IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 13, Issue 10 Ser. I (October. 2019), PP 09-15
www.iosrjournals.org

Effects of Zinc Oxide Nanoparticles on Chlorophyll Content, Growth Attributes, Antioxidant Enzyme Activities And Bioaccumulation of Common Bean (*Phaseolus Vulgaris L.)* Grown In Soil Medium

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Abstract: *The use of conservative products to improve growth and productivity of plants is as old as man. Zinc oxide nanoparticles (ZnONPs) use smart/controlled release and targeted delivery system to improve alot of parameters in plants including yield, thereby making them suitable as fertilizers. This study therefore assessed the effects of ZnONPs on the physiology, chlorophyll content, biochemical activities, antioxidant enzyme activities and bioaccumulation potential of Phaseolus vulgaris after 90 days of exposure to ZnONPs following standard methodologies. The growth parameters examined in this research revealed that there was significant increase at all evaluation times at P <0.05 in all ZnONPs treated plants. Furthermore, there was increase in yield especially at low concentration of 300 mg/kg for all the parameters examined. Flowers and fruits took longer to emerge at high doses of 600 and 1000mg/kg confirming the phytotoxicity tendencies of ZnONPs at higher concentration. Exposure of Phaseolus vulgaris to ZnONPs treatment increased productivity, promoted uptake and bioaccumulation of Zn in the leaf and roots tissues of Phaseolus vulgaris. Futhermore, ZnONPs enhanced the activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidise (APX) and catalase (CAT).*

Keywords: *Enzyme activity, zinc oxide, nanoparticles, phytotoxicity, plant stress*

Date of Submission: 18-09-2019 Date of Acceptance: 05-10-2019

I. Introduction

Zinc is one of the nutrients needed by plants in a minute form for its growth and metabolic processes. Adamson et al. 2000 established that Zn/ZnO shortage is the most widespread micronutrient deficiency and the fourth most fundamental yield or output limiting nutrient after nitrogen, phosphorus, and potassium. Due to the rise in the use of Zn/ZnO in end user products, it is quite likely that through both accidental release and conscious appliance of these materials there may be an escape into atmospheric environments, whether terrestrial or aquatic. Zn/ZnO induces conspicuous effect on many organisms, especially on plants, which are a crucial base ingredient of all ecosystems (Hussein et al. 2002).

Beans (*Phaseolus vulgaris* L.) is also identified to be the common bean, green bean or kidney bean which is an herbaceous annual plant that originated in central and south America but is now cultivated in many parts of the world (Petry et al. 2012) broadly grown for its tasty seeds which add aroma and protein to the diets of millions of the populace all over the globe. This prehistoric crop belongs to the family Leguminosae or Fabaceae and like many other legumes it has a capability to fix nitrogen from the air by a process known as symbiosis with bacteria inhabiting the root nodules. As a result, common bean is high in protein and in many parts of the world it is considered the 'meat of the poor' (Mackinder et al. 2001). The remarkable diversity of colours, textures and tastes of the common bean make it an accepted choice for the common citizens in the world over.

Some notable researchers have described the key role of Zn/ZnO nanomaterials for plant growth, yield, enzyme activity and phytotoxicity (Akanbi-Gada et al. 2019, Hussein et al. 2002, and Zhao et.al 2012). For instance, higher plant mostly absorbs Zn as a divalent cation (Zn^{2+}), which acts either as functional, structural, or as the metal part of enzymes or a regulatory cofactor of several enzymes. However, few literatures are available on the use of zinc-oxide nanoparticles on food crops especially beans. This research therefore is aimed at assessing the effects of zinc-oxide nanoparticles on the physiology, chlorophyll content, and antioxidant enzyme activities of beans.

II. Materials And Methods

Experimental Design

Potted experiment was carried out in a screenhouse at the Botanical Garden of the University of Ilorin, Ilorin, Kwara State, Nigeria. The experiment is a Completely Randomised Block Design (CRBD). Healthy seeds of beans were grown in pots containing 2kg of sandy loamy soil spiked with ZnONPs. The treatments consisted of four different concentrations (0 mg/kg, 300 mg/kg, 600 mg/kg and 1000 mg/kg), each was replicated thrice to make a total of 12 pots. The germination rate of beans was determined according to the method of Bolaji et al. (2015) with slight modifications. Determination of seed germination of beans commenced 2 weeks after planting and continued fortnightly. Measurement of growth parameters such as leaf length, stem girth, number of leaves and leaf area also commenced 2 weeks after planting and continued fortnightly till plants were harvested.

Determination of chlorophylls and enzymes activity

Chlorophyll contents were determined by acetone incubation method, which was done after 15 days and 30 days of growth according to the method described by Makeen et al. (2007). Briefly, fifty milligram (50 mg) of the leaf tissue was measured and placed in a sample bottle. Five millilitres (5mls) of 80 % acetone was added. The sample bottle was shaken from time to time, and later placed in a refrigerator for 48 hrs. The extracted liquid was filtered through glass wool to remove leaf pieces and transferred to another graduated tube. Absorbance were read using spectrophotometer and Arnon's equation was used to convert absorbance measurements to mg chl g^{-1} using V-3000PC Spectrophotometer.

Plant parts (roots and shoot) were harvested and homogenise with liquid nitrogen. One ml (1ml) of phosphate buffer was added per 100mg plant (fresh weight). The homogenate was centrifuged at 4000 × g at 4°C for 10 min to remove gross plant debris. The supernatant was diluted two-fold in phosphate buffer (this was kept aside for enzyme activities analysis).

Determination of Malondialdehyde (MDA) and Hydrogen Peroxide (H_2O_2)

Lipid peroxidation of the formed MDA in the sample was determined according to the method of Li (2000). The plant material (leaf or root) was homogenized using pestle and mortar with 3 ml of 0.5 % thiobarbituric acid in 20 % thiochloroacetic acid (w/v).The reacting mixture was incubated at 100°c for about 30 min and the reaction was terminated in cooled water. The sample was centrifuged 10,000 g for 10min.Absorbance was measured spectrophotometrically at three different wave lengths. MDA concentration was determined by the following formula: MDA (µmol/L) = 6.45 (A_{532}-A_{600}) - 0.56A_{450}

Determination of Hydrogen Peroxide was by the methods of Nakano and Asada (1981).The root or leaf parts were harvested and homogenized with liquid nitrogen, about 1 ml of phosphate buffer was added per 100 mg plant (fresh weight). The homogenates were centrifuged at 4000 × g at 4°C for 10 min to remove gross particles plant debris, then the supernatant was diluted in two-fold in phosphate buffer. To 100 µl of the supernatant, 450 µl of 17m M $H_2O_2$and 450 µl of 25 Nm ascorbate was added. Absorbance was read at 290 nm for 3 min. One unit of enzyme activity is defined as a decrease in absorbance of 0.001/min at 290 nm

In determining ascorbate peroxidase (APX), to 100µl of the supernatant, about 450 µl of 17mM $H_2O_2$and 450 µl of 25Nm ascorbate was added and absorbance was read at 290 nm for about 3 min (Nakano and Asada, 1981). A decline in absorbance of 0.001/min at 290nm is defined as one unit of enzyme activity.

Catalase (CAT) activity in the plant was determined according to the method described by Aebi (1974). To about 666µl of the initial supernatant, 334 µl of 73Mm H_2O_2 was added and absorbance was read at 240 nm for 3 min. One unit of enzyme activity is defined as a decrease in absorbance of 0.001/min at 240nm

Superoxide dismutase (SOD)was determined according to the method of Beyer and Fidovich (1987) 1 g of fresh leaf and root samples was homogenized under ice cold conditions in 3ml extraction buffer, containing 50 Mm phosphate buffer (pH 7.4). One gram (1 g) of Polyvinyl pyrrolidone (PVP) and 0.5 % (v/v) Triton X-100 at 4°C was added, this was centrifuged at 10,000 ×g for 20 min. The supernatant fraction for the assay was used. To about 50-150µl extract, 3.5 ml of O_2 generating solution containing 14.3 Mm methionine, 82.5 µM NBT and 2.2 µM riboflavin was added. The extract was adjusted to a final volume of 0.3 ml with 50Mm K-phosphate (pH 7.8 and 0.1 Mm Na_2 EDTA. The reaction was allowed to run for 10 min and ended by switching off the light. The change in Nitro blue tetrazolium (NBT) was determined by reading absorbance at 560 nm. One unit of SOD is defined as the amount of enzyme that produced a 50 % inhibition of NBT reduction under assay conditions. All enzymes were assessed using U-V/ visible spectrophotometer (model 752N).

Determination of Zn contents

Root/shoot was dried in an oven at 70 ° C for 24 hrs and one gram (1g) was measured, it was grinded with pestle and mortar then weighed into a clean digestion flask. About 9 ml of Conc. HNO_3 and 3 ml of Conc. HCl was added (3:1). The sample was heated on a hot plate and data were obtained using Atomic Absorption Spectrophotometer at 350 nm.

Determination of Protein, Total Soluble Sugar, and Starch To determine crude protein content, the conventional method of Micro Kjedahl was used as described AOAC (2000) .

Total soluble sugar, reducing sugar and starch contents were assessed using the methods of Verma and Dubey (2001) and as reported by Mukherjee et al. (2016) with little modifications. 100mg of dried plant sample was ground in 2ml of 80% ethanol and then heated in a water bath for 30 min. After cooling to room temperature, the extracts were centrifuged at 14000 rpm for 30min.This process was repeated twice. All supernatants were combined and the total soluble sugar content was determined spectrophotometrically at λ=490nmusing Beckman Coulter DU 730 UV-Visible spectrophotometer.

For determination of starch, the remains from total sugar extraction was used to assess the starch content. The precipitate was dried up at 70˚C for 24 hrs.2mls of doubled distilled water was poured into the content and the mixture was heated in a water bath at 90˚C for 15 min. After cooling to ambient temperature, 1 ml of strong tetraoxosulphate VI acid was added. The suspension was stimulated for 15 min and the final volume was increased to 5 ml using doubled distilled water. The supernatants starch content was quantified based on the method of AOAC (1990) and expressed in mg/100g dry weight.

Statistical Analysis

Data were subjected to Analysis of Variance (ANOVA) to check if there was significant difference in the results obtained. Duncan multiple range test (DMRT) was used to separate the means at 0.05 level of significance using SPSS version 20 software. Origin 7.0 was also used in plotting graphs.

III. Results and Discussion

The properties of the used ZnONPs in this study have been previously described by Akanbi-Gada et al. (2019). The ZnO NPs has particle sizes of <100 nm with irregularly shaped structures which are generously proportioned and more diverse. It also has large amount of oxygen of about 21.51 % and highly crystalline in nature (Fig 1).The presence of oxygen could be as a result of impurities or presence of oxides which could be during synthesis or storage of the nanoparticles or as a result of the surfactants used during preservation.

Table 1 shows the growth parameters of beans after 30& 60 days of exposure to ZnONPs. There was an improvement in growth parameters examined in plants exposed to ZnONPs especially at low concentrations (300 mg/kg) compared with the control. Leaf length was statistically significant with the value of 6.00 cm ±0.58 at $p \leq 0.05$. Similarly leaf breadth, leaf area, number of leaves and stem girth showed tremendous improvement over control. This could be because of the efficiency of nutrient uptake which is associated with nanostructured formulated zinc oxide fertilizers which increases nutrient uptake to plants and save nutrient resource. The result slightly disagrees with that of Olga et al. (2016) who reported simultaneous stimulating and inhibitory action of the effect of Cu and Zn nanoparticles at their individual and combined application on growth parameters of *Pistia stratiotes.*

Table 2 shows the chlorophyll contents after 15 & 30 days of exposing beans to ZnONPs. Zinc oxide had no effect on the chlorophyll content of beans after 15 days of exposure. The result of this agrees with the work of Mukherjee *et al.*(2013) where chlorophyll content was not impacted upon after 15 days of ZnONPs application. This shows that zinc started to produce toxicity when the plants were at middle age. The implication of this is that there was substitution of the central metal atom (Mg^{2+}) with Zn which has damaged the mechanism of chlorophyll formation in stressed plants (Kupper et al.1996),this could be that the substitution of the central atom has prevented photosynthetic light harvesting in the affected chlorophyll molecules which could result in a breakdown of photosynthetic process. Furthermore, there was a reduction in chlorophyll content after 30 days of exposure of beans to zinc oxide nanoparticles (Table 2).

Productivity parameters of beans were examined by considering yield based on the data obtained in pod length, number of pod per plant, number of seed per pod and number of seed per plant (Table 3). There was a higher yield in all plants exposed to the nanoparticles. There was a significant increase and tremendous improvement at all concentrations in all these parameters in comparison with the control except in number of seeds per pod where the control was significantly higher at 0.05 level of significance. The roots of plants exposed to zinc oxide nanoparticles had roots which were larger than that of the control and this is similar to reports of Mukherjee et al.2013 who reported that the root of green pea had significantly larger roots compared to the stem. This could be as a result of zinc concentration which had reached toxic level that reduced stem elongation.

Table 4 shows the number of days of germination before flower and fruit emergence. The phytotoxicity tendency of ZnONPs was shown, this is because flower and fruit emerged earlier in control plants than in all the ZnONPs treated plants. This means that ZnONPs delayed or inhibited flowering and fruiting because of less bioavailability of ZnONPs to plants due to its large particle size and also less solubility (< 100 nm zinc oxide nanoparticles was used for this research). Furthermore, dose dependent response was observed with the lowest number of days for flower emergence occurring in control (43.00days±0.57cm), 300 mg/kg (55.00days±0.00cm)

and 1000mg/kg (60.00days±0.00cm). Similar trend was also observed in fruiting. Similar dose dependent response was recorded by authors like Lin and Xing. (2007) and He et al. (2011).

The risk of using ZnONPs as a fertilizer to enhance plants productivity could be a potential stressors on beans and this could trigger the production of reactive oxygen species called (ROS). ROS molecules such as hydroperoxyl radical (HO_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), singlet oxygen (O_2) and superoxide radicals (O_2^-) (Apel and Hirt 2004; Karuppanapandian et al. 2011). To overcome the stress, plants will produce antioxidants to counter, suppress or overcome the effect of the stressors. Examples of such antioxidants are catalase (CAT), Ascorbate peroxidise (APX), and Superoxide dismutase (SOD). The following stress attribute in plants were examined and further analysed; lipid peroxidation inform of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2).

There was concentration dependent increase in lipid peroxidation in the leaves as determined by (malondialdehyde) production with a correspondingly similar increase in the production of hydrogen peroxide (Table 5). CAT and APX activities were reduced in ZnONPs treated leaves as compared to control (Table 6). This result disagrees with that of Mukherjee et al.2013. SOD was also high at elevated zinc oxide concentration which may be as a result of enhanced ROS scavenging process and low oxidative stress in leaves (Table 6). This corroborates the work of Wang et al. 2018 where tomato plant was exposed to ZnONPs. Similar trend was observed in the enzyme activities of the roots except that it was on a higher magnitude in the root (Table 6). This also agrees with the result of Ogunkunle et al. (2018). Figure 2 shows zinc concentration in the root and shoot of beans exposed to ZnONPs treatment after 90 days of exposure. Zinc contents increased in roots linearly as concentration ZnONPs increased .This could be because the first point of contact of plant during germination is the root. Thounaojam et al. (2012) reported that roots play a significant role in nutrient uptake since they are the first contact with minerals from soil. There was about 3 times fold increase in the accumulation of zinc by roots in comparison with the shoot. Similar dose dependent zinc accumulation was also observed in bean shoots but with a lesser magnitude compared to root. Several reports corroborate the result of this study. For example, Mukherjee et al. (2013) observed similar low translocation of zinc in the shoot of plants exposed to zinc oxide nanoparticles. Similarly, Ogunkunle et al. (2013); (2018) corroborate the result of this aspect of the research.

Table 7 shows the parameters used in evaluating seed quality after harvest. The exposure of the seed to ZnONPs generally had little effect on the quality of the seed when compared with the result of the control. The quality of the seeds was determined by determining protein, total soluble sugar, and total starch. There was significant difference at all evaluation time with respect to control at $p \leq 0.05$level of significance. This aspect of food science in nanotechnology is still open for debate. It became imperative therefore to further re-examine the safety of the nanoparticles-exposed plants so as to ascertain its safety before consumption as there are varied opinions of researchers on this aspect of ecotoxicological studies.

IV. Conclusion

This study has been able to show that ZnONPs has a tendency to promote growth and productivity responses in beans especially at low dosage. This will eventually translate to higher yield or bumper harvest. This makes ZnONPs an emerging tool in agriculture for improved agricultural practices. People consume beans basically because it provides man with protein among other nutrients and it is a major building block of the body tissues. The result on seed quality revealed that accumulation of zinc was down regulated in comparison with the control. These findings could only mean that ZnONPs can enter the food chain through edible plants tissues like leaf, stem and seeds which may affect the food quality of beans.

V. Recommendation

It is recommended by the authors of this work that the seeds and fruits from this research should be further subjected to *in-vivo* testing so as to ascertain their safety for human and animal consumption.

CONFLICT OF INTEREST

The authors wish to say that there is no conflict of interest.

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TABLE 1: Growth parameters of beans after 30 and 60 days of exposure to ZnONPs

No of Days	Treatments	Leaf Length (cm)	Leaf Breadth (cm)	Leaf Area (cm)	Number of Leaves(cm)	Stem Girth (cm)
30	Control	1.53±0.18^{b}	2.03±0.33^{b}	2.62±60.45^{c}	9.67±1.86^{c}	1.00±0.00^{b}
	300 mg/kg	6.00±0.58^{a}	3.30±0.15^{a}	14.98±2.06^{a}	11.67±3.84^{a}	2.52±0.27^{a}
	600 mg/kg	4.50±0.76^{a}	2.20±0.2^{b}	7.65±2.04^{b}	6.33±0.67^{b}	2.81±0.29^{a}
	1000 mg/kg	3.40±1.5^{a}	1.40±0.57^{b}	4.87±3.10^{b}	5.33±2.03^{b}	3.05±0.25^{a}
60	Control	3.33±0.33^{b}	1.56±0.66^{b}	3.95±0.57^{b}	4.33±0.43^{b}	1.40±1.52^{b}
	300 mg/kg	6.27±0.98^{a}	3.40±0.83^{a}	17.20±6.89^{a}	12.67±0.24^{a}	2.755±0.43^{a}
	600 mg/kg	3.70±0.30^{b}	1.53±0.20^{b}	4.33±0.84^{b}	5.33±0.60^{a}	2.05±0.24^{b}
	1000 mg/kg	3.53±1.41^{b}	1.30±0.46^{b}	4.42±2.65^{b}	7.00±1.54^{b}	2.86±0.69^{b}

Values with the same letter (s) along the same column are not statistically different at $p \leq 0.05$

TABLE 2: Chlorophyll content after 15 and 30 days of exposure to ZnONPs

Number of days	Treatments	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total Chlorophyll (mg/g)
15	Control	0.57±0.33^{a}	0.70±0.5^{a}	1.27±0.67^{a}
	300 mg/kg	0.73±0.38^{a}	0.60±0.31^{a}	1.33±0.22^{b}
	600 mg/kg	0.60±0.2^{a}	0.60±0.30^{a}	1.20±0.26^{b}
	1000 mg/kg	1.01±0.21^{a}	0.47±0.15^{a}	1.47±0.13^{a}
30	Control	0.18±0.08^{a}	0.57±0.09^{a}	0.74±0.09^{a}
	300 mg/kg	0.50±0.25^{a}	0.93±0.59^{a}	1.43±0.84^{b}
	600 mg/kg	0.05±0.20^{a}	0.20±0.06^{a}	0.25±0.07^{b}
	1000 mg/kg	0.27±0.40^{a}	0.30±0.10^{a}	0.57±0.06^{a}

Values with the same letter (s) along the same column are not statistically different at $p \leq 0.05$

Table 3: Yield parameters of beans exposed to ZnONPsafter 90 days

Treatments	Pod length(cm)	Number of pod/plant (cm)	Number of seeds/pod (cm)	Number of seeds/plant (cm)
Control	3.27±0.15^{b}	2.33±0.33^{b}	3.67±0.33^{a}	8.67±1.76^{a}
300 mg/kg	3.74±0.85^{a}	3.00±0.00^{a}	2.67±0.33^{b}	8.00±1.00^{a}
600 mg/kg	2.58±0.63^{b}	2.33±0.58^{b}	2.30±0.58^{b}	4.67±0.67^{b}
1000 mg/kg	2.17±0.19^{d}	2.00±0.00^{b}	1.00±0.00^{c}	2.00±0.00^{c}

Values with the same letter (s) along the same column are not statistically different at p≤0.05

Table 4: Days of Flowers and Fruit emergence after 90 days of exposure to ZnONPs

Treatments	Days to flowering	Days to fruiting
Control	43.00±0.57^{c}	47.00±0.57^{c}
300mg/kg	55.00±0.00^{b}	56.66±3.33^{b}
600 mg/kg	60.00±0.00^{a}	65.00±0.00^{a}
1000 mg/kg	60.00±0.00^{a}	65.00±0.00^{a}

Values with the same letter (s) along the same column are not statistically different at p≤0.05

TABLE 5: Stress attributes in beans leaf and root after 90 days of exposure to ZnONPs

BEANS	TREATMENTS	Malondialdehyde (MDA)	Hydrogen peroxide (H_2O_2)
LEAF	CONTROL	0.15.±0.01^{a}	7.29±0.03^{a}
	300mg/kg	0.04±0.01^{c}	3.35±0.13^{b}
	600mg/kg	0.14±0.00^{a}	3.53±0.28^{b}
	1000mg/kg	0.09±0.00^{b}	0.08±0.01^{c}
ROOT	Control	0.15±0.0^{b}	7.45±0.17^{b}
	300 mg/kg	0.55±0.08^{ab}	8.71±0.20^{a}
	600 mg/kg	0.88±0.083^{ab}	9.05±0.33^{a}
	1000 mg/kg	0.99±0.14^{a}	8.67±0.20^{a}

Values with the same letter (s) along the same column are not statistically different at p≤0.05

TABLE 6 : Antioxidant enzyme activities in beans leaf and root after 90 days of exposure to ZnONPs

	Treatments	APX	CAT	SOD
LEAF	Control	165.4±2.62^{c}	114.79±1.27^{c}	89.67±4.91^{c}
	300 mg/kg	293.17±1.48^{b}	66.37±1.03^{d}	123.42±1.55^{b}
	600 mg/kg	294.56±1.50^{b}	756.93±5.62^{a}	417.83±3.75^{a}
	1000 mg/kg	534.46± 3.28^{a}	678.04±3.27^{b}	123.90±0.47^{b}
ROOT	Control	162.93±2.58^{b}	116.23±0.66^{a}	27.68±0.16^{c}
	300 mg/kg	238.12±14.00^{a}	364.84±82.21^{a}	138.01±56.62^{b}
	600 mg/kg	123.40±1.76^{b}	205.18±0.03^{a}	266.45±7.17^{a}
	1000 mg/kg	247.48±37.3^{a}	569.93±250.76^{a}	149.29±0.43^{b}

Values with the same letter (s) along the same column are not statistically different at p≤0.05

Table 7 : Food Quality of beans after 90 days of exposure to ZnONPs

Treatments	protein (mg/kg Dw)	Total soluble sugar(mg/kg Dw)	Total starch (mg/kg Dw)
Control	12.87±0.05^{a}	0.07±0.01^{a}	3.77±0.01^{a}
300 mg/kg	10.47±0.05^{b}	0.05±0.01^{b}	3.46±0.01^{b}
600 mg/kg	10.41±0.00^{c}	0.04±0.00^{b}	2.45±0.99^{c}
1000 mg/kg	10.40±0.00^{c}	0.04±0.03^{b}	3.52±06^{a}

Values with the same letter (s) along the same column are not statistically different at p≤0.05

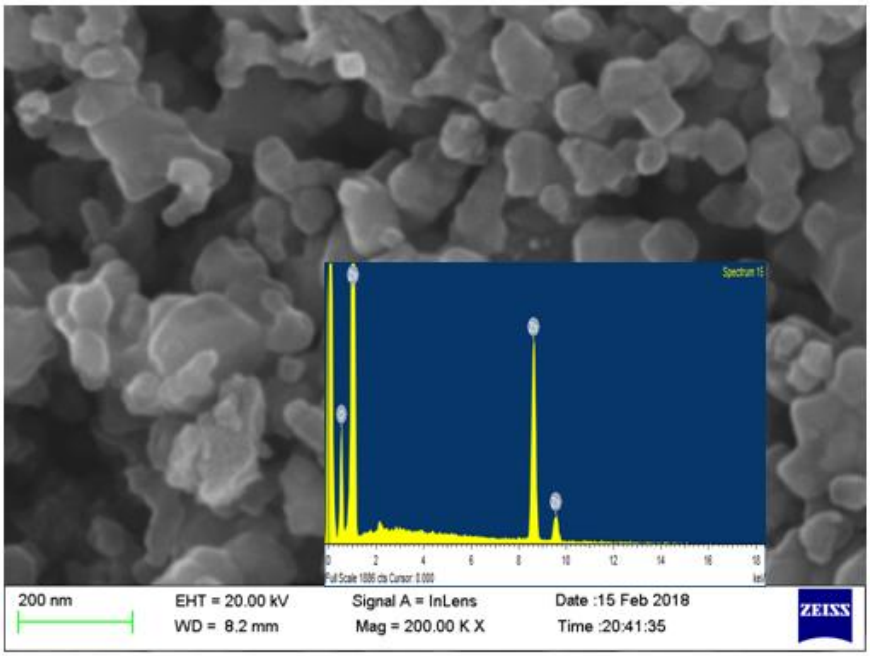

Figure: 1: FESEM micrograph of ZnONPs coupled with energy dispersive X-ray spectroscopy (EDX) (Akanbi-Gada et al.,2019)

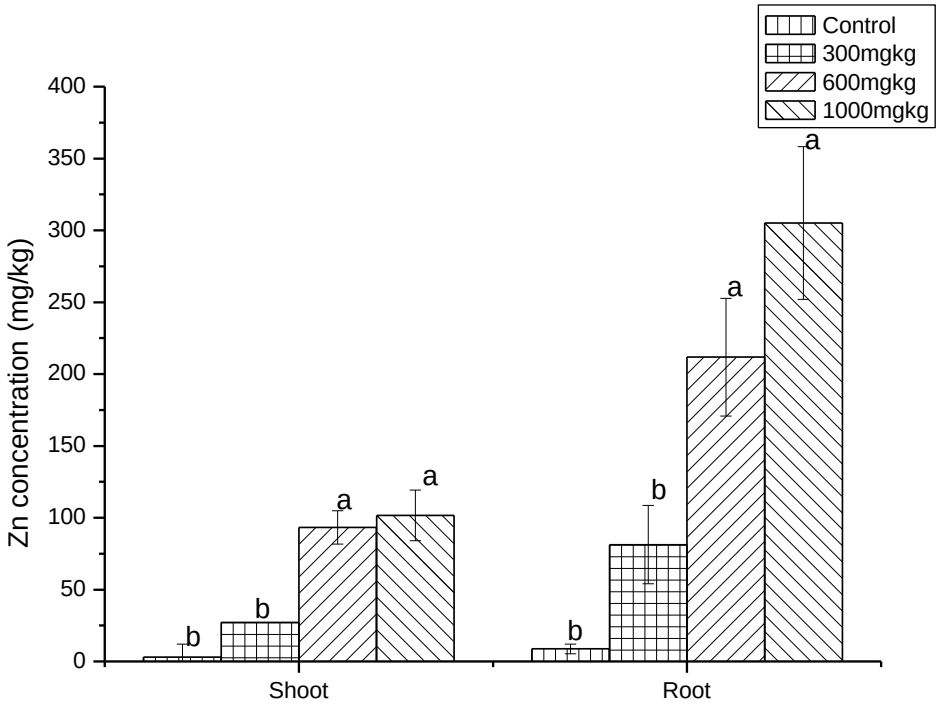

Fig 2: Bioaccumulation Potential of Beans after 90 days of exposure to ZnONPs

Mariam.A .Akanbi-Gada. " Effects of Zinc Oxide Nanoparticles on Chlorophyll Content, Growth Attributes, Antioxidant Enzyme Activities And Bioaccumulation of Common Bean (Phaseolus Vulgaris L.) Grown In Soil Medium." IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT) 13.10 (2019): 09-15.