Annals. Food Science and Technology
2017

BIOACCUMULATION OF COPPER AND TISSUE PARTITIONING OF SELECTED MINERAL ELEMENTS IN GERMAN ROUND PEA (*Kleine Rheinlanderin*) UNDER THE INFLUENCE OF COPPER NANOPARTICLES

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Abstract

Increased use of copper-based nanoparticles in agriculture has generated safety concerns for living organisms. In this study, copper nanoparticle (nCu) was exposed to round pea for 49 days via soil substrate at different levels of 0, 125, 500 and 1000 mg/kg. At harvest, plants were processed and parameters such as seed emergency, chlorophyll contents and protein content were determined. Furthermore, Cu content and selected minerals (Mg, Mn, Zn, Co, Fe and K) in root and shoot tissues of round pea were determined by Aqua-regia digestion and analyzed using Flame atomic absorption spectrophotometer and Flame photometer. Results showed that the highest nCu treatment (1000 mg/kg) enhanced seed emergency, chlorophyll contents (chlorophyll a and b) but reduced soluble protein content in the plant. Furthermore, nCu treatment also increased the uptake of Cu from soil substrate but drastically restricted the translocation to above-ground biomass, though biomass Cu concentrations in treatments were still within recommended concentration range in plant. Cobalt accumulation was significantly reduced in both root and shoot tissues, Fe content only reduced in roots and not shoots, while Zn was the only mineral element that was significantly accumulated in the plant. In summary, results showed that copper nanoparticles portend both beneficial and detrimental effects on round pea plant.

Keywords: Mineral elements, bioaccumulation, phytotoxicity, nanoparticles, translocation, round pea

Received: 19.10.2017 Reviewed: 05.12.2017 Accepted: 13.12.2017

1. INTRODUCTION

Nanoparticles are nano-scopic materials with range of 1 to 100 nm in size with physico-chemical properties that can be manipulated compared to corresponding bulk material (Nel *et al.*, 2006).They can occur naturally (e.g. volcanic dust, mineral composites), incidentally (due to man-made activities, e.g. diesel exhaust, coal combustion, welding fumes) or manufactured (carbon nanoparticles, Au nanoparticles, Cu nanoparticles, TiO_2 nanoparticles etc.) (Ruffini-Castiglione and Cremonini, 2009). Reports from various researches suggested that nanoparticles can portend both beneficial and detrimental effects on growth, metabolism and development of plants (Ma *et al.*, 2010). The uptake, translocation and accumulation of nanoparticles in plants depends on their composition, concentration, size, and physical and chemical properties as well as plant species (Ma *et al*. 2010; Alimohammadi *et al.*, 2011).The unique properties of nanoparticles contributes to its potential to promote metabolism in plants (Giraldo *et al.*, 2014), and its bioaccumulation (both essential and non-essential) above particular thresholds in plant could lead to toxicity in plants (Ke *et al.*, 2007).

Studies have indicated the possibility of using metal-based nanoparticles such as Cu nanoparticles as effective agent against pathogenic fungi. This novel usage of Cu nanoparticles may likely promote intensive use of Cu nanoparticles and CuO nanoparticles in agricultural practices, and creating high possibility of Cu-based nanoparticles entrance into the food chain through contamination of agricultural products. Few researches on physiological and biochemical mechanisms of plants in relation to Cu nanoparticles have been carried out. For instance, Lee *et al*. (2008) studied the uptake and translocation of Cu nanoparticles in mungbean (*Phaseolus radiata*) and wheat (*Triticum aestivum*) *in vitro* and showed that they could pass the cell membrane and accumulate in the cells. Stampoulis *et al*. (2009) also studied the effect of silver, copper and zinc oxide nanoparticles and their corresponding bulk counterparts on seed germination, root elongation, and biomass production of *Cucurbita pepo* plants and observed phytotoxicity to the plant exposed to Ag and Cu nanoparticles. Recent study by Hong *et a*l. (2015) and Zhao *et al*. (2016) suggested that Cu nanoparticles did not only reduced the size of the plants but also altered nutrient contents in plants.

Therefore, this study aims to investigate the influence of copper nanoparticles on copper bioaccumulation and selected mineral nutrients in German round pea (*Pisum sativum*).

2. MATERIALS AND METHODS

2.1. Materials and planting

Copper nanoparticles (nCu) were supplied by Ionic Liquids Technologies GmbH, as powder. The nCu, as described by the manufacturer has average particle size of 25 nm (particle range 0-60 nm), 99.9% purity, specific surface area of 30-50 m^2/g and spherical in shape. The particle size and morphology of the nCu particles were checked using scanning electron microscopy (Thermo Scientific- JSM-6510) equipped with Microanalysis Noran System 7 energy dispersive X-ray spectroscopy (EDS) with Noran System 7 Software version 4.0. Briefly, approximately 10 mg of the nCu particles were dispersed by ultrasonic vibration in 30 ml of isopropanol for 1 min. Afterward, a drop of the mixture was placed on the SEM sample holder and allowed to dry at 70^{o}C in a chamber prior to SEM examination. The elemental content of the nCu particles to assess the purity was determined by EDX at 25Kev energy level with dwell time of 28 s.

Sand (Terrasan Haus and Gartenberdarf GmbH) and compost (Spielsand Sahara, WECO GmbH) used as substrate for plant growth in this study were purchased from a commercial store in Wuppertal, Germany. The substrate was mixed in ratio 1:1 w/w of sand and compost to make a substrate mass of 1.5 kg. The substrates were spiked with copper nanoparticles (nCu) in the following levels in five replicates: 0 (control); 125 mg/kg (T1), 500 mg/kg (T2) and 1000 mg/kg (T3). Seeds of German round pea- Kleine Rheinlanderin (*Pisum sativum*) were purchased from a commercial outlet in Wuppertal, Germany. The seeds were tested for viability before planting in treated soil in 3 seeds per pot and arranged in triplicate per treatment. The plants were allowed to grow for 49 days (7 weeks) under normal ambient condition in a screen house of the University of Wuppertal, Germany before plants were harvested, washed and processed for analysis.

2.2. Chemical analysis

Contents of chlorophyll and total soluble proteins of leaves were determined according to Anon (1949) and Lowry *et al*. (1951) respectively. The optical density (OD) for both chlorophyll and total soluble protein was read on a UV-VIS spectrophotometer (Thermos scientific- Genesys10S).For chemical analysis of plant samples, 1 g of freeze-dried sample (root or shoot) was digested using aqua-regia method and metal analysis was done using Perkin Elmer AAnalyst 200 while K was determined by Flame Photometer-PFP7Jenway. Quality control and assurance was put in place by replicate digestion and analysis, use of blank and internal standards.

2.3. Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and significant means were separated by Duncan multiple range test (DMRT) at $P<0.05$. Data for figures were analyzed by Student-t test and presented by Origin 8 software. Translocation index (TI) (that shows the mobility of element in plants) was calculated as the ratio of metal content in shoot (mg/kg DW) to metal content in root (mg/kg DW) according to Baker (1981) and Ghosh and Singh (2005).

3. RESULTS AND DISCUSSION

Characteristics of copper nanoparticles (nCu)

Scanning electron micrographic (SEM) examination of the nCu particles gave results that conformed to the specifications from the manufacturer. The morphology of nCu dispersed in isopropanol as solvent is shown in Figure 1a. Particles of nCu have a spherical shape with average particle size of 25 nm and 87% metal purity. The particles formed hetero-aggregations in form of flocs of few nanometers in diameter in isopropanol as solvent. The degree of aggregation of the nCu was high, possibly because of the small particle sizes resulting in the observed agglomeration. The Energy dispersive X-ray (EDX) of the nCu particles presented in Figure 1b showed the presence of small amounts of carbon and oxygen. The carbon could possibly come from surfactant used by the manufacturer to stabilize the nanoparticles while the presence of oxygen could be from oxidation during processing.

Effects of copper nanoparticles on germination, chlorophyll and soluble proteins

The germination percentage of round pea increased with increasing concentration of nCu in the study (Table 1). Copper is a well-known important nutritional need of plant for metabolism and development, especially in cell formation and protein synthesis (McCauley *et al.*, 2011). In this study, application of nCu positively influenced germination/emergence of round pea seed. Similarly, Lee *et al*. (2008) has also reported that copper nanoparticles increased germination percentage of wheat and mung bean.

Significant increase ($P<0.05$) in contents of chlorophyll a and b was observed when pea was exposed to 1000 mg nCu/kg (T3) whereas at 125mg nCu/kg, significant decrease ($P<0.05$) was obtained. Copper nanoparticles at 500 mg/kg (T2) showed no influence on the chlorophyll contents as compared to the control (Table 1). Compared to control, the soluble protein content was found to be significantly higher at 125 mg/kg and 500 mg/kg whereas at 1000 mg/kg, there was a significant reduction in the protein content (Table 1). This study showed that copper nanoparticles favoured photosynthesis, as its application enhanced photosynthesis which is very important in the plant growth and development. Copper, as an essential element helps in photosynthetic electron transfer, and metal nanoparticles can boost the efficacy of chemical energy production in photosynthesis (Govorov and Carmeli, 2007; Raven *et al.*, 1999).Protein was positively affected though as the copper nanoparticles increased, the protein reduced. This showed that there is a threshold level of copper at 125 mg/kg for round pea. Copper is required for protein synthesis in plants (McCauley *et al.*, 2011) and from this study, it was observed that Cu uptake boosts protein synthesis in round pea.

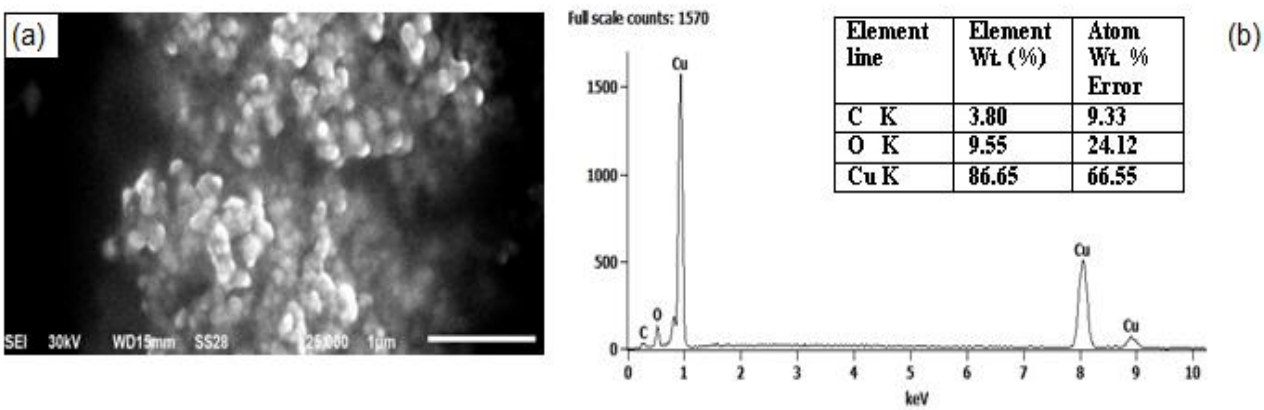

Fig. 1. (a) Scanning electron micrograph showing morphology of nCu dispersed in isopropanol (b) Energy dispersive X-ray spectroscopy analysis of nCu (EDX at 25Kev with Take- off angle of 35.3°)

Table 1. Chlorophyll and soluble protein contents of leaves of round pea exposed to Cu nanoparticles

Treatment	Emergency (%)	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Soluble proteins (mg/g FW)
Control	84 ±7.48^{c}	0.677±0.035^{b}	0.325±0.015^{b}	6.43±0.408^{c}
T1	88±4.90^{b}	0.477±0.029^{c}	0.250±0.019^{c}	8.04±0.561^{a}
T2	96±4.09^{ab}	0.752±0.075^{b}	0.299±0.027^{b}	7.15±0.096^{b}
T3	96±4.09^{ab}	1.085±0.042^{a}	0.470±0.013^{a}	6.50±0.187^{c}

Values are means±SD (n=3). Same letters mean no statistical difference among treatments at DMRT test (p<0.05)

Uptake of copper in round pea

Table 2 presents Cu contents in root and shoot tissues of round pea plant with the translocation index. Significant uptake of Cu by the root tissues was observed at all treatments (125, 500 and 1000 mg/kg) compared to control. Contents of Cu in the roots were 3.75, 11.9 and 19.5-fold higher than control content respectively for 125, 500 and 1000 mg/kg treatments (T1, T2 and T3). Similar trend of increasing Cu concentrations was also observed in shoot tissues, though Cu contents in shoot were significantly lower than root contents. This is an indication of low translocation of copper in round pea plant, and also consistent with the reports of Ogunkunle *et al*. (2016) on *Vigna unguiculata* grown on nCu-amended soil. Lee *et al*. (2008) reported the uptake and translocation of copper nanoparticles in mungbean (*Phaseolus radiata*) and wheat (*Triticum aestivum*) and suggested that it could be due to copper passing through the cell membrane and accumulating in plant cells. Similarly, Zheng *et al*. (2004) reported *Chrysanthemum* sp. to be more sensitive to copper toxicity in the root system than shoot. Similar studies also reported that plants' exposure to CuO nanoparticles result to adsorption of copper on root surfaces and transfer to the shoots (Dimkpa *et al.*, 2013; Wang *et al.*, 2013).

Translocation index (TI), which is an indication of mobility in plants was very low in all treatments and it was also observed that as concentration increased, amount of Cu translocation into the shoot reduced. For instance, 16.1%, 12.9% and 9.6% of Cu contents in root tissue were translocated into the shoot respectively for 125, 500 and 1000 mg/kg treatment. It means that Cu was restricted to the root tissues as indicated by the translocation index (TI), thereby signaling the potential of round pea to restrict or prevent toxic effect of Cu in the upper plant biomass. Copper, which was referred to as immobile elements (McCauley *et al.*, 2011)) could contribute to its low transfer to the shoot area. Plants are classified into accumulators and excluders based on the metal content in the root and aerial parts (Baker, 1981). Excluders retain high level of metal in their roots (with TI value<1.0) and prevent transfer of such metal to their aerial parts over a wide range of metal concentration in the soil while accumulators transfer metal from root zone to the shoot zone, with TI always greater than 1.0 (Baker 1981). Results from this study show that round pea could be a potential excluder of Cu with TI value that was less than 1.0 and in fact, recommended range of Cu in plants is 2-50 mg/kg (Epstein and Bloom, 2005).

Table 2. Copper uptake and accumulation (mg/kg dw) in round pea exposed to Cu nanoparticles

Soil treatment (mg/kg dw)	Root	Shoot	Translocation index (TI)
Control	16.0±0.00^{d}	8.75±0.35^{c}	0.57
T1	60.5±0.71^{c}	9.75±0.35^{c}	0.17
T2	191.2±1.06^{b}	24.7±0.35^{b}	0.15
T3	312.7±86.6^{a}	30.0±0.71^{a}	0.11

Values are means±SD (n=3). Same letters mean no statistical difference among treatments at DMRT test (p<0.05)

Influence of Cu nanoparticles on tissue partitioning of mineral nutrients

Figure 2 presents the partitioning pattern of Mg in the tissues of round pea plant. Shoot Mg contents of plant in control and 125 mg/kg treatments were significantly higher than the root content whereas there was no difference in the root tissue contents as nanoparticle concentration increased up to the highest concentration of 1000 mg/kg (T3). Copper nanoparticles treatment showed no significant influence on tissue partitioning and concentration of Mn in root and shoot tissues except that, at 1000 mg/kg (T3), Mn content reduced compared to control (Figure 3). Hong *et al*. (2015) have also reported that Cu nanoparticles have no influence on uptake and bioaccumulation of Mg and Mn in lettuce and alfalfa exposed for 15 days. In addition, the content of Mn in the shoot of round pea after exposure to nCu was still within the threshold of adequate concentration of Mn (50 mg/kg) in plants according to Epstein and Bloom (2005). Contrarily to this report, Zhao *et al*. (2016) found that nano-Cu decreased Mg uptake in roots of cucumber.

For Zn content (Figure 4), different pattern of partitioning was observed in the plant-round pea. Concentration of Zn in the root was higher than shoot content for all the treatments, control inclusive. This indicates that nCu treatment has no influence on the partitioning pattern of Zn in the plant but significantly increased ($P<0.05$) the root and shoot contents at higher treatments (500 and 1000 mg/kg) and 500 mg/kg respectively. This was contrary to observation of Hong *et al*. (2015) where Cu nanoparticles did not alter the uptake Zn in lettuce and alfalfa exposed for 15 days. However, Zhao *et al*. (2016) investigated the response of cucumber plants to Cu nanoparticles at 10 and 20 mg L^{-1} and reported an interference in the uptake of Zn in cucumber plant.

Copper nanoparticles treatment to round pea did not alter the partitioning pattern of Co in round pea plant but significantly influenced tissue accumulation at higher concentrations (125 and 1000 mg/kg) as shown in Figure 5. Cobalt level decreased significantly ($P<0.05$) with increasing nCu concentration in the root tissue while shoot tissue showed imbalance in the level of Co for all treatments (125, 500 and 1000 mg/kg), though control treatment recorded highest level of Co in both root and shoot tissues of round pea. Increased nCu concentration (125, 500 and 1000 mg/kg) significantly decreased ($P<0.05$).

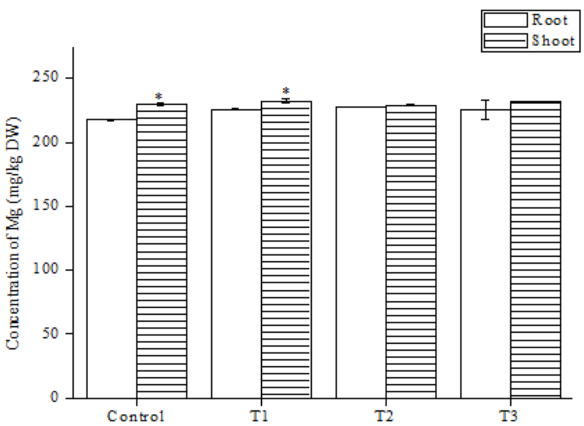

Fig. 2. Concentration of Mg (mg/kg dw) in tissues of round pea after Cu nanoparticles exposure (Note: '*' denotes statistically higher content between root and shoot)

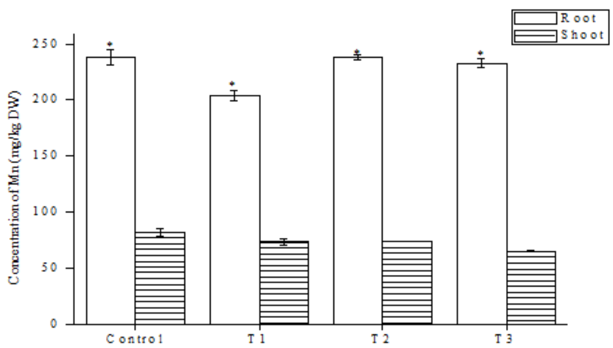

Fig. 3. Concentration of Mn (mg/kg dw) in tissues of round pea after Cu nanoparticles exposure (Note: '*' denotes statistically higher content between root and shoot)

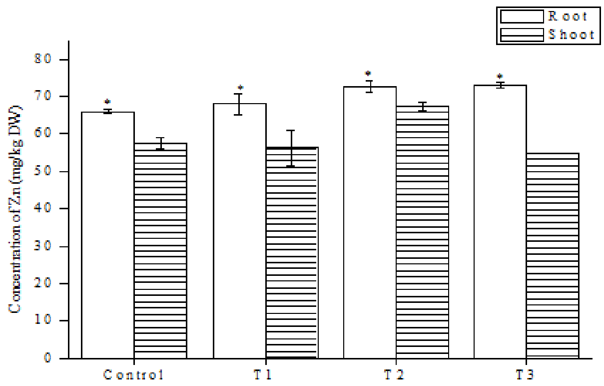

Fig. 4. Concentration of Zn (mg/kg dw) in tissues of round pea after Cu nanoparticles exposure (Note: '*' denotes statistically higher content between root and shoot)

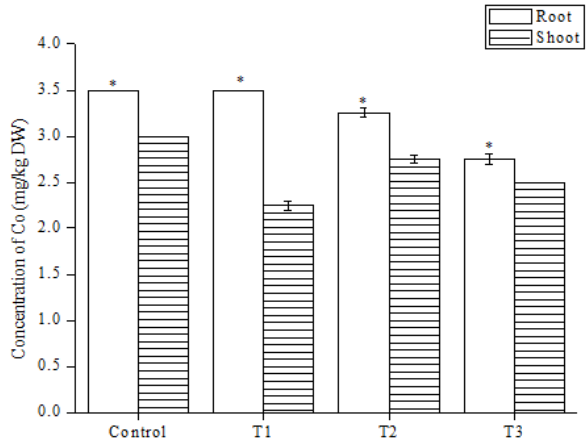

Fig. 5. Concentration of Co (mg/kg dw) in tissues of round pea after Cu nanoparticles exposure (Note: '*' denotes statistically higher content between root and shoot)

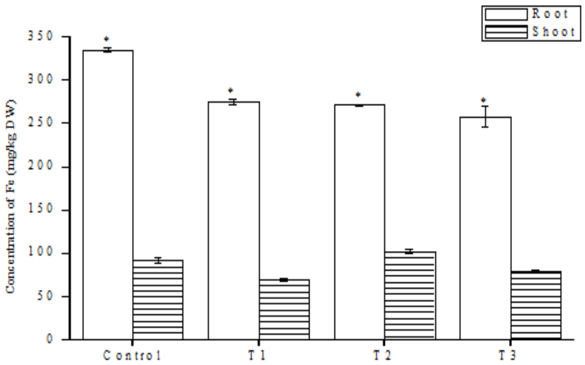

Fig. 6. Concentration of Fe (mg/kg dw) intissues of round pea after Cu nanoparticles exposure (Note: '*' denotes statistically higher content between root and shoot)

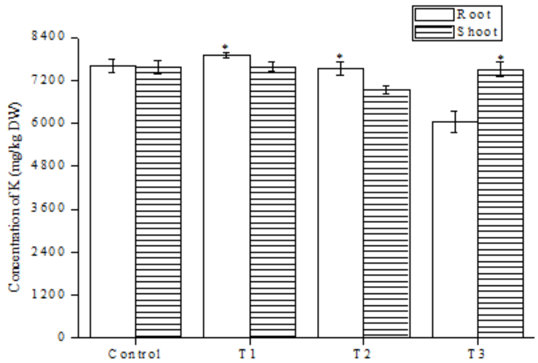

Fig. 7. Concentration of K (mg/kg dw) in tissues of round pea after Cu nanoparticles exposure (Note: '*' denotes statistically higher content between root and shoot)

Fe concentration in root tissues of round pea whereas Fe concentrations in shoot tissue displayed imbalance (Figure 6).

Concentration of Fe in shoot at 125 mg/kg and 1000 mg/kg decreased compared to control while there was a significant increase ($P<0.05$) at 500 mg/kg.It seems a possibility that Fe that is a more active element in plants than Cu could have formed insoluble hydroxides in the roots, thereby restricting the translocation to aerial biomass. This results is similar to the report of Hong et al. (2015) on alfalfa and lettuce, and several previous studies have also shown that uptake of Fe is decreased in by excess Cu (Ouzounidou *et al.*, 1995; Cotruvo *et al.*, 2015).

Imbalance of potassium was observed in root and shoot tissues of round pea under nCu influence (Figure 7). Root and shoot content of K showed no significant difference ($P>0.05$) in control whereas at 125 and 500 mg/kg (T1 and T2) treatments, K concentrations in root tissues were significantly higher ($P<0.05$) than shoot. A twist in tissue partitioning of K was observed at 1000 mg/kg (T3); K concentration in shoot was significantly higher ($P<0.05$) than root content, though statistically similar to root content of control.

In addition, at highest concentration (1000 mg/kg), K concentration decreased in the root tissue whereas, no difference in K content of shoot was observed compared to control. The decrease of K concentration at 1000 mg/kg (T3) may be the result of leakage mediated by ion channels as reports by Murphy *et al*. (1999) demonstrated that Cu promotes K^+ efflux rather than inhibiting K^+ uptake in Arabidopsis seedlings. Zhao *et al*. (2016) reported a decline in K accumulation due to nCu exposure in cucumber.

4. CONCLUSION

In summary, round pea was found to uptake significant concentrations of Cu from Cu nanoparticles into the root system, however it was noted that translocation of same was restricted, portraying round pea as an excluder plant. Copper nanoparticles treatment also enhanced seed emergency and chlorophyll contents but negatively affected the protein contents in the shoot of the plant. Cu nanoparticles treatment reduced Co accumulation in both root and shoot tissues, and Fe accumulation in only root tissue of the round pea, while Zn concentration increased in the roots.

5. ACKNOWLEDGEMENT

This material is a part of the study sponsored by the TWAS-DFG Cooperative Visit at Bergische Universtat Wuppertal and Delta X Facility, Dortmund, Germany under Fellowship agreement- TWAS-3240287207. C. O. Ogunkunle wishes to thank Dr. Dirk Leutzenkirchen-Hecht (Physics Department) of BergischeUniversitat Wuppertal, Germany for the provision of round pea seeds and copper nanoparticles, Dr. Ben Bornmann (Physics Department) for assistance in the use of SEM; and Prof Lohaus and Mr. Tim Kreutzer of the Department of Biology and Chemistry (Bergische Universitat Wuppertal, Germany) for making the screen house available and technical supports at the University.

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