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Addition-omission of zinc, copper, and boron nano and bulk oxide particles demonstrate element and size -specific response of soybean to micronutrients exposure

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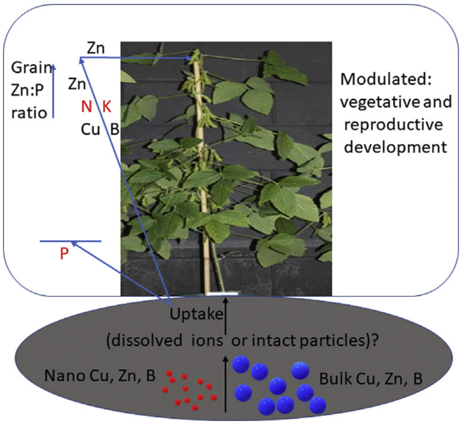
HIGHLIGHTS

- Particle-size and element-specific effects studied by addition-omission strategy
- Nano and bulk particle mixtures promoted grain yield and modulated nutrient uptake.
- Nano exposure specifically stimulated vegetative responses and N and K accumulation.
- Omitting each nutrient evoked element and particle size-specific responses.
- Zn evoked the most responses with implications for environmental and human health.

GRAPHICAL ABSTRACT



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ABSTRACT

Plant response to microelements exposure can be modulated based on particle size. However, studies are lacking on the roles of particle size and specific microelements in mixed exposure systems designed for plant nutrition, rather than toxicology. Here, an addition-omission strategy was used to address particle-size and element-specific effects in soybean exposed to a mixture of nano and bulk scale oxide particles of Zn (2 mg Zn/kg), Cu (1 mg Cu/kg) and B (1 mg B/kg) in soil. Compared to the control, mixtures of oxide particles of both sizes significantly ($p < 0.05$) promoted grain yield and overall (shoot and grain) Zn accumulation, but suppressed overall P accumulation. However, the mixed nano-oxides, but not the mixed bulk-oxides, specifically stimulated shoot growth (47%), flower formation (63%), shoot biomass (34%), and shoot N (53%) and K (42%) accumulation. Compared by particle size, omission of individual elements from the mixtures evoked significant responses that were nano or bulk-specific, including shoot growth promotion (29%) by bulk-B; inhibition (51%) of flower formation by nano-Cu; stimulation (57%) of flower formation by bulk-B; grain yield suppression (40%) by nano-Zn; B uptake enhancement (34%) by bulk-Cu; P uptake stimulation by nano-Zn (14%) or bulk-B (21%); residual soil N (80%) and Zn (42%) enhancement by nano-Cu; and residual soil Cu enhancement by nano-Zn (72%) and nano-

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B (62%). Zn was responsible for driving the agronomic (biomass and grain yield) responses in this soil, with concurrent ramifications for environmental management (N and P) and human health (Zn nutrition). Overall, compared to bulk microelements, nanoscale microelements played a greater role in evoking plant responses.

1. Introduction

Nitrogen (N), phosphorus (P) and potassium (K) are the most used nutrients in fertilizers in terms of volume and frequency. However, soils in many parts of the globe have become non-responsive to NPK fertilization, due in part to the depletion of trace or microelements such as zinc (Zn), copper (Cu), boron (B), manganese (Mn), and iron (Fe). Non-responsive soils are common in regions of the world where low use of balanced fertilizers containing both macro- and micro -nutrients, and/or pervasive lack of replenishment of micronutrients in continuously cropped smallholder farms have stripped the soil of these elements (Vanlauwe et al., 2010; Jones et al., 2013). Conversely, crop production in more advanced agricultural systems is characterized by high applications of NPK and use of genetically improved plant varieties. This has resulted in crops with high nutrient demands, higher biomass production, and consequently, biomass-driven micronutrient dilution. Under both fertilizer use scenarios, the net outcome is soils depleted of micronutrients, and harvested crops with decreased nutritional value (Thomas, 2003; Jones et al., 2013; Dimkpa and Bindraban, 2016).

Micronutrients provide multiple benefits under different crop production conditions. Such benefits include influencing the uptake and use efficiency of N; increasing crop biomass production above NPK levels; and promoting tolerance to abiotic (e.g., drought and salinity) and biotic (pests and diseases) stresses by enhancing water use efficiency, plant health, and systemic response (Servin et al., 2015; Dimkpa and Bindraban, 2016; Angle et al., 2017). In addition, micronutrients increase the nutritional quality of food crops by supplying nutrients such as Zn and Fe that are critical for human health but often lacking in diets around the globe (Dimkpa and Bindraban, 2016; Smith and Myers, 2018). Notably, these benefits could be achieved at relatively low or reduced NPK application rates (Dimkpa et al., 2017a; Das et al., 2018). A reduced N application rate has implications for greenhouse gas (GHG) emissions, given correlations between high N rate and GHG production (Wang et al., 2016). In contrast to N, the effects of micronutrient fertilization on P acquisition have been conflicting. On the one hand, application of metallic micronutrients under high P fertilization may impede P and micronutrient use efficiencies by lowering nutrient bioavailability to the plant (Watts-Williams et al., 2014; Dimkpa et al., 2017a, 2017b, 2018a). Conversely, mobilization of P in native unfertilized soils with low P bioavailability could be enhanced by Zn and Fe due to micronutrient-induced modifications in microbial community structure, enzyme activity, or soil chemistry (Zahra et al., 2015; Raliya et al., 2016).

Informed by these advances, calls are being made for a paradigm shift towards more balanced fertilizers containing macro- and micro -nutrients, as well as for rethinking the design and formulation of nutrients in fertilizers (Bindraban et al., 2015, 2018). Relative to conventional (bulk-scale or ionic) micronutrients, nanoscale (≤100 nm in at least one dimension) micronutrients are increasingly being evaluated as fertilizers for quantitative and qualitative crop improvement (Dimkpa and Bindraban, 2018). Nanoscale materials are more reactive than their conventional counterparts, with potential for both beneficial and inhibitory outcomes for plants and other terrestrial species. The heightened reactivity associated with nanoscale materials is due to their possession of greater surface area than bulk materials. Several recent studies have compared the effects of nanoscale vs. conventional micronutrients in soil-grown plants, in the context of fertilizer-nutrients, rather than as phytotoxicants (reviewed in Liu and Lal, 2015; Tolaymat et al., 2017, and Dimkpa and Bindraban, 2018). The studies reported positive responses due to exposure to nanoparticles, the degree of which depended on the specific nanoparticle chemistry, exposure dose, specific soil chemistry, and crop species. However, terrestrial plants are often exposed to multiple elements simultaneously, either as nutrients or toxicants in soil. Thus, plant exposure to nanoparticles is likely to occur in mixed, rather than single, element situations. In one instance involving soybean, mixed formulations of nanoparticle and ionic Zn, Cu, and B were evaluated under drought stress, and positive or negative plant responses were recorded on growth, yield and uptake of specific nutrients (Dimkpa et al., 2017b). Notably, these studies were based on inclusion of micronutrients in a plant fertilization regime. Nonetheless, assessing the role of specific nutrient elements in mixed systems is essential to understanding their benefits in crops. This can be achieved by a systematic omission of each constituent from the fertilizer regime. This strategy is especially applicable in situations where standard soil testing capabilities are limited or cost prohibitive, as is the case in many parts of the globe (Dimkpa et al., 2017c). To date, there has been no comparative assessment of the effects of omitting nano- vs. bulk-scale micronutrients in fertilizer amendments to agricultural crop plants. Thus, the objectives of the present study were to (i) evaluate differences in plant response to exposure to mixed nano vs. conventional (bulk) oxides of Zn, Cu and B in soybean, and (ii) assess the effects of omission of nano and bulk Zn, Cu, and B from the balanced-nutrient fertilizers on the plant responses. While few prior studies have evaluated mixed nanoparticles, or single nanoparticles vs. conventional trace elements in plant systems (e.g., Dimkpa et al., 2015; Jośko et al., 2017; Pagano et al., 2017), to the best of our knowledge, this is the first study assessing the response of an agricultural crop plant to nanoparticles using an addition-omission strategy in the context of nutrition.

2. Materials and methods

2.1. Chemicals

Commercial nanoscale powders of zinc oxide (ZnO; 18 nm), and copper oxide (CuO; 40 nm) were purchased from US Research Nanomaterials, Inc., Houston, Texas, USA. Boron oxide (B_2O_3; <100 nm) nanoscale powder was purchased from American Element, Los Angeles, California, USA. Bulk (>1000 nm) powders of ZnO, CuO and B_2O_3 were purchased from Sigma-Aldrich, St Louis, Missouri, USA. Each dry powder was weighed out based on the active element (Zn, Cu, or B) corresponding to 2 mg Zn/kg soil, 1 mg Cu/kg soil, and 1 mg B/kg soil, mixed together for uniformity, and stored dry in plastic vials until needed. Twice more Zn than Cu or B was used, to reflect the physiological needs of these nutrients by soybean.

2.2. Soil preparation

The soil involved in this study is a sandy loam. Prior to use for plant growth, the soil was analyzed for bioavailable nutrient contents. The analysis indicated levels of the test elements (Zn, Cu, and B) that ranged from critically deficient (Zn), borderline-deficient (B), to slightly-sufficient (Cu). In addition, plant available N, P, and S levels were particularly low (Table 1, upper row). However, the total N (including inorganic; hence, bioavailable + organic; hence, immediately nonbioavailable) level was 400 mg/kg. The organic N fraction, upon mineralization, would eventually become available for plant use.

Table 1
Bioavailable levels of nutrients in the test soil prior to planting (upper row); and description of the study treatments (lower row).

Element	N	P	K	Mg	Ca	S	Zn	Fe	Mn	B	Cu
Concentration in soil (mg/kg)	4	2	246	150	1174	2.7	0.1	4	6	0.5	0.4
Treatment description	Balanced nutrition			Nutrient omission							
	Control	All nano	All bulk	Minus (−) nano Zn	Minus (−) nano Cu	Minus (−) nano B	Minus (−) bulk Zn		Minus (−) bulk Cu	Minus (−) bulk B	

Furthermore, the soil has a pH of 6.87, a low organic matter content of 0.92%, and a correspondingly low CEC of 7.88 cmol/kg. Eight kilogram of the dry soil was loaded into replicate pots (10 l volume), and basal NPK at the rates of 10:100:275 (mg/kg soil) was mixed into the soil for all treatments. Because monocalcium phosphate and sulfate of potash were used as the sources of P and K, respectively, Ca (5.8 mg/kg) and S (5.4 mg/kg) were also amended into the soil following P and K application. Basal amendments of the mixtures of nanoscale or bulk oxide powders were made to each pot containing the dry soil, corresponding to 16 mg Zn/pot, 8 mg Cu/pot, and 8 mg B/pot. The pots were then tumbled on a mechanical mixer, to facilitate uniform distribution of the particles into the soil. As described in the treatment description in Table 1 (lower row), these added elements constitute the balanced-nutrient fertilizer (addition) henceforth described in this study. Conversely, the exclusion of each micro element implies its omission from the balanced nutrient mix, separately for the nanoscale and bulk oxides. Collectively, nine treatments were involved, including a control treatment with only an N-P-K-S-Ca amendment (Table 1).

2.3. Plant material and growth conditions

A potted plant growth study was conducted at the International Fertilizer Development Center's greenhouse in Muscle Shoals, Alabama, USA, using soybean [*Glycine max* (L.) var. Stonewall; life cycle: 120–140 d] and the soil described above, during June to September. Notable summer greenhouse conditions for this study were: average temperature, 30 °C; relative humidity, 44–96%; and daylight duration, 13–15 h. Prior to seed establishment, the soil was watered to field moisture capacity. Subsequently, soybean seeds inoculated with the rhizobium, *Bradyrhizobium japonicum* (3.13 g/kg seed), were sown at three seeds per pot. At emergence, seedlings were thinned down to one per pot. The experiment was assigned a randomized complete block design comprising 3 replicated plants per treatment. Watering and other greenhouse growth management practices were conducted as required, following routine operations. During the study, selected plant vegetative growth variables, including shoot height, number of shoot branches, and number of flowers, were measured. From two weeks post germination, shoot height was measured periodically, up to 4 times during the growth cycle; two of those were coincident with flowering time and cessation of vegetative growth (end-point). Upon full maturity, plants were harvested and separated into shoot (leaf + stem), and grain for further analysis.

2.4. Plant and soil analyses

Harvested plant tissues were subsequently oven-dried at 60 °C until constant weight was achieved, and then ground into powder by means of a Model 4 Thomas Wiley Laboratory Mill (Pennsylvania, USA) for shoot tissues, or by a ZM 100 Retsch grinder (Retsch GmbH, Haan, Germany) for seeds. The ground tissues were acid-digested in a solution of sulfuric acid (3 ml) and H_2O_2 (1 ml), followed by heating (1 h at 350 °C), cooling to room temperature, and equilibration with distilled H_2O. Sub-samples of the prepared tissues were subsequently subjected to Skalar segmented flow analysis for N and P, or to inductively coupled plasma-optical emission spectroscopy (ICP-OES; model Spectro Arcos, SPECTRO Analytical Instruments GmbH, Kleve, Germany) for K, Zn, Cu, and B. Soil samples were also collected from the harvested pots for each treatment, to determine pH and bioavailable levels of N, P, Zn and Cu. To this end, roots were carefully removed from the soil, and soil devoid of any root particles was collected for each treatment. Detailed procedures for the soil extraction and analyses for these elements were as previously described (Dimkpa et al., 2018a).

2.5. Data analysis

A two-way analysis of variance (ANOVA; OriginPro 2018) was used to determine significant differences in plant responses to the treatments and in block effects for each variable, including vegetative and reproductive development, and nutrient contents of plant and soil samples. A Fisher LSD mean comparison was performed to further explore the differences with significant ($p < 0.05$) ANOVA.

3. Results and discussion

In this study, soybean was subjected to micronutrient addition-omission treatments using nano and bulk Zn, Cu and B oxide particles. This strategy allowed to investigate plant response to mixed elements, and the specific role of each element at two particle size scales. Where a significantly different response occurred between the mixture of one particle size (but not the other particle size) and the control, or between the particle sizes (“All” nano vs. “All” bulk), such response was considered size-specific. For the omissions, where a significantly different response occurred due to omitting one element, but not the others, such was considered element-specific; and when between the nano and bulk omission of a specific element, such was considered size-specific.

3.1. Soybean vegetative growth and flower production are influenced by nano and bulk scale micronutrients

Compared to the control, exposure to an “All” nanoscale mixture of ZnO, CuO and B_2O_3 enhanced soybean growth. Starting from 4 weeks post-germination, plants treated with the nanoscale mixture demonstrated a significant increase in shoot height that was maintained until maturity. Plants treated with an “All” bulk mixture of ZnO, CuO and B_2O_3 had median shoot heights that did not significantly differ from either the control, or the nanoscale treatment (Fig. 1). With micronutrient omission, the shoot height of the nanoscale omissions when compared with the “All” nano treatment was significantly lower for nano Cu (week 4), nano Cu and nano Zn (week 6), and for all three nano omissions (week 8). This suggests that all three nutrients, consistently so Cu, contributed to stimulating shoot elongation in the plants. In contrast to the nano omissions, no strong differences were observed among bulk Zn and Cu omissions and the “All” bulk treatment. However, shoot elongation of the bulk B omission plants was significantly greater during weeks 2 and 4, compared to the “All” bulk treatment. Notably, the shoot height of plants under B omission was strongly different between the nano and bulk particles from week 4 until maturity, suggesting a size-specific, growth-suppressing effect of bulk B on soybean shoot elongation (Fig. 1).

Shoot branching was significantly increased by the mixture of nano or bulk particles, compared to the control (Table 2). However, omission of nano or bulk Zn from each balanced nutrient mixture reduced shoot

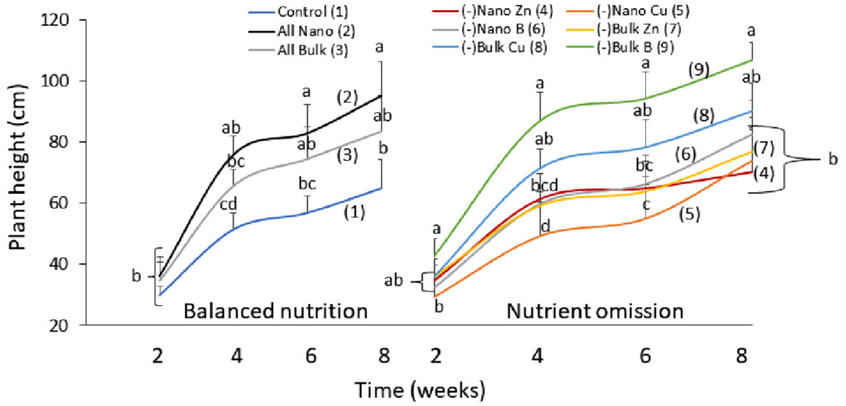

Fig. 1. Effects of balanced fertilization and omission of nanoscale or bulk Zn, Cu or B on the shoot height of soybean in soil. Values are means and SDs, and different letters at each time point indicate statistical differences in the shoot height among all treatments analyzed separately for each time point ($p < 0.05$; n = 3). Treatments were compared together, regardless of spatial separation of graph into "balanced nutrition" and "nutrient omission".

branching to a similar level as the control. In contrast, omission of Cu and B had no effect on shoot branching, regardless of the particle size (Table 2). Regarding shoot biomass, the "All" nano treatment increased shoot dry weight of soybean, compared to the control, while no significant increase was recorded for the "All" bulk treatment (Table 2). Notably, omission of Zn from the mixture both as nanoscale and bulk particles significantly reduced shoot dry weight to the control level. Contrarily, omission of other micronutrients had no effect on shoot biomass production, compared to "All" nano and "All" bulk treatments. However, dry biomass values with these omissions were all significantly greater than the control. Taken together, these findings confirm reports in the literature (e.g., Dimkpa and Bindraban, 2016) that plants often require other nutrients in addition to NPK, specifically micronutrients, to maximize their growth and development potential. Few prior studies using nanoscale Zn, Cu and/or B at low fertilizer-compliant rates in soil have demonstrated their positive effects on soybean vegetative growth (Ngo et al., 2014; Dimkpa et al., 2017b). However, at rates higher than usual for micronutrient fertilizers (≥100 mg/kg), largely deleterious effects have been observed, dependent on the element and exposure concentration (Priester et al., 2012; Yoon et al., 2014; Gautam et al., 2016). At such high levels, presenting these nanoparticles in mixed forms has been shown to mitigate their inhibitory effects in different plant species, compared to single nanoparticle exposure (Dimkpa et al., 2015; Jośko et al., 2017). In the present study, although the plants' vegetative responses were strongly positive towards the "All" nano micronutrients, the responses were not overwhelmingly different between the "All" nano and "All" bulk treatments. This minimizes a role for particle size in the mixed treatments on the measured vegetative variables. It is conceivable that mixing of the particles might have engendered specific three-nutrient interactions that negated size-specific effects, including possible binary or ternary metal occlusions, heteroaggregation, and precipitation. Omitting one nutrient, however, could reconfigure the patterns of nutrient interactions in the mixed system, leading to different plant responses. Based on nutrient omission, it was demonstrated that not all the micronutrients in the mixed nutrient condition evoked responses to the same degree. Data in Table 2 suggest that Zn was predominantly responsible for influencing the growth response. This is likely related to its original low level in the test soil; whereby, Zn is the most limiting nutrient, relative to Cu and B. We point out though that the Zn exposure concentration was double that of Cu and B. This was by design, as soybean requires more Zn than Cu or B for *in planta* metabolism, based on micronutrients removal rates from soil (https://www.spectrumanalytic.com/support/library/ff/Soybean_Growth.htm).

Flower formation was also affected by balanced nutrient and nutrient omission, whereby, the "All" nano, but not the "All" bulk, treatment significantly increased the number of flowers, compared to the control (Table 2). Furthermore, compared to the "All" nano treatment, omission of nano Cu significantly reduced flower number, whereas omission of nano Zn or nano B did not affect flower number. In contrast to nano B, omission of bulk B significantly increased flower number, relative to the "All" bulk treatment; whereas omission of bulk Zn and bulk Cu did not significantly alter flower number (Table 2). Based on these results, Cu and B appear to be more specifically involved in flower formation, whereby Cu omission reduced, while B omission increased flower number. The effect for Cu agrees with reports in the literature concerning the role of Cu in flowering (e.g., Adams et al., 1975). However, the contrasting outcomes for flower formation between nanoscale and bulk Cu are intriguing. In prior studies involving ionic Cu, it was demonstrated that Cu deficiency reduces flower formation in plants (Graves and Sutcliffe, 1974; Adams et al., 1975; Bhakuni et al., 2009). A critical level of Cu for optimum flower formation in daisy (*Chrysanthemum morijiolium*) was reported as 5.5 mg/kg (Graves and Sutcliffe, 1974). The role of Cu in flower formation is thought to be related to its activation of polyphenol and indole acetic acid (IAA) oxidases, enzymes involved in the oxidation of IAA; in the absence of Cu, IAA inhibits flower production (Adams et al., 1975; Bhakuni et al., 2009). For the "All" nano treatment, an amount of Cu that was likely optimum for flower formation in soybean would have been accumulated (see Section 3.2), which improved flower number, and which, when

Table 2
Effects of balanced fertilization and omission of nanoscale or bulk Zn, Cu or B on the vegetative variables of soybean. Values are means and SDs, and different letters after values indicate statistical differences among all treatments, separately for each growth variable ($p < 0.05$; n = 3).

Treatment/variable	Balanced nutrition			Nutrient omission					
	Control	All nano	All bulk	(−) nano Zn	(−) nano Cu	(−) nano B	(−) bulk Zn	(−) bulk Cu	(−) bulk B
Num. shoot branches	3.8c ± 0.8	5.4a ± 0.9	5.6a ± 0.9	4.4bc ± 1.1	6.2a ± 0.4	5.4a ± 0.5	4.4bc ± 0.5	5.2ab ± 0.8	5ab ± 0.7
Shoot dry weight (g/plant)	13.6bc ± 2.2	18.2a ± 2.9	17.4ab ± 4.4	12.7c ± 1.6	19.5a ± 4.2	20.5a ± 3.1	13.1c ± 2.4	18.5a ± 4.8	18.4a ± 3.2
Num. flowers	9.6d ± 0.5	15.6abc ± 1.3	12.2bcd ± 1.3	10.4cd ± 1.8	8d ± 1.9	10.2cd ± 2.1	11.6bcd ± 2.7	16.2ab ± 4.2	18.2a ± 1.8

omitted, caused Cu level in the plant to fall below a threshold that negatively affected flower production. As indicated above, the presence of bulk Cu in the "All" bulk treatment did not increase flower numbers, relative to the control. Thus, its omission also did not affect flower number, when compared to the "All" bulk treatment. Notably, the levels of plant Cu in these treatments are similar. Moreover, plant Cu level in the "All" nano treatment was higher than the "All" bulk treatment (see Section 3.2). Taken together, these findings, perhaps, could explain the lack of effect on flower formation by bulk Cu. We speculate that these opposite outcomes for Cu could be related to the different bioavailability of the Cu types exposed to the plants. Compared to nanoscale CuO, bulk CuO is less likely to release Cu ions in planted systems, as well as less likely to be taken up as intact CuO particles (Dimkpa et al., 2013; Peng et al., 2017). In the case of B, its omission in bulk form increased, rather than reduced, flower formation, relative to "All" bulk treatment. Curiously, this outcome was not observed with nanoscale B. Unfortunately, very limited prior research has been conducted using boron oxide particles as a nutrient for plant growth (Dimkpa et al., 2017b); virtually all the reports of B effects on plants have involved ionic B (boric acid). The role of B in flower formation, much like Cu, is related to hormonal regulation (Adams et al., 1975). Boron deficiency retarded flower formation both quantitatively and qualitatively in black gram (*Vigna mungo*), whereby seed B was between 37% and 50% lower than in B sufficient plants (Pandey and Gupta, 2013). However, an earlier study showed that B deficiency alone did not affect flower formation in daisy, but worsened the lack of flower development, when simultaneously deficient with Cu (Adams et al., 1975). Our observation for B does not align with these prior reports, as it appears that the presence of B negatively interfered with flower formation in the "All" bulk treatment. Hence, flower number was highest upon omission of bulk B. Notably, the plant B level was significantly reduced upon B omission, compared to the "All" bulk treatment (see Section 3.2). As noted, this was not the case for the plant B level due to nano B omission, relative to the "All" nano treatment. The reason for the different response to flower formation by B based on particle size is unclear at this time. It would, thus, be interesting to further understand the effect of B types on flower formation in agricultural plants.

3.2. Above-ground Cu and B uptake is differently affected by nano and bulk particles

As expected, inclusion of Cu and B as nanoscale or bulk particles in the balanced nutrient formulation significantly increased their uptake into soybean above-ground (shoot and grain) tissues, compared to the control treatment. For Cu, the increase was stronger with nanoscale than with bulk particles, while the opposite was observed for B. Upon omission of nanoscale Cu, Cu accumulation was decreased. In contrast, omission of bulk Cu did not alter Cu uptake in the plant. In the case of B omission, absence of the nanoscale compound did not significantly alter B accumulation; whereas, bulk B omission strongly reduced above-ground tissue B content (Table 3). Thus, there was a nano-specific preference for Cu uptake by the plant that was not observed with B. Previously, soybean exposed to an "All" nanoscale mixture of these micronutrients had more B, but similar Cu content in the grain, when compared to the control treatment (Dimkpa et al., 2017b). However, unlike the present study, those experiments were conducted under drought conditions that affected overall nutrient bioavailability and uptake. Here, the higher Cu content in the "All" nanoscale treatment than in the "All" bulk treatment suggested greater bioavailability of Cu from the smaller-sized particles under conditions where water supply is not limiting. Conversely, the data for B precludes any nano-size benefit, as nanoscale B was less bioaccumulated than the bulk B. Unfortunately, no studies have yet compared the solubility and plant uptake patterns of nanoscale vs. bulk B oxides. However, looking at B levels in all the B-containing treatments in Table 3, they were significantly higher, or tended to be higher, in the bulk, than the nano, treatments. Interaction among nutrients in the rhizosphere is known to affect their mutual uptake into the plant, and as such, nutrient ratios play a critical role. A synergistic interaction of Cu and Zn, as well as of B and Zn, mutually favoring nutrient uptake has been reported in plants (Agarwala et al., 1995; Çikili et al., 2015). In the present study, omission of nanoscale Zn reduced the plant Cu content, relative to the "All" nano scale plants, while the omission of bulk Zn lowered the level of B, relative to the "All" bulk treatment. These findings suggest that the higher level of Cu or B in the "All" nano scale or "All" bulk treatments, respectively, could be related to a beneficial Zn interaction. Nevertheless, why such Cu-Zn or B-Zn interactions occurred at the observed particle size scales, but not the other, remains unknown.

3.3. Omission of Zn negatively affects soybean grain yield

Compared to the control treatment, grain yield was significantly increased by the "All" nano scale and "All" bulk treatments; however, grain yield was not different between the "All" nano scale and "All" bulk particle mixtures (Fig. 2). When Zn was omitted from the respective balanced nutrient mixtures, there were significant declines in yield, compared to the "All" nano or "All" bulk mixture. The decline was significantly stronger with nanoscale Zn omission than with omission of bulk Zn. In contrast to Zn, omissions of nanoscale and bulk Cu or B did not depress grain yield, compared to the respective balanced nutrient mixtures (Fig. 2). Taken together, these findings indicate that Zn is responsible for stimulating grain yield in this Zn-deficient soil. In previous reports, exposure to Zn or Cu nanoparticles positively affected soybean seed yield in soil (Priester et al., 2012; Ngo et al., 2014). Moreover, we reported that foliar exposure to a mixed nanoscale Zn, Cu and B oxide treatment increased soybean yield under drought stress in the same soil used in the present study (Dimkpa et al., 2017b). In that study, a correlation of grain nutrient uptake with yield suggested that Zn, rather than Cu and B, was influencing grain yield. Here, by using a nutrient omission strategy, the specific role of Zn in stimulating soybean grain yield in this soil was confirmed. However, an equally interesting observation is the finding that omission of nanoscale Zn was more detrimental to grain yield than omission of bulk Zn. Therefore, given the lack of comparative studies on the effects of nanoscale vs. bulk ZnO particles in soybean grown to full maturity in soil, the present result represents a novel insight on nano-specific effects of Zn omission on crop yield. Notable also is the overall improvement in soybean yield by Zn, under a low N application rate.

3.4. Zn fertilization improves soybean Zn accumulation and grain Zn concentration

Soybean grain yield increases occurred with concurrent significant increases in the shoot Zn accumulation from both nanoscale and bulk

Table 3
Effects of balanced fertilization and omission of nanoscale or bulk Zn, Cu or B on above-ground accumulation (mg/plant) of Cu and B in soybean. Values are means and SDs, and different letters after values indicate statistical differences among all treatments, separately for each nutrient ($p < 0.05$; n = 3).

Treatment/variable	Balanced nutrition			Nutrient omission					
	Control	All nano	All bulk	(−) nano Zn	(−) nano Cu	(−) nano B	(−) bulk Zn	(−) bulk Cu	(−) bulk B
Cu	0.33d ± 0.04	0.45a ± 0.03	0.39bc ± 0.06	0.39bc ± 0.03	0.34cd ± 0.01	0.44ab ± 0.06	0.36cd ± 0.03	0.36cd ± 0.03	0.38bc ± 0.04
B	1.62e ± 0.1	2.78bc ± 0.3	3.67a ± 0.3	2.27ecd ± 0.2	2.48bcd ± 0.3	2.35cd ± 0.3	2.91bc ± 0.2	3.32ab ± 0.5	2.17ed ± 0.7

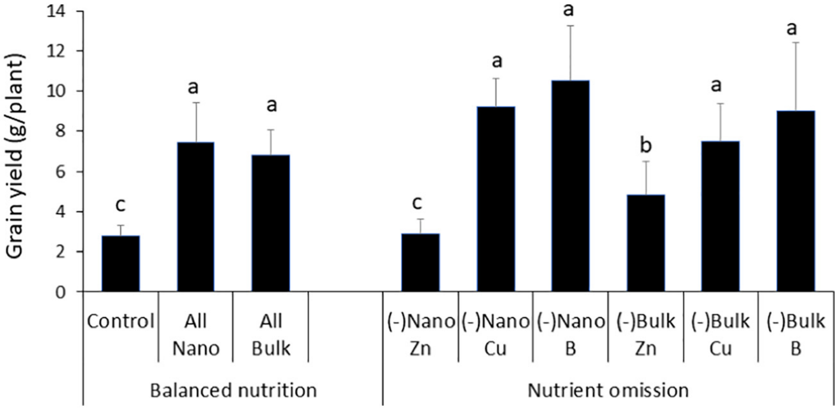

Fig. 2. Effects of balanced fertilization and omission of nanoscale or bulk Zn, Cu or B on the grain yield of soybean in soil. Values are means and SDs, and different letters on bars indicate statistical differences among all treatments ($p < 0.05$; n = 3). Treatments were compared together, regardless of spatial separation of graph into "balanced nutrition" and "nutrient omission".

oxide mixed treatments, compared to the control. Hence, omitting each Zn type significantly reduced shoot Zn uptake, relative to the "All" nano or "All" bulk treatment. Omissions of Cu and B did not affect Zn uptake in the shoot, regardless of particle size (Fig. 3). The initial pH of the soil used in this study was near neutral (6.87). After plant growth, soil pH was lowered in all the treatments to between 6.37 and 6.57 (see Section 3.9), presumably by the combined actions of organic acids contained in root exudates and the metabolism of ammonium used as the N source. However, the pH was not significantly different among the treatments. Thus, no significant particle-size dependent differences in Zn uptake were evident in the plants. This agrees with García-Gómez et al. (2017), who also reported similar shoot Zn uptake from nanoscale and bulk ZnO by bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*), separately in acidic (pH 5.39) and alkaline (pH 8.46) soils. Thus, while soil pH could modulate Zn dissolution and plant uptake (Watson et al., 2015), whether it does so differently between nano and bulk oxides remains to be seen.

Due to the human health implication of fortifying edible plant tissues with Zn, the Zn content of the soybean grains was analyzed separately from the shoot content. As shown in Fig. 3, grain Zn concentrations of plants treated with the "All" nano and "All" bulk mixtures were significantly increased, compared to the control. However, as with observation in the shoot Zn uptake, the subsequent omission of Zn resulted in a strong reduction in grain Zn concentration for the nanoscale Zn, but a less strong reduction for the bulk Zn, each compared to their respective balanced nutrition treatment. The obtained grain Zn concentrations with fertilization indicated that particulate Zn (nano or bulk oxide) can enhance grain levels of the nutrient within the human dietary needs, while still being below the upper tolerable limit for toxicity (Priester et al., 2012; Ebbs et al., 2016). Comparing patterns of grain yield and grain nutrient content show that high Zn accumulation upon Zn fertilization circumvented grain nutrient (in this case Zn) dilution that occurs as grain yield increases. Thus, as with conventional (bulk or ionic) Zn, nano Zn under judicious exposure can be used to fortify plants with Zn, without the expectation of any negative impact on the grain Zn nutritional quality due to heightened nanoscale reactivity. Interestingly, relative to the control, 205% and 196% more Zn were bioaccumulated in the plant shoots in the "All" nano scale and "All" bulk treatments, respectively. However, Zn grain translocation factor (TF; ratio of grain Zn to shoot Zn) was higher in the control plants, compared to the "All" nano scale and "All" bulk treatments. The TFs were 1.79 for the control, and ~0.87 for both the "All" nano and "All" bulk treatments. The higher TF in the control treatment could be explained by the likelihood of Zn-starved plants responding to low Zn supply more efficiently, in which case more of the shoot Zn in these plants was translocated and partitioned in the grain, as compared to the other treatments. It would appear that in soybean, as in certain rice cultivars (Impa et al., 2013a, 2013b), mechanisms associated with Zn translocation to the grain can

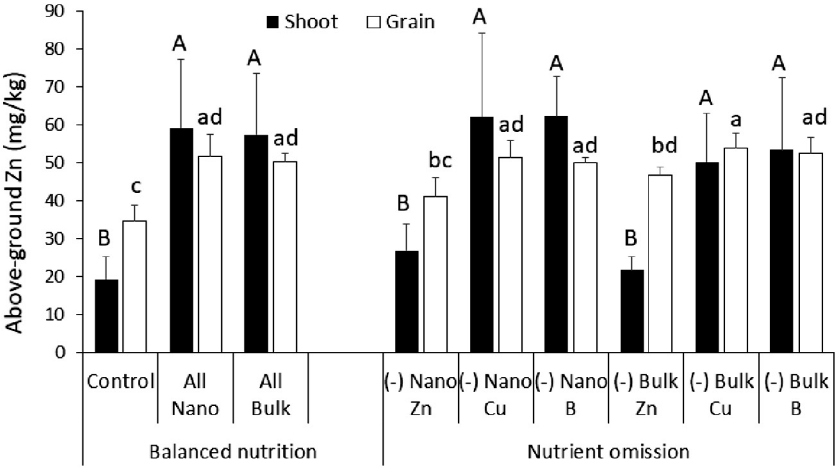

Fig. 3. Effects of balanced fertilization and omission of nanoscale or bulk Zn, Cu or B on Zn accumulation in soybean. Values are means and SDs, and different letters on bars indicate statistical differences among all treatments, separately for shoot (upper case) and grain (lower case) ($p < 0.05$; n = 3). Treatments were compared together for each variable, regardless of spatial separation of graph into "balanced nutrition" and "nutrient omission".

be modulated to prevent excessive grain Zn accumulation under high shoot Zn. It did not seem that the co-presence of Cu and B in the shoots affected the efficiency of Zn translocation to the grain, given that the levels of grain Zn in the Cu omission (having B) and B omission (having Cu) were similar to the "All" nano and "All" bulk treatments. This is the case, given the lower ratio of Cu/B to Zn in the exposures, which reduces Cu competitiveness for plant uptake relative to Zn. Importantly, this finding appears to be crop species-dependent. For example, calculations using data from Peralta-Videa et al. (2014) and Mukherjee et al. (2016) based on the sum of leaf and stem Zn contents support the present observation, whereby grain Zn TFs in soybean and green pea were higher in control than in the nanoscale (50 mg/kg or 250 mg/kg) and bulk ZnO (250 mg/kg) treatments. Notably, as with the present study, the TFs for the nanoscale and bulk ZnO were similar to the study of Mukherjee et al. (2016) involving green pea and nano and bulk ZnO. Contrary to these legumes, for sorghum and wheat, we previously observed higher Zn TFs under nanoparticle and ionic Zn exposures, than in the control treatment (Dimkpa et al., 2017a, 2018b).

3.5. Omission of nano Zn and nano cu inhibits N accumulation

Above-ground (shoot + grain) N uptake was significantly increased by the "All" nanoscale treatment, compared to both the control and "All" bulk treatments; in contrast, the "All" bulk treatment had no effect on N accumulation, relative to the control (Fig. 4). Exclusion of nanoscale Zn and Cu significantly reduced above-ground N, indicating that these elements were important for stimulating N uptake. In contrast, omission of each of the test nutrients from the "All" bulk treatment had no significant impact on N level. This indicates a particle size-dependent response of the plants to N acquisition. Stimulation of N uptake in soybean by the micronutrients is one of the key outcomes of this study, considering the critical importance of improving plant N use efficiency to minimize GHG (nitrous oxide) emission, and to increase the sustainability of agriculture in general. However, few studies have investigated the effect of nanoscale micronutrients on N acquisition by plants. For instance, exposure to a fresh mixture of nanoscale or ionic Zn-Cu-B with N, as well as to N-Zn alone, increased N uptake in shoot and/or grain tissues in several crop plants, including soybean, sorghum, and wheat (Dimkpa et al., 2017a, 2017b, 2018b). However, in the case of the mixed formulation, Zn rather than Cu or B was thought to be involved in the effect. In rice, omission of ionic Zn, Cu or B from an all-nutrient mixture resulted in a significant (12%), and insignificant (8% or 7%), respectively, averaged decreases in N accumulation from two soils (Singh et al., 2018). The results from the current study corroborate the earlier findings as they concern Zn and Cu, but also demonstrate for the first time a lack of similar outcome with bulk Zn and Cu oxide. Notably, there was significantly higher level of Cu in the soil of the nanoscale Zn omission, and a correspondingly high level of Zn in the nanoscale Cu omission, relative to omission of their corresponding bulk counterparts (see Section 3.9). It would, thus, appear that these two metals acted in concert at the nanoscale to modulate N-related activities in the root. That said, the mechanism underlying metal influence on N uptake is hinged upon their potential to alter microbial ammonification or nitrification rates via influencing urease, dehydrogenase, and nitrification enzymes activities (Chaperon and Sauvé, 2007; Grohs et al., 2011), leading to reduced N losses as ammonia and/or nitrous/nitric oxide. For instance, Khariri et al. (2016) demonstrated in rice soils that a urea-Zn-Cu formulation could lower ammonia loss by 35%, while omission of Zn or Cu lowered ammonia loss by 20% or 28%, respectively. In the same study, the urea-Zn-Cu formulation lowered N_2O emission by 28%; and omitting Zn or Cu lowered N_2O emission by 22% or 18%, respectively. Because ammonium nitrate was the form of N used in the present study, ammonium transformation via nitrification would have occurred. Taken together, although these prior studies did not directly correlate reductions in gaseous N losses to increased N uptake by plants, intuitively, we postulate that reduced N loss allows for more N to be available in the rhizosphere for plant uptake. This assumes that overall loss from nitrate leaching is also reduced by a decreased nitrification rate. Notably, the reports described above for N loss mitigation were observed with ionic micronutrients. Research over whether similar effects can also be observed with nanoscale micronutrients is currently underway.

3.6. Addition-omission of nano Zn and bulk B modulate above-ground P accumulation

Exposure to the micronutrients significantly lowered above-ground P accumulation in the plants, irrespective of micronutrient particle size. With nutrient omission, above-ground P level was restored to the control level by nanoscale Zn and bulk B. In contrast, omissions of nanoscale Cu and nanoscale B, as well as bulk Zn and bulk Cu, did not affect above-ground P levels, relative to the respective particle mixtures (Fig. 5). This indicates that Zn in the nano mixture and B in the bulk mixture were responsible for inhibiting P uptake. The negative interaction between P

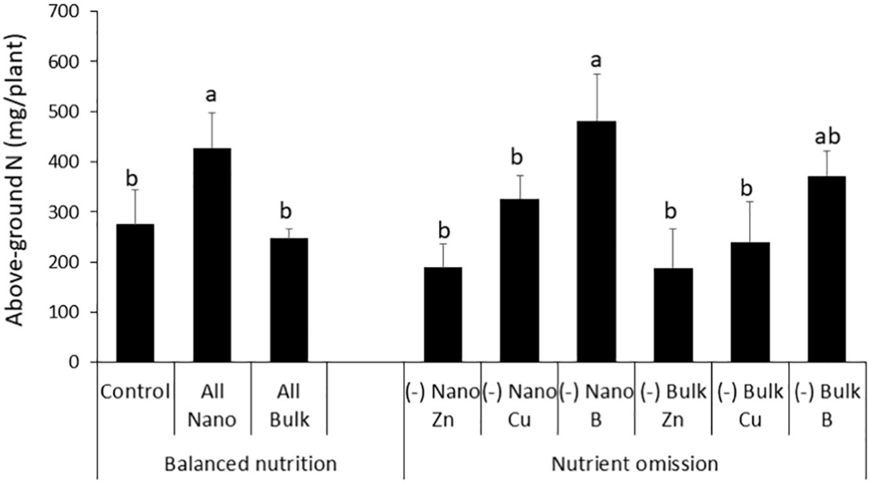

Fig. 4. Effects of balanced fertilization and omission of nanoscale or bulk Zn, Cu or B on nitrogen accumulation in soybean. Values are means and SDs, and different letters on bars indicate statistical differences among all treatments ($p < 0.05$; n = 3). Treatments were compared together, regardless of spatial separation of graph into "balanced nutrition" and "nutrient omission".

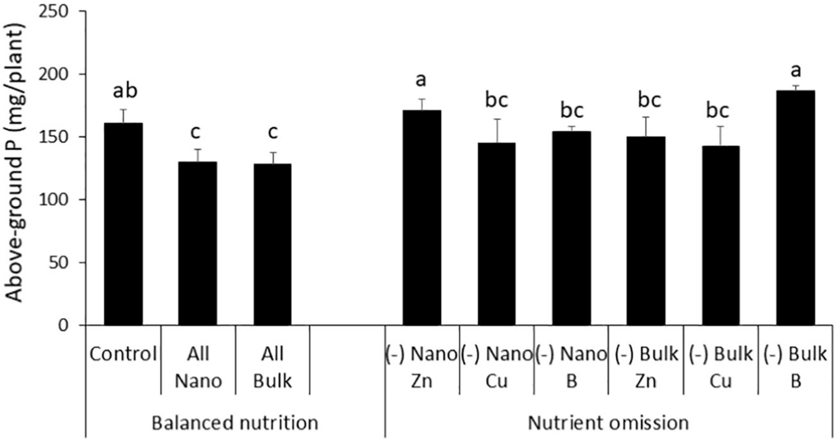

Fig. 5. Effects of balanced fertilization and omission of nanoscale or bulk Zn, Cu or B on phosphorus accumulation in soybean. Values are means and SDs, and different letters on bars indicate statistical differences among all treatments ($p < 0.05$; n = 3). Treatments were compared together, regardless of spatial separation of graph into "balanced nutrition" and "nutrient omission".

and Zn is well reported in the literature, and is based primarily on the formation of Zn-phosphate or Zn-phytate complexes that occur especially under high P intensity (Lv et al., 2012, 2015; Dimkpa et al., 2013; Zhang et al., 2018). Zn-phosphate is highly immobile, and when it occurs in the rhizosphere, can limit the uptake of P by plants (Watts-Williams et al., 2014; Dimkpa et al., 2017a). This inhibition was noticeable in the current study with the "All" nano and "All" bulk mixtures containing ZnO. However, omission of nanoscale but not of bulk ZnO negated the inhibition, indicating a size-specific role for Zn in P uptake interference in soybean. Soil with the "All" nano treatment had a slightly higher amount of post-harvest Zn than the "All" bulk treatment (see Section 3.9). It is, therefore, likely that the higher reactivity of nanoscale than bulk ZnO in terms of dissolution rates (McBeath and McLaughlin, 2014) played a role in these outcomes, since formation of Zn-phosphate occurs primarily with Zn ions dissolved from the particles (Lv et al., 2012). We demonstrated in a previous study that soybean shoot accumulation of P was decreased by foliar exposure to a mixture of Zn, Cu and B nanoscale oxides at low rates comparable to those used in the present study (Dimkpa et al., 2017b). Also, in a single-element soil exposure situation, Peralta-Videa et al. (2014) reported inhibition of above-ground P accumulation in soybean by ZnO nanoparticles (50 mg/kg). Intact ZnO nanoparticles that penetrate root cells (Bandyopadhyay et al., 2015) may undergo transformation, potentially with root P, and obstruct P translocation to shoot. In contrast, ZnO nanoparticles from soil amendment did not affect the above-ground accumulation of P in wheat (Dimkpa et al., 2018b), and only did so in sorghum when P levels were high (Dimkpa et al., 2017a). Notably, omission of bulk B negated the reduction in P uptake observed with the "All" bulk treatment; but this was not observed with the nano B treatment. The bulk B omission plants contained 2.17 mg of B, and there was a negation of the P inhibition observed in the "All" bulk treatment. In contrast, the nano B omission plants contained 2.35 mg B, and there was no negation of the P inhibition caused by the "All" nano treatment. The differences in the plant B contents between the "All" nano and nano B omission (0.43 mg) vs. "All" bulk and bulk B omission (1.5 mg) suggest, perhaps, that a critical amount of additional B (≥1.5 mg) in the above-ground plant tissue might play a role in impeding P acquisition in soybean. Like soybean, maize plants exposed to low B (from ionic B) contained higher tissue P than plants with higher B exposure (Chatterjee et al., 1990). In the present study, the "All" bulk treatment had 32% more B than the "All" nano treatment; yet, both treatments inhibited P uptake similarly. The mechanism for the size-dependent effect of B on P accumulation upon B omission deserves further investigation.

3.7. High grain P-to-Zn ratio is reduced by Zn fertilization

The reduced amount of P in the tissues of plants exposed to "All" nano and "All" bulk treatments prompted us to investigate the ratios of P and Zn in the grain, with the intention of determining to what degree the relative grain P and Zn levels might influence grain Zn bioavailability for subsequent human/animal nutrition. Fig. 6 demonstrates that grain P content was 224 times greater than grain Zn content in the control plants. However, the presence of Zn in the "All" nano treatment significantly lowered the P to Zn ratio to 139. Similarly, the presence of Zn in the "All" bulk treatment also lowered the ratio of P to Zn, though to a slightly lesser extent (157) than the nanoparticle treatment. Phosphorus exists in plant tissues principally in the form of phytate (or phytic acid). Previous studies have demonstrated that Zn in the plant tissue will bind to this P form, leading to the formation of Zn-phytate, an insoluble complex that greatly affects the dietary bioavailability of Zn (Raboy and Dickinson, 1984; Dimkpa et al., 2013, 2018b; Magallanes-López et al., 2017; Zhang et al., 2018). Data in Fig. 6 suggests that Zn fertilization with nano and bulk ZnO, coupled with its translocation to the grain, lowered P-Zn ratios, thereby minimizing the effect of the overwhelmingly high grain P to Zn ratios on the formation of Zn-phytate. This allows for some availability of free Zn not complexed with P, thereby potentially improving Zn bioavailability for human nutrition.

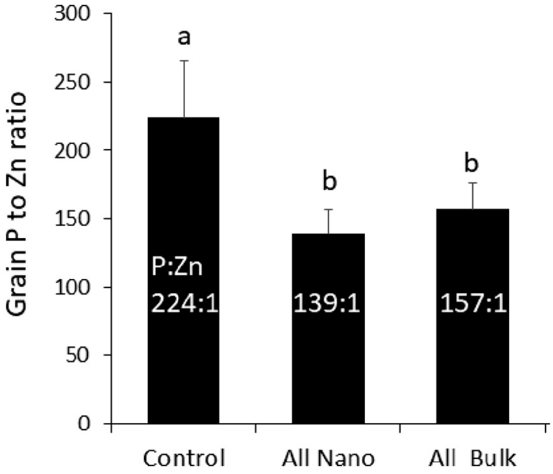

Fig. 6. Effects of nanoscale or bulk oxide Zn, Cu and B on the phosphorus/zinc ratios in soybean grain. Values are means and SDs, and different letters on bars indicate statistical differences among all treatments ($p < 0.05$; n = 3).

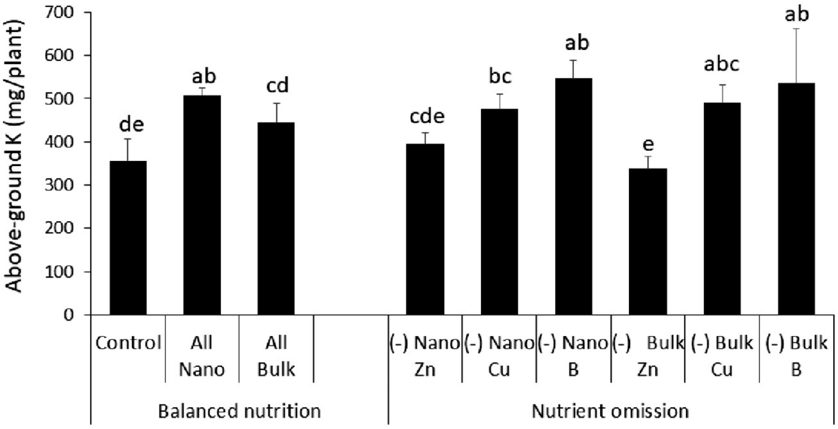

Fig. 7. Effects of balanced fertilization and omission of nanoscale or bulk Zn, Cu or B on potassium accumulation in soybean. Values are means and SDs, and different letters on bars indicate statistical differences among all treatments ($p < 0.05$; n = 3). Treatments were compared together, regardless of spatial separation of graph into "balanced nutrition" and "nutrient omission".

3.8. Omission of Zn reduces K accumulation

The level of above-ground K accumulation in the control treatment was strongly promoted by the presence of Zn, Cu, and B in the "All" nano treatment; in contrast, the effect of the "All" bulk treatment was not significant, relative to the control. Notably, the "All" nano-treated plants also contained significantly more K than the "All" bulk-treated plants (Fig. 7). Omission of nano and bulk Zn significantly reduced K uptake by the plant, reverting the K levels to the control level. In contrast to Zn, K uptake upon omission of Cu and B was not significantly affected, regardless of Cu and B particle size (Fig. 7). These findings imply that the presence of Zn was directly influencing the stimulation of K uptake in the mixed exposure plants. Exposure to ZnO nanoparticles has previously been reported to increase K accumulation in several crops, including wheat, sorghum, and bean (the last crop example depended on dose and soil) (Stewart et al., 2015; Dimkpa et al., 2017a, 2017b; Medina-Velo et al., 2017). This outcome was not different between nanoscale and bulk Zn for bean (Medina-Velo et al., 2017), as was also observed for soybean in the present study, based on the omission treatments. Interestingly, omission of Zn (ionic) from an NPK fertilization depressed K accumulation in rice (Dash et al., 2015), as it did here for soybean. The mechanism surrounding the potential uptake interaction between Zn and K is unresolved. However, being a competitive divalent metal, it seems plausible that Zn ions released from the particles could alter potentials across the root plasma membrane, thereby facilitating K uptake.

3.9. N and P levels in post-harvest soil are influenced by micronutrients omission

Due to the dynamics of N and P in soil in terms of potential below-ground losses (nitrate-N or P leaching) or fixation (in case of P), the potentially bioavailable fractions of N and P in the treated soils were analyzed upon plant harvest. For N, the post-harvest soil N levels were not strongly affected by the presence of micronutrients, irrespective of particle size, when compared to the control treatment (Table 4). With nutrient omission, the level of post-harvest soil N was significantly greater in the absence of nanoscale Cu, compared to the "All" nano treatment. In contrast to Cu, the absence of other micronutrients had no significant effect on soil N, irrespective of particle size, when compared to "All" nano or "All" bulk treatment, respectively. Notably, while omission of nanoscale Zn and Cu each depressed N accumulation in the plant, relative to the "All" nano treatment (Fig. 4), only in the case of Cu omission was soil N higher than the "All" nano scale exposure. Thus, it seems that Zn and Cu may have slightly different mechanisms of action on N use by the plant: Zn facilitated N uptake more than Cu, while Cu acted to preserve more N in the soil; importantly, both acted in concert to minimize N loss.

For P, soil level was highest in the "All" nano treatment, albeit non-significantly, compared to the control. However, significantly more P was present in the soil with the "All" nano, than with the "All" bulk treatment. All of the nanoscale omissions significantly reduced post-harvest P levels, relative to the "All" nano scale treatment; while all of the bulk omissions did not affect P levels, relative to the "All" bulk treatment (Table 4). Furthermore, the P scenarios presented a noteworthy observation in terms of potential Zn/Cu interaction at the nanoscale that was not observed at the bulk scale in the omission treatments. A trend for low soil available P corresponding to high soil Zn or Cu is evident, suggesting, as previously noted, the possible formation of non-bioavailable metal-P complexes that are not captured by the P extraction method used in this study, whereby only the soluble P fraction is extracted and subsequently measured (Menon et al., 1996).

3.10. Post-harvest Zn and Cu soil levels and their potential interactions

The residual Zn and Cu levels were also measured in the soil. As indicated in Table 4, soil Zn levels in the "All" nano and "All" bulk treatments were, as expected, significantly higher than the level in the

Table 4
Detectible (mg/kg) levels of nutrients in soil, and soil pH after harvest of plants exposed to balanced fertilization and omission of nanoscale or bulk Zn, Cu or B. Values are means and SDs, and different letters after values indicate statistical differences among the treatments, separately for each nutrient and pH ($p < 0.05$; n = 3).

Treatment/variable	Balanced nutrition			Nutrient omission					
	Control	All nano	All bulk	(−) nano Zn	(−) nano Cu	(−) nano B	(−) bulk Zn	(−) bulk Cu	(−) bulk B
N	4.61ab ± 1.2	3.54b ± 0.7	4.19ab ± 0.9	3.86ab ± 1.2	5.61a ± 0.7	4.16ab ± 1.0	2.84b ± 0.3	3.12b ± 0.3	5.49a ± 1.1
P	26.8ab ± 8.5	34.5a ± 6.1	24.5b ± 8.8	20.1b ± 6.7	19.5b ± 2.9	19.1b ± 6.4	26.0ab ± 7.5	22.2b ± 4.2	21.6b ± 5.6
Zn	0.23d ± 0.09	0.67ab ± 0.06	0.58bc ± 0.17	0.19d ± 0.06	0.69a ± 0.14	0.64ab ± 0.15	0.15d ± 0.02	0.49bc ± 0.12	0.53b ± 0.10
Cu	0.18c ± 0.02	0.35b ± 0.07	0.36b ± 0.10	0.62a ± 0.14	0.25bc ± 0.05	0.59a ± 0.26	0.36b ± 0.10	0.21bc ± 0.02	0.37b ± 0.03
pH	6.41a ± 0.2	6.40a ± 0.2	6.37a ± 0.1	6.57a ± 0.1	6.47a ± 0.1	6.43a ± 0.2	6.40a ± 0.2	6.56a ± 0.1	6.50a ± 0.2

control treatment. As also expected, omission of nano or bulk Zn significantly lowered residual Zn levels, while Cu or B omission did not affect residual Zn levels. The Zn data in all the Zn-containing treatments presented a clear trend for more soluble Zn to be present in the nanoscale than in the bulk treatments, ranging between 16% ("All" nano vs. "All" bulk), 41% (minus nanoscale Cu vs. minus bulk Cu), and 21% (minus nanoscale B vs. minus bulk B). While the values may not be statistically significant in all cases, it supports previous reports that smaller-sized ZnO particles release more Zn ions than larger-sized materials in planted systems (Dimkpa et al., 2012; McBeath and McLaughlin, 2014). However, other studies have also indicated no difference, or even greater Zn ion release from bulk than nano ZnO (Dimkpa et al., 2013; Bandyopadhyay et al., 2015; García-Gómez et al., 2017). Nevertheless, due to their smaller size, undissolved ZnO nanoparticles can penetrate root cells (Bandyopadhyay et al., 2015), thereby confounding overall bioavailable Zn levels in the rhizosphere under nanoscale exposures. For Cu, levels in the "All" nano and "All" bulk treatments were significantly higher than in the control treatment, but did not differ between each other. Not surprisingly, omission of Cu reverted soil Cu levels to the control value, irrespective of particle size. However, omission of nano Zn or B strongly increased Cu levels in the soil, relative to the "All" nano treatment, while, omission of bulk Zn or B did not affect Cu level, relative to the "All" bulk treatment (Table 4). It is plausible that in both mixed particle systems, micronutrient interactions could have occurred that obfuscated Cu solubility, regardless of particle size. Hence, Cu levels were similar between the "All" nano and "All" bulk treatments. However, the levels were significantly higher in the nano than the bulk treatments, when Zn or B was omitted. Higher solubility for nano CuO than bulk CuO is suggested in previous reports (Shi et al., 2011; Dimkpa et al., 2013; Peng et al., 2017). The high Cu content upon omission of Zn or B in the nanoscale treatments indicated an antagonistic interaction of Zn or B towards Cu in the "All" nano treatment. In the case of Zn, this would not be surprising, given the ratio (2:1) of Zn to Cu in the treatments. Hence, Zn′s influence will dominate over Cu in terms of extractible Cu upon formation, if any, of Cu-Zn oxide complexes. Surprisingly, such antagonistic interaction was not observed with omission of bulk Zn or B, indicating it was nano-specific. We point out that the soil organic matter level and CEC are both low, which could have an overall negative effect on the dissolution, bioavailability, and uptake of amended nutrients. How soil organic matter and CEC influence Zn and Cu dissolution from nano vs. bulk oxide particles is presently being investigated.

Table 5
Summary of variables showing statistically significant (x) and insignificant (−) particle size-specific responses. In the balanced fertilization scenario, comparisons were between "All" nano and the control, and between "All" bulk and the control. In the nutrient omission scenarios, comparisons were made between nano and bulk particles, separately for each nutrient (n = nano; b = bulk).

Balanced Fertilization	**All Nano**	**→ compared to control ←**	**All Bulk**
Effect on:			
Shoot height	x		-
Flower number	x		-
Shoot dry weight	x		-
N uptake	x		-
K uptake	x		-

Nutrient Omission Effect on:	nZnO	bZnO	nCuO	bCuO	nB_2O_3	bB_2O_3
Shoot height						x
Flower number			x			x
Grain yield	x					
B uptake				x		
P uptake	x					x
Post-harvest soil N			x			
Post-harvest soil Zn			x			
Post-harvest soil Cu	x				x	

4. Conclusions

In this study, a micronutrient addition-omission strategy was applied to evaluate the element and size -specific effects of particulate micronutrients on crop growth and development, grain productivity, and plant-soil nutrient dynamics. These effects are summarized in Table 5, indicating that compared to the control, the "All" nano, but not the "All" bulk, treatment stimulated shoot elongation, flower formation, shoot biomass production, and N and K accumulation in soybean. These particular responses are clear indications of nano size-specific effects, under a mixed exposure scenario. However, when the individual micronutrients are compared based on particle size in the omission scenarios, there were both nano and bulk size-specific responses. As presented in Table 5, these responses include: (i) promotion of shoot growth by bulk B omission; (ii) inhibition of flower formation by nanoscale Cu omission; (iii) stimulation of flower formation by bulk B omission; (iv) more severe depression of grain yield by nanoscale Zn omission; (v) enhancement of B accumulation by bulk Cu omission; (vi) stimulation of P accumulation by nanoscale Zn omission; (vii) stimulation of P accumulation by bulk B omission; (viii) increase in residual soil N by omission of nanoscale Cu; (ix) increase in residual soil Zn by omission of nanoscale Cu; and (x) increase in residual soil Cu by omissions of nanoscale Zn and B. Importantly, the use of micronutrients as a multi-responsive amendment for simultaneously addressing agronomic (enhancement of biomass and grain production), environmental (N use management), and human health (Zn biofortification of edible grains) needs was clearly demonstrated in this study. Overall, compared to the bulk microelements, the nanoscale microelements played a greater role in realizing these benefits. Conversely, the well-known antagonism between Zn and P in plants was reaffirmed. The evidence that Zn was primarily responsible for driving the agronomic responses is likely due to it being more limiting in the soil, relative to Cu and B. Interestingly, unlike Cu and B omissions in which specific significant responses were observed at one or the other particle scale, all the significant size-specific responses observed with Zn were due to nanoscale ZnO omission. Nevertheless, regardless of particle size, Zn fertilization of food crops is a realistic strategy to concomitantly increase agricultural productivity, address global human Zn deficiency, and manage N use in cropping systems, contingent upon finding the appropriate conditions for the use of Zn, given its negative effect on P management.

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