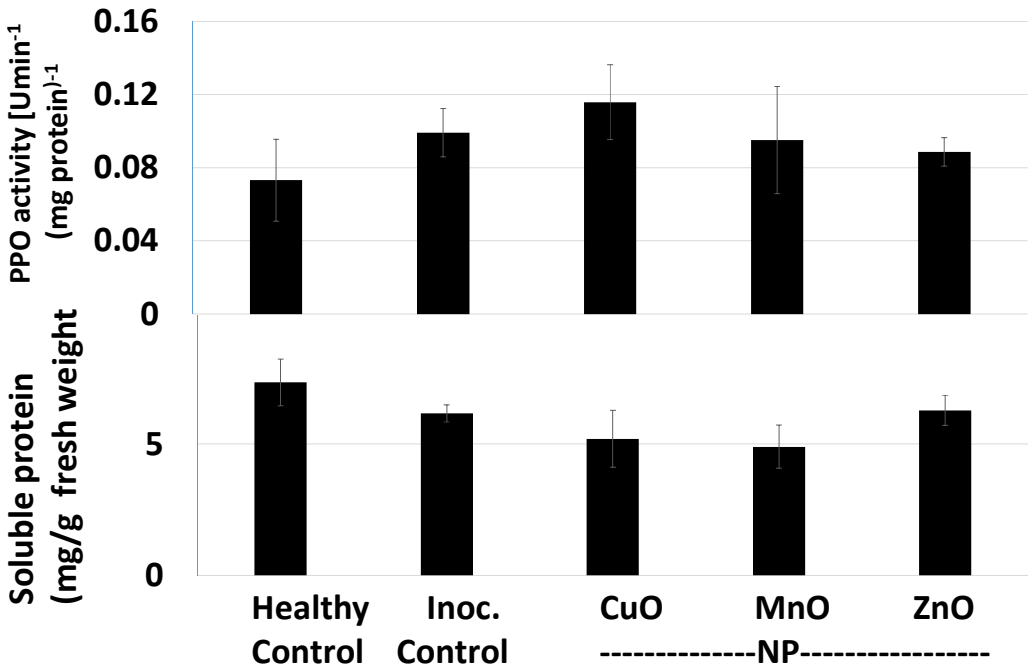
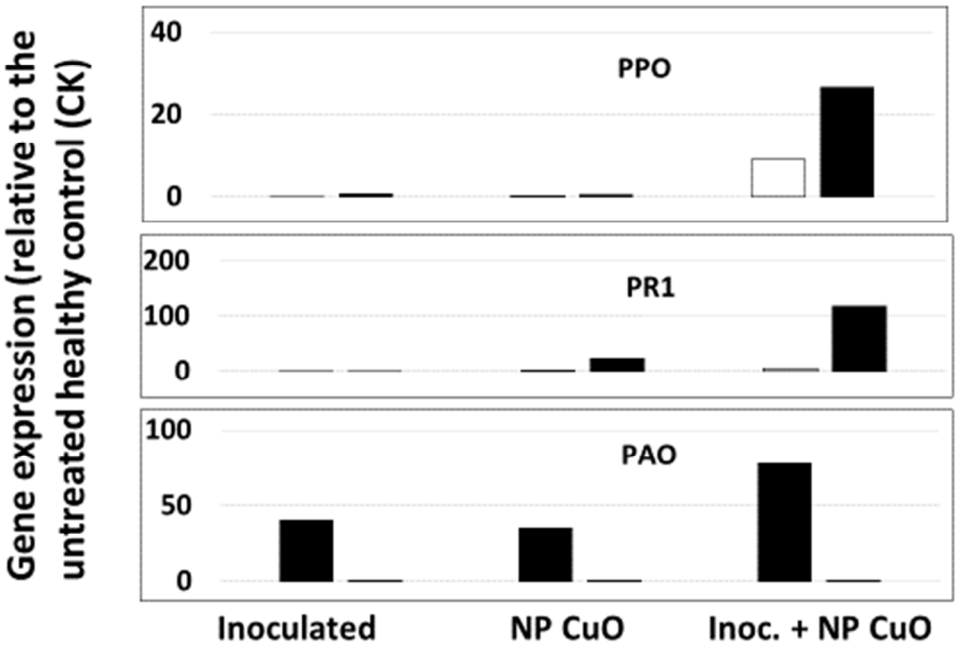


Fig. 11. Levels of polyphenol oxidase (PPO) activity and total soluble protein from roots of watermelon plants treated with foliarly applied applications of CuO, MnO or ZnO and grown in soil infested with *Fusarium oxysporum* f. sp. *niveum.*



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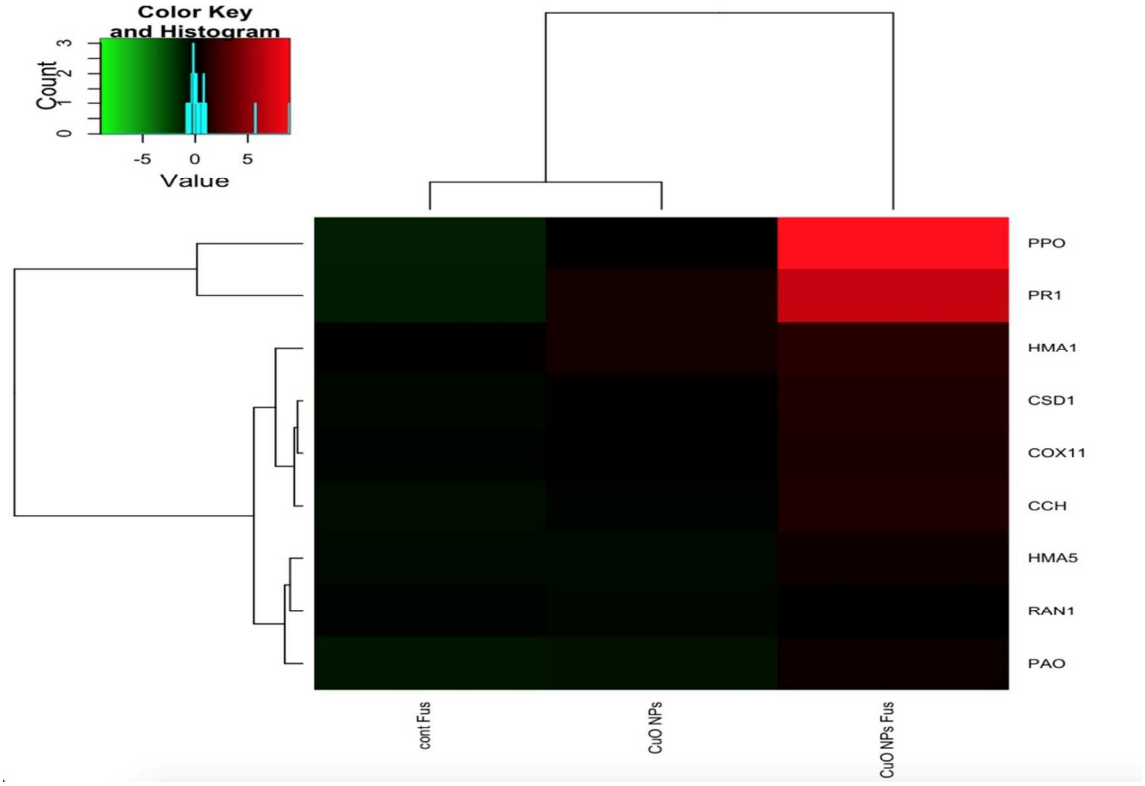

2

3 **Fig. 12a**. Gene expression levels of polyphenol oxidase (PP0) (top), Pathogenicity related

4 proteins (PR1) (middle), and Polyamine oxidase (bottom) from roots of watermelon plant treated

with or without NP of CuO and grown in non-infested soils. Values are based on values from untreated control plant grown in non-infested. **Fig. 12b.** Heatmap response of watermelon roots treated with NP of CuO and grown in non-infested soils. Values are based on untreated control or soil infested with *Fusarium oxysporum* f. sp. *niveum.* Signals were normalized on the untreated control (data not shown). In the heatmap, down-regulated genes are reported in green, whereas upregulated genes are shown in red. Genes not significantly different from the expression levels of the untreated control are reported in black.