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Original Article

Curbing the Growth of Wax Bean (*Vigna unguiculata* L.) *via* a Novel Complex of Nano Zinc Oxide/Vermicompost

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Abstract

Vermicompost (VC) samples were prepared from manure and spent mushroom compost (SMC) and were impregnated with zinc oxide nanoparticles (ZnO NPs), giving ZnO NPs/VC complexes that were added into the soil in which wax beans (*Vigna unguiculata* L.) were then planted. The study was carried out through a factorial experiment in a randomized complete block design with three factors. The experimental factors included: ZnO NPs (0, 0.4, 0.8 and 1.2 mg kg^{-1}), two substrate types (cow manure and SMC) and VC (2.5, 5 and 7.5 weight percentages). To the substrate types, adult earthworms (*Eisenia fetida*) were added. Specifically, after three months, the prepared VC was soaked in ZnO NPs solutions, mixed with soil (according to cultivation substrate weight), then employed in wet plantation of wax beans. The obtained results showed that with increasing ZnO NPs, leaves' chlorophyll, grains number per pod, stem length, hundred grains weight, grain yield, and the grain protein content significantly decreased. In general, the usage of these NPs in the applied amounts could curb the undesired growth of this species.

Keywords: cow manure, chlorophyll, grain yield, vermicompost, ZnO NPs

Introduction

Application of nanoparticles (NPs) has made significant advances in biomedical, pharmaceutical, semiconductor materials, cosmetics, composites, coatings, fabrics, scratch resistant fabrics, stains etc. (Biswas *et al*., 2005). Among them, ZnO NPs have attracted much attention for their anti-bacterial, photo catalytic and piezoelectric and deodorizing properties (Bouvy *et al*., 2007). Application of ZnO NPs decreased seed germination percentage of perennial ryegrass and maize, halted root growth in radish, rapeseed, rye, lettuce, corn and cucumber (Lin and Xing, 2007). Also its application shriveled root tips, destroyed epidermis, and outer root cells (Lin and Xing, 2008).

Zinc itself is an essential trace element for all higher plants and has various metabolic roles. Zn is a vital element for the synthesis of enzymes and a precursor of indole acetic acid (IAA) (Lindsay, 1972). Also, it is a component of carbonic anhydrase (Sarmadnia and Koocheki, 1996). Increasing the amount of Zn and Cd applied to the crop, increased their concentration in leaves, but decreased in the stem (Van Assche *et al*., 1988). In 2010, Lopez and his colleagues investigated the ZnO NPs uptake and accumulation by soybean seeds and observed that the absorbed Zn by buds was significantly more at the concentration of 500 mg l^{-1}. However, in higher doses their uptake and absorption reduced, due to ZnO NPs accumulation. Also by increasing their concentration, root length decreased.

Dimitrioss *et al*. (2009) evaluated the effects of five nano materials (multi-walled carbon nanotubes, Ag NPs, Cu NPs, ZnO NPs and nano silica) on germination, root length and biomass of zucchini (*Cucurbita pepo*). Compared to the

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control, application of Cu NPs decreased the root length. Also, dosed with zinc and silver NPs and carbon nanotubes, plant biomass decreased.

All this considered, the purpose of the hereby study was to probe the cytotoxicity of a new nano zinc oxide/vermicompost complex on wax bean (*Vigna unguiculata* L.).

Materials and Methods

This experiment was conducted as a factorial experiment in a randomized complete block design with three factors including: four levels of ZnO NPs (0.0, 0.4, 0.8 and 1.2 mg kg^{-1}), two levels of substrate type (cow manure and SMC) and three levels of vermicompost (2.5, 5 and 7.5 percent weight of substrate) in three replications.

Earthworms (*Eisenia fetida*), cow manure, SMC and soil were obtained from Tarbiat Modares University research farm.

ZnO NPs were acquired from the Research Institute of Petroleum Industry (RIPI). The properties of the consumed materials are listed in Tables 1 and 2.

The soil had loamy texture, with the acidity of 7.2, EC of 1.6 ds m^{-1} and the amounts of copper and zinc were 2.80 and 1.09 mg kg^{-1}, respectively.

Wax bean seeds (*Vigna unguiculata* L.) were obtained from Plant Breeding and Grain Institute, Karaj, Iran.

To start this experiment, initially the composted and screened cow manure was completely soaked in tap water for one week to release its leach. SMC was also completely submerged and aerated for 10 days for ammonia gas and salts removal with the purpose of impeding the worms' death. Then, the prepared substrates were poured into the pots weighing almost 6 kilograms. Next, 30 adult worms with clitellum segments and the weight of almost 500 to 800 mg were selected and added to the substrates. For vermicompost production, the earthworms were kept at the temperature of 20 to 25 °C (ambient) and moisture of 60 to 80 percent (substrate) which provided a suitable living condition for the earthworms for three months (Edwards,1998). Later, the treated vermicomposts with ZnO NPs ratios of 2.5, 5 and 7.5 weight percentage were completely mixed with soil and placed in the pots.

In order to plant wax beans with the density of 40 plants per square meter (Ghanbary and Taherii, 2004), 6 seeds which had already been disinfected by Mancozeb were wet planted. After seed germination and at the multi leaf stage when plant loss risk was at its minimum, thinning operation was undertaken and three seedlings were placed in each pot.

During the plant growth, all crop cares such as hand weeding, pest and plant diseases fighting and irrigation were provided.

To measure the leaves' chlorophyll before flowering, three leaves were randomly selected from each pot and their chlorophyll was measured using SPAD-502.

After the browning of approximately two third of the pods, the irrigation was terminated and they were harvested at several stages considering their asynchronous maturation. Later, the number of pods and grains per pod were counted and averaged for each bush.

The mean of yield and weight of hundred grains based on 14% moisture were measured by a digital scale. After harvesting the pods, stem length was calculated and averaged for each bush. To measure the biological yield, all bushes at the maturity stage were cut from the crown and dried, weighed and averaged. The harvest index was calculated based on the ratio of the economic yield to the biological yield from the following formula :Harvest index: grain yield / biomass yield × 100.

To measure the grain protein, one gram of each grinded sample was dissolved in 15 cc of sulfuric acid 98% (Merk) and supplemented to the desired quantity by adding 5 g catalyzer. Then, the proteins were determined using Kjeldahl model Analyzer 1030.

To measure the grain Zn concentration, one gram of the obtained grains was grinded and dissolved in one gram nitric acid 65% (Merck - 100 456) and the Zn concentration was calculated by flame atomic absorption instrument model AA670 (Ahmad and Shuhaimi, 2010).

Data analysis was conducted by SAS software in GLM procedure and for mean comparison Duncan's test (α = 5%) was used.

Results and Discussion

Leaf chlorophyll and weight of hundred grains

Analysis of variance showed that the main, mutual and triple interactions were statistically significant on leaves chlorophyll and the main and mutual interactions (except the NPs and vermicompost interaction) were statistically significant on weight of hundred grains (Table 3). With the application of ZnO NPs leaves chlorophyll at the levels of 2.5, 5 and 7.5 percent vermicompost produced from cow manure and SMC reduced to 15, 12 and 16 percent and 4, 19 and 9 percent respectively (Table 4). The highest chlorophyll content was obtained at the first level of ZnO NP with the usage of 5 percent vermicompost produced from SMC. According to Van Assche and Clijsters (1990), significant reduction was observed in photosynthetic pigments such as chlorophyll *a, b* and subsidiary pigments such as carotenoids, as a result of the usage of heavy metals like copper, zinc and lead in many plant species. Also, the factors producing oxidative stress such as heavy metals stress may reduce the chlorophyll content by disturbing the balance in the iteration of complex proteins of optical system II (Laspina *et al*., 2005). Therefore, it seems that the significant decrease in photosynthetic pigments in stressed plants is generally due to their increasing degradation (Vassilev *et al*., 2003). In another study by Bonnet *et al*. (2000), Zn excess reduced chlorophyll synthesis in *Lolium Perenne* L.

Table 1. Properties of cow manure and SMC

EC	12.5	12.37
pH	7.5	7.6
Na (ppm)	62	392
K (ppm)	1,700	2,500
Ca (ppm)	3,400	36
Cu (ppm)	31	18
Zn (ppm)	95	192
O.C (ppm)	15.9	16.61

Table 2. Properties of ZnO NPs

Property	Index value
Purity	99%
Particle mean size	50 nm<
Specific surface area	80 $m^2 g^{-1}$ <

In the current experiment, the results of the triple interactions revealed that with the ZnO NPs increase at the same levels of vermicompost produced from the cow manure and SMC weight of hundred grains decreased to 34, 23 and 18 and 24, 36 and 35 percent respectively. The lowest weight of hundred grains was obtained at the level of 2.5 percent vermicompost produced from cow manure and 1.2 mg kg^{-1} ZnO NP (Table 4). Increasing the use of vermicompost produced from SMC at the same levels of ZnO NPs did not have a significant effect on weight of hundred grains, but the same levels of ZnO NPs (except the first level) increase in the vermicompost usage produced from cow manure increased the weight of hundred grains. A reason for the effect of vermicompost produced from cow manure on the weight of hundred grains could be the availability of nutrients and also the soil biological, physical and chemical properties improvement, since vermicompost usage makes optimal growing conditions during grain filling and more dry substance production causes heavy grains and weight of weight of hundred grains increase.

The replacement of central magnesium ion in chlorophyll by heavy metals (such as Zn) is one of the injuries which prevents light trapping in photosynthesis and therefore, causes chlorophyll loss and photosynthetic activity reduction (Prasad and Strzalka, 1999). On the other hand, Zn is essential in auxin activity, protein synthesis, production and development speed of grain (Malakoti and Lotfollahi, 1998). Therefore, probably the Zn excess could cause disruption in the chlorophyll production of plants and photosynthesis reduction. Also by disrupting grain production and development, the product quantity reduces. According to the results of Gerwing *et al.* (2003), Zn fertilization had no effect on soybean yield. Also, in another study by Chaney (1993), Zn caused yield reduction in Poaceae family.

Protein and zinc concentration in grain

Analysis of variance of grain protein and zinc content showed there was a significant difference among the main, mutual and triple interactions (Table 3). In the case of ZnO NPs usage in the levels of 2.5, 5 and 7.5% of vermicompost produced from cow manure and SMC, the grain protein decreased to 3, 0.5 and 5 and 0.4, 3 and 6 percent respectively. The highest amount of grain protein was obtained using 7.55 vermicompost produced from SMC without ZnO NPs (0 mg kg^{-1}) (Table 4). Given the Zn role in protein synthesis (Malakoti and Lotfollahi, 1998), probably an increase in Zn absorption and uptake causes protein synthesis disruption and protein reduction in grain. These researchers concluded that the disorder in Zn amount may prevent the antioxidant enzymes activity, which results in oxidative damages to protein molecules, chlorophyll and nucleic acids (Cakmak, 2000). In the current experiment, ZnO NPs usage at the levels of 2.5, 5 and 7.5% of vermicompost produced from cow manure and SMC, grain Zn concentration increased to 14, 15 and 12% and 21, 26 and 35% respectively (Table 4). The amount of Zn in leguminous crops and cereals grain is about 20 to 25 ppm (FAO/HWO, 2002); according to data obtained and summarized in Table 4, the Zn amount in grain was more than the critical level and this reflects a better ZnO NPs absorption by plant, which caused toxicity in wax bean.

For metal based NPs (e.g. ZnO), permeability increase and even pores' development in bacterial cell walls with similar size to plant cell wall was reported (Stoimenov *et al.*, 2002). Thus, NPs by passing through the plant cells could accumulate in plant tissues. The results of an investigation by Rengel and Graham (1995) showed that with the application of 0.8 mg Zn to soil, its concentration rose from 12 to 18 mg kg^{-1} in wheat grains.

Table 3. Analysis of variance for some related growth parameters of wax bean at different levels of ZnO NPs

		Mean of Square (MS)								
S.O.V	Df	Chlorophyll (Spad)	100 seed weight	Seed protein	No. of pod. $plant^{-1}$	No. of seed. pod^{-1}	Zinc content in seed	Biological yield	Seed yield	Harvest index
Replication	2	2.68*	3.20 ns	0.002 ns	0.21 ns	0.65**	0.00 ns	44.72 ns	59.79 ns	97.77 ns
A factor (NPs)	3	191.03**	941.41**	2.505**	3.27**	1.167**	190.85**	5487.94**	1794.05**	761.79**
B factor (substrate types)	1	37.55**	109.19**	0.05**	0.62 ns	0.30 ns	7.24**	794**	59.88 ns	709.70**
C factor (Vermicompost)	2	136.58 **	157.98 **	0.936 **	1.10 ns	0.20 ns	44.20 **	1393.03 **	201.86**	120.35 ns
A×B	3	0.84 ns	15.00 *	0.281 **	0.57 ns	0.16 ns	16.74 **	254.45 *	50.14 ns	3.44 ns
A×C	6	6.70 **	4.29 ns	0.512 **	0.57 ns	0.22 ns	1.95 **	230.24 **	19.16 ns	63.62 ns
B×C	2	0.97 ns	90.19 **	0.382 **	0.07 ns	0.42 *	8.42 **	599.47 **	90.25 *	64.16 ns
A×B×C	6	11.11 **	26.97 **	0.252 **	0.48 ns	0.06 ns	2.81 **	144.38 ns	47.16 ns	69.46 ns
Error	46	0.82	5.14	0.001	0.47	0.12	0.00	70.42	27.74	39.43
(cv%)	-	2.30	7.02	0.168	12.34	13.72	0.17	10.81	20.24	18.90

ns Non- significant, ** and * significant at the 1% , 5% probability levels, respectively

Table 4. Mean comparison interaction effect of the NPs, substrate and vermicompost on wax bean

Substrate Types (g kg^{-1})	SMC			Cow manure		
	Levels of vermicompost (%)					
	7.5	5	2.5	7.5	5	2.5
mg kg^{-1}(ZnO NPs)	Chlorophyll (Spad)					
0.0	40.03 ^{cd}	50.06 ^{a}	40.76 ^{def}	41.40 ^{cde}	44.96 ^{b}	42.63 ^{c}
0.4	37.87 ^{hij}	44.63 ^{b}	40.13 ^{efg}	36.70 ^{jkl}	41.46 ^{cde}	38.56 ^{ghi}
0.8	35.30 ^{lm}	39.16 ^{fgh}	38.36 ^{hi}	35.80 ^{klm}	39.46 ^{fgh}	35.90 ^{klm}
1.2	35.90 ^{klm}	37.36 ^{ijk}	38.93 ^{ghi}	32.26 ^{n}	38.43 ^{hi}	34.60 ^{mn}
	Weight of hundred grains (g)					
0.0	41.72 ^{ab}	43.14 ^{ab}	39.56 ^{bc}	41.13 ^{bc}	45.43 ^{a}	37.22 ^{cd}
0.4	30.93 ^{ghf}	32.46 ^{gef}	33.40 ^{def}	39.25 ^{bc}	40.30 ^{bc}	28.93 ^{ghi}
0.8	26.46 ^{ijk}	26.71 ^{hijk}	29.20 ^{fghi}	33.37 ^{def}	35.26 ^{de}	23.02 ^{kl}
1.2	23.89 ^{ijk}	24.23 ^{jkl}	27.09 ^{hijk}	27.51 ^{hij}	29.33 ^{fghi}	21.40 ^{lm}
	Grain protein (%)					
0.0	23.81 ^{a}	23.14 ^{c}	22.38 ^{e}	23.39 ^{b}	22.97 ^{d}	22.94 ^{d}
0.4	22.85 ^{b}	22.81 ^{b}	22.34 ^{c}	22.31 ^{c}	22.92 ^{a}	22.33 ^{c}
0.8	22.14 ^{d}	22.20 ^{c}	22.34 ^{b}	22.32 ^{b}	22.96 ^{a}	22.17 ^{cd}
1.2	22.07 ^{c}	22.11 ^{b}	22.19 ^{b}	22.07 ^{c}	22.66 ^{a}	22.02 ^{c}
	Zinc concentration in grain (mg kg^{-1})					
0.0	29.87 ^{r}	29.54 ^{s}	29.16 ^{t}	32.72 ^{o}	31.56 ^{p}	30.91 ^{q}
0.4	38.30 ^{c}	36.77 ^{h}	34.35 ^{m}	35.26 ^{k}	34.81 ^{l}	33.23 ^{n}
0.8	40.30 ^{b}	37.21 ^{f}	35.46 ^{j}	36.90 ^{g}	36.29 ^{i}	35.36 ^{j}
1.2	42.06 ^{a}	37.48 ^{e}	36.30 ^{i}	37.82 ^{d}	37.50 ^{e}	36.87 ^{g}

Means by the uncommon letter in each row and column are significantly different according to Duncan tests ($p < 0.05$).

Table 5. Mean comparison of ZnO NPs effect on some properties of wax bean

NPs (mg kg^{-1})	No. of pod. $Plant^{-1}$	No. of grain $.Pod^{-1}$	Grain yield (g in bush)
0.0	6.06 ^{a}	2.86	6.97 ^{a}
0.4	5.76 ^{ab}	2.70 ^{ab}	5.36 ^{b}
0.8	5.49 ^{b}	2.50 ^{bc}	3.88 ^{c}
1.2	4.98 ^{c}	2.27 ^{c}	2.77 ^{d}

Means by the uncommon letter in each column are significantly different according to Duncan tests ($p < 0.05$).

Table 6. Mean comparison of interaction effect of substrate type and vermicompost on wax bean

Substrate types (mg kg^{-1})	SMC			Cow manure		
Vermicompost (%)	7.5	5	2.5	7.5	5	2.5
No. of grain. Pod^{-1}	2.22 ^{a}	2.38 ^{b}	2.40 ^{b}	2.73 ^{a}	2.60 ^{ab}	2.61 ^{ab}
Grain yield (g in bush)	4.78 ^{bc}	4.63 ^{bc}	4.34 ^{bc}	5.10 ^{ab}	5.67 ^{a}	3.96 ^{c}

Means by the uncommon letter in each row are significantly different according to Duncan tests ($p < 0.05$).

Number of pods per plant and grains per pod

According to the variance analysis, the effect of ZnO NPs on the pods per plant number was significant (Table 3). With the ZnO NPs increase, the pods per plant number reduced by 11% (Table 5). The grains per pod number were significantly affected by the interaction of the substrate types and vermicompost (Table 3). By increasing the ZnO NPs amount, the number of grains per pod decreased (Table 5). Like other heavy metals, when Zn accumulates in soil and ultimately in plant tissue, some metabolic processes alter, depending on the plant species and thereby interferes with the growth and development of plants (Stoyanova and Doncheva, 2002). Also Zn toxicity causes reduced production, stunted growth, or leaves chlorosis, due to iron deficiency, chlorophyll synthesis reduction, chloroplast decomposition and disturbance in phosphorus, magnesium and manganese absorption (Chaney, 1993). Therefore, it negatively affects the yield components.

By increasing the vermicompost produced from SMC, the number of grains per pod increased. On contrary, vermicompost produced from cow manure had no significant effect on this characteristic (Table 6). In a study by Sagar *et al.* (2009), the application of SMC had a positive effect on the growth and yield in tomatoes, peas, potatoes, ginger, garlic, wheat, rice, maize and apple. It seemed that in the hereby study the positive effect of vermicompost produced from SMC on yield components was related to supplying plant's nutrients, in addition to increasing the soil porosity and property modification.

Yield and harvest index

The main interaction of NPs and vermicompost and the mutual interaction of substrate type and vermicompost on grain yield were significant (Table 3). By increasing the amount of ZnO NPs, the grain yield decreased by 42% (Table 5). According to the results of Lopez *et al.* (2010), probably ZnO NPs reduced soybean (*Glycine max*) root growth and nutrient uptake and also as the result of leaves chlorosis, photosynthesis and plant growth reduced. John *et al.* (2009) reported that the bud's growth reduction under Cu or Zn stress may result from the water potential reduction, hampered nutrient uptake and secondary stress such as oxidative stress, that eventually leads to decrease in yield and yield components. According to Table 6, increase in vermicompost produced from SMC had no significant effect on grain yield. However, vermicompost produced from cow manure increased the grain yield. The reason of significance of the vermicompost effect on grain yield could bet the produced grain yield per unit area as a dependent variable, reliant on yield component. As it was observed in the current experiment, the weight of hundred grains was under the vermicompost influence. Thus, it may be one of the reasons for the grain yield increase. Pirdashti *et al.* (2010) reported that the vermicompost usage, separately and combined with chemical fertilizer, improved the soybean yield. In another experiment by Jat and Ahlawat (2006) on corn and bean, it was found that vermicompost usage caused increased biological yield, grain yield and quality compared with the control.

Conclusions

Regarding the determined amount of Zn in plants, such as established by FAO, the excess amount of it in plants under experiment and the induced toxicity, which disrupted the metabolic and physiological processes, finally led to the decreased plant growth. The reasons of this toxicity may be the solubility of ZnO NPs, as well as their absorption and uptake in the plant tissues. Therefore, in the case of toxicity, the use and entry of NPs into the environment and their effects on plants, more researches are needed to be conducted. Above all, the positive effect of vermicompost produced from cow manure and SMC on yield, yield components and bean grain protein was clearly observed in the current experiment. It seemed that vermicompost provided better conditions for the improvement of helpful microbial activity in the soil and mineral nutrients through optimal absorption by the roots of beans, thus with a beneficial effect on plant growth and development. According to the results of this research it could be concluded that organic fertilizers such as vermicompost can improve yield without environmental impact.

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