Hue University Journal of Science: Natural Science; ISSN 1859-1388

Vol. 126, No. 1C, 2017, P. 47-56; DOI: 10.26459/hueuni-jns.v126i1C.4524

EFFECT OF METAL NANOPARTICLES ON THE GROWTH OF NGOC LINH GINSENG (*Panax vietnamensis* Ha *et* Grushv.) LATERAL ROOTS CULTURED *IN VITRO*

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Abstract. Ngoc Linh ginseng plays important in pharmaceutical industry because its triterpenoid saponin drugs could improve health and treat many diseases. Metal nanoparticles revealed completely new properties based on specific characteristics (size, distribution and morphology) compared to metal ions or salts, especially, their potential applications in plant tissue culture. Effects of metal nanoparticles (0.5–2.5 mg/L nZnO, 1–3 mg/L nAg, and 1–3 mg/L nCu) in free-hormone-MS medium were studied on the Ngoc Linh ginseng root culture. Results showed that nAg and nCu increased positive effects on the lateral root formation and growth at different concentrations. Except for, the root abnormal anatomical morphology revealed that cell layers of xylem, endodermis and epidermis thicken and darken, or vascular bundle expand, or some black points appeared in root caps and dorsal bundles. The optimal metal nanoparticle for the root growth is nCu; and the highest growth indexes were obtained at 1.5 mg/L nCu with 99.3% lateral root formation). The ginseng root grew at 2.5 mg/L nAg better, but more abnormalities. The inhibition growth and negative impacts on the ginseng root were recorded in the medium containing nZnO (0.5–2.5 mg/L) and the highest metal nanoparticle concentration (above 2.5 mg/L nCu and nAg).

Keywords: *in vitro*, lateral root culture, metal nanoparticle, MS medium, Ngoc Linh ginseng

1 Introduction

Nanotechnology, an emerging field, widely used in many medical, pharmaceutical, cosmetic, biosensors and agriculture and agricultural products 11. The metal nanoparticles (NPs) are microscopic particles (1–100 nm), made from molecules or atoms 24. Because of their surface effect and the critical size, nanoparticles have unique physical and chemical properties such as quick reaction and catalysis compared with conventional mass materials 24. Therefore, NPs have a better interaction with plants. NPs can create physical or chemical effects on the organs of plants to improve plant growth 11. Usually, NPs enter the tree through the lateral root system and through the cortex and the lining of the xylem system of main roots 6. In particular, some NPs with a small size and surface properties that are suitable for the size of the holes in the cell membrane easily enter the cell and participate in metabolic reactions within the cell 24. After entering the cell, the NPs mixed with metal ions and react with sulfhydryl groups, carboxyl

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Submitted: 26-9-2017; Revised: 26-10-2017; Accepted: 2-11-2017

groups and ultimately alter the activity of plant proteins. These effects may be beneficial for the growth and development of plants such as enhanced antimicrobial activity and the ability of plants to grow roots and shoots 11.

Nanoparticles of silver (Ag), copper (Cu), zinc (Zn), and zinc oxide (ZnO) have found applications in agriculture. Although rooting and mineral nutrition are intimately related, few studies have attempted to characterize the effects of specific minerals, especially metal nanoparticles on each of the three phases of the rooting process. Copper – an essential transition metal – is involved in many physiological processes in plant rooting due to its multiple oxidation states *in vitro* 3. Copper is added to the medium in the form of nitrates with low concentrations to improve root growth 3. Copper nanoparticles (nCu) enhance root growth and mode of application to yield plant maximization 3. Silver nanoparticles (nAg) have not only strong antibacterial activity but also improve plant rooting. They increase the root growth of chrysanthemum in the micro hydroponic system 9. The root number of *in vitro* culture is affected by zinc 11. The addition of Zn to the culture media improves the mass of roots 1213. Nano zinc oxide also increases the development of the lateral root system on field 414.

Ngoc Linh ginseng, a precious medicinal plant of Vietnam, contains many compounds that are highly medicinal in the treatment of blood pressure, cardiovascular, blood sugar, nerves, liver and kidney or to produce functional products to nourish and enhance health 8. In order to harvest large amounts of biomass in a short time, many studies have shown that root culture is optimal for ginseng root growth and accumulation of saponins 81023. However, in order to come to ginseng mass production, the evaluation of the effect of nanomaterials is necessary. Understanding the benefits and harms of the nanoparticles can find a new and safe pathway to improve the growth of lateral roots in the *in vitro* culture. Therefore, in this study, we evaluated the stimulation of copper, zinc, and silver nanoparticles in the growth of Ngoc Linh ginseng roots.

2 Materials and methods

2.1 Materials

Plant material

Lateral root explants (length of 1 cm, weight about 10 mg) were separated from adventitious roots that were cultured in the MS medium with 5 mg/L IBA within 56 days. The culture was carried out at the Department of Molecular Biology and Plant Breeding of Tay Nguyen Institute for Scientific Research.

Materials synthesis and characterization

As previously stated, silver nanoparticles, zincs oxide nanoparticles, and copper nanoparticles were tested for potential rooting on *in vitro* chrysanthemum shoots, tomato, soybean and oat breeding 111218. The synthesis procedure for nAg, nCu, and nZnO was presented in the supplementary information from Institute of Environmental Technology in Hanoi. The particle size, morphology and crystallinity were investigated using scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), and Infrared (IR) spectroscopy.

Silver nanoparticles with diameters smaller than 20 nm were manufactured using $AgNO_3$ (750–1000 ppm), β-chitosan (250–300 ppm), $NaBH_4$ (200 ppm). The mole ratio of [$NaBH_4$]/[$AgNO_3$] was ¼. The $NaBH_4$ drip speed was 10–12 droplet/min 9.

Copper nanoparticles (the average diameter) were synthesized in the presence of chitosan via reduction of $Cu(NO_3)_2$ with sodium borohydride with the ratio of 4 mL 2.0 mg/mL chitosan: 2mL $Cu(NO_3)_2$ 10 mM:1 mL $NaBH_4$ 0.1 M in aqueous solution. Chitosan molecules absorbing on the surface of prepared copper nanoparticles formed the corresponding copper nanoparticle-chitosan 22.

The synthesis of nZnO by the sol–gel method showed wurtzite construction, purity about 99.9%, and lattice parameters (50 nm in average diameter and 100–200 nm in length). First, 0.5 M zinc acetate solution was transferred into TEA solution and stirred for 20 min at temperature of 120°C. Then, 6 mL of NH_4OH (25%) was slowly dropped into the solution with aluminum foil to form nZnO after 30 min 15.

2.2 Methods

Effect of metal nanoparticles on the growth of Ngoc Linh ginseng lateral root

Lateral root explants (length of 1 cm) were cultured in the hormone-free MS medium without plant growth regulator. The medium contained 30 g/L sucrose and 8 g/L agar. To evaluate the effect of metal nanoparticles (NPs) on the growth of lateral root, silver nanoparticles, zinc oxide nanoparticles, and copper nanoparticles were added to the culture medium at the difference concentrations: 0, 0.5, 1, 1.5, 2 and 2.5 mg/L for nZnO; 0, 1, 1.5, 2, 2.5, and 3 mg/L for nCu and nAg before sterilizing.

Growth conditions: The media were poured into 250-mL glass bottles containing 30 mL culture medium and sterilized using an autoclave at 121°C, 1 atm in 20 minutes. Lateral root cultures were incubated in a growth cabinet in darkness and at the controlled temperature of 25 ± 2°C with the humidity of 55–60%.

Morphological and anatomical studies of Ngoc Linh ginseng lateral root

Lateral roots were obtained from living specimen of *in vitro* Ngoc Linh ginseng root culture and sectioned with a razor blade. The thickness of the sections was 10–12 μm. After the preparation of root sections, samples were washed with distilled water and placed in a 5 % sodium hypochlorite solution for 20 min for clearing and rinsed with distilled water. Then, the samples were washed in 10% acetic acid for 2 min. Subsequently, the sections were stained with methyl blue and carmine for lignin and cellulose, respectively. The sections were embedded and mounted in glycerol. Thin cut sections were observed under a microscope fitted with digital camera Nikon SMZ 800 (Nikon, Japan) with 10 times of magnification 16.

Statistical Analysis

Each treatment was repeated three times and the explants were arranged in a randomized complete bottle design with five root explants per treatment and ten bottles. The data of *in vitro* cultures were recorded on the 56 day of culture.

The data (rate of formed lateral roots, fresh weight (mg), dry weight (mg), means of number roots, and root length (cm) were analyzed using one-way ANOVA. When significant differences occurred ($p < 0.05$), the interaction of various factors prior to Duncan's multiple range was tested using the SPSS 20.0 software 7.

3 Results and discussion

3.1 Effect of silver nanoparticle on the growth of *in vitro* Ngoc Linh ginseng lateral root

The effect of different concentrations of nAg (0, 1, 1.5, 2, 2.5, and 3 mg/L) on lateral roots of Ngoc Linh ginseng is presented in Table 1 and Fig. 1a. The results showed the significance difference when the ginseng root culture contained nAg. Combined analysis results showed that all concentrations simultaneously affected the mean of root number, dry weight and percent of lateral root formation ($p < 0.05$). The silver nanoparticles supplemented in the culture medium had a clearly impact on increasing the growth of Ngoc Linh ginseng roots; especially, high percent of lateral root formation (over 50% in nAg supplemented treatments) compared with control only 26.7% without nAg) (Table 1). The anatomy morphology of the ginseng lateral root containing nAg induced abnormalities as well as cell wall thickening and darkening and vascular bundle expanding; or some black point showed up in root cap, xylem, and endodermis (Fig. 1f). The optimal concentration was 2 mg/L nAg with the highest growth indexes such as root number (35.3 roots), root length (3.3 cm), fresh weight (67.0 mg), and dry weight (10.7 mg).

Table 1. Effect of silver nanoparticle on the growth of *in vitro* Ngoc Linh ginseng lateral root

nAg Conc. (mg/L)	**Root number**	**Root length** (cm)	**Fresh weight** (mg)	**Dry weight** (mg)	**Lateral root formation** (%)
0	16.7^{d*}	1.0^{c}	25.3^{d}	2.5^{d}	26.7^{d}
1	20.0^{cd}	1.2^{c}	25.3^{d}	3.2^{cd}	55.0^{c}
1.5	23.7^{c}	2.2^{b}	36.0^{c}	4.0^{c}	73.3^{b}
2	**35.3^{a}**	**3.3^{a}**	**67.0^{a}**	**10.7^{a}**	**90.0^{a}**
2.5	30.3^{b}	2.5^{b}	54.3^{b}	7.7^{b}	83.3^{ab}
3	16.7^{d}	1.1^{c}	42.3^{c}	3.8^{c}	80.0^{ab}

*Different letters in a column indicate significant differences at $p < 0.05$ by Duncan's multiple range tests.

Ethylene inhibits lateral root development; thus, when nAg inhibited the production of ethylene, percent of lateral root formation was improved significantly and optimized at 2 mg/L nAg (Table 1). Bleecker and Kende (2000) also showed that ethylene would be substantially weakened by Ag^{+}, led to accumulate auxin and develop lateral root system 2. Besides, nAg could change the balance of oxidation systems and further affect the homeostasis of water and other small molecules within the plant body 11. However, a potential mechanism of absorbent characterization of nAg on the root surface could disrupt the structure of the thylakoid membrane and decreased cell plant growth 11. Therefore, determining the optimum concentration of nAg on plant growth was necessary for cultures *in vitro* and on field. On the fifth day of nAg exposure with treatments (10 and 100 mg/L) in water hyacinth, at the high concentration (100 mg/L), nAg improved plant growth on field 21. *In vitro* culture, using concentration is lower than on field, such as 2 mg/L Ag is the best for developing the ginseng root. Some other research revealed the negative effect of nAg, such as inducing toxicity on the root elongation of *Arabidosis thaliana* at high concentration 12. In our results, the decrease of root growth initially occurred at 3 mg/L nAg, but the weight was not lower than control for containing some black points of nAg in root cell at epidermis or cortex area (Fig. 1f).

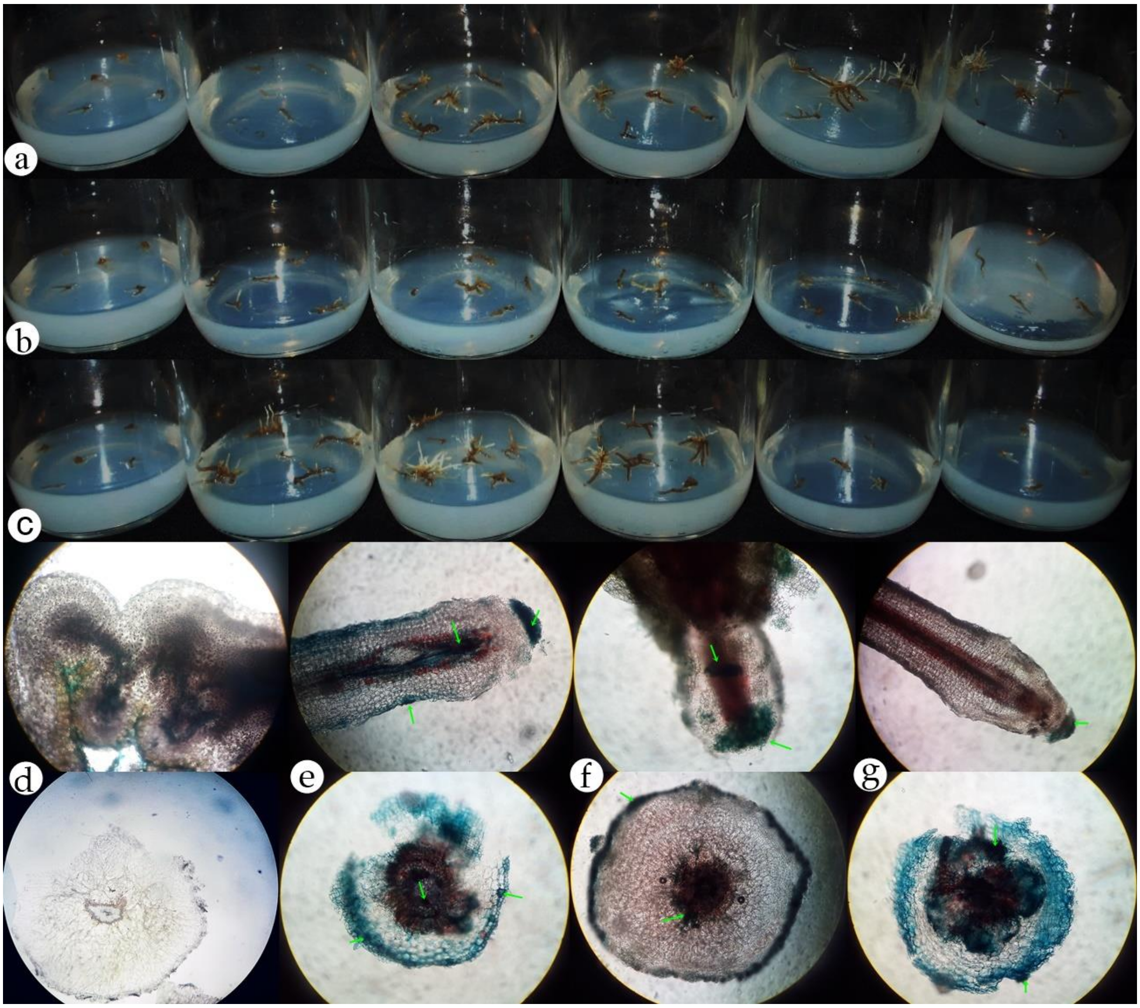

Fig. 1. Effect of metal nanoparticles on the growth of *in vitro* Ngoc Linh ginseng lateral root: **a**. nAg (1–3 mg/L); **b**. nZnO (0.5–2.5 mg/L); **c**. nCu (1–3 mg/L); **d**. Horizontal and vertical anatomy of lateral root controls, **e**. Horizontal and vertical anatomy of lateral root on MS medium containing nZnO, **f**. Horizontal and vertical anatomy of lateral root on MS medium containing nAg, **g**. Horizontal and vertical anatomy of lateral root on MS medium containing nCu.

3.2 Effect of zinc oxide nanoparticle on the growth of *in vitro* Ngoc Linh ginseng lateral root

After 60 days of treatment supplemented with different concentrations of nZnO in free-hormone-MS medium, the results showed that mean of number of roots, root length, fresh weight, and dry weight were statistically different in various concentrations of nZnO (Table 2, Fig. 1b). Other researches also showed the impacts of the different nZnO concentration on root growth 19. Root length in foliar sprayed plants also increased with 250 mg/L ZnO; concentrations above 250 mg ZnO decreased root length on the tomato 19. Beside, Pokhrel and Dubey (2013) showed that nZnO and nAg are lower toxic to the early root growth in maize and cabbage, albeit than their specified ionic salts 18. Among them, 2 mg/L nZnO reached best effects to *in vitro* Ngoc Linh ginseng lateral root formation and growth which manifested in the best of number of roots (11.0 lateral roots), root length (2.6 cm), fresh weight (37.7 mg), and dry weight (4.0 mg). Preliminary observations were made for morphological characterization of Ngoc Linh ginseng lateral root (Fig. 1f). Thinner vascular tissue or cortex area, more black points in root cap area, but thicker endodermis, and more cell xylem than control were observed.

Table 2. Effect of zinc oxide nanoparticles on the growth of *in vitro* Ngoc Linh ginseng lateral root

nZnO Conc. (mg/L)	**Root number**	**Root length** (cm)	**Fresh weight** (mg)	**Dry weight** (mg)	**Lateral root formation** (%)
0	16.7^{a*}	1.0^{c}	25.3^{c}	2.5^{c}	26.7^{d}
0.5	1.3^{d}	0.7^{c}	17.0^{e}	1.4^{d}	28.3^{d}
1	2.7^{d}	1.0^{c}	21.0^{d}	2.1^{c}	46.7^{c}
1.5	7.7^{c}	1.6^{b}	33.0^{b}	3.2^{b}	73.3^{ab}
2	$\mathbf{11.0^{b}}$	$\mathbf{2.6^{a}}$	$\mathbf{37.7^{a}}$	$\mathbf{4.0^{a}}$	$\mathbf{80.0^{a}}$
2.5	7.0^{c}	2.1^{a}	34.7^{b}	3.3^{b}	66.7^{b}

*Different letters in a column indicate significant differences at $p < 0.05$ by Duncan's multiple range tests.

In *Lolium perenne*, considerable effects of nZnO in the root epidermis, endodermis and cortex, with internalization of NPs in the endodermal and vascular tissues were observed 13. In this study, the effects increased remarkably lateral roots by nZnO, but the main root explant was dark brown similar to the supplement of nAg in culture medium (Fig. 1). Although Raliya and Tarafdar (2013) revealed that the positive impact of nZnO on the growth and yield of clusterbean; nZnO was increased biomass, shoot length, root length, root area, chlorophyll content and total soluble leaf protein on field 20, our results nZnO is harmful for the ginseng root growth. Like the anatomy results, notably, the strategic investigations of root anatomy revealing cellular alterations in apical meristem, zone of elongation, and meta-xylem count, coupled with metal bio-uptake, moisture content, germination, and root elongation under NPs versus ionic salt treatments showed differential potential of metal-based NPs and their ions for developmental toxicity in agriculturally important crop plants 18.

3.3 Effect of copper nanoparticle on the growth of *in vitro* Ngoc Linh ginseng lateral root

Copper (Cu) is an essential micronutrient element in the ginseng root growth, as it is cofactors of many enzymes and play an important role in electron transport, redox reactions, and in a variety of metabolic pathways 15. Biomolecular responses to oxidative stress, similar to reactions used to abiotic synthesize Cu nanostructures of controlled size and shape, likely causing the transformation 1. This newly identified mode of copper bio-mineralization by plant roots under copper stress may be common in oxygenated environments. The knowledge of biochemical changes had explained why plants adopt against oxidative stress induced by accumulated metal ions. The key assessing effects of nCu in ginseng roots *in vitro* was a primary objective of this study. Our results revealed that nCu enhanced the lateral root formation and growth of Ngoc Linh ginseng roots compared with control (Table 3, Fig. 1c). In Cu nanoparticle treatments (1–3 mg/L), after 60 days, 1.5 mg/L nCu was the best with 31 lateral roots in 2.3 cm length, 57.7 mg fresh weight, 9.3 mg dry weight, and 99.3% lateral root formation (Table 3). The speculation that Cu nanoparticles adhered to the root, because complex formation may not be true, as there must be some complexing agent exuded by the root hairs. The adsorption of CuO nanoparticles by wheat root was concentration dependent 11. The authors have concluded that Na_4EDTA increases the solubility of CuO nanoparticles 15. Therefore, lateral roots could grow better in medium containing nCu.

Table 3. Effect of copper nanoparticle on the growth of *in vitro* Ngoc Linh ginseng lateral root

nCu (mg/L)	Root number	Root length (cm)	Fresh weight (mg)	Dry weight (mg)	Lateral root formation (%)
0	16,7^{d*}	1.0^{b}	25.3^{f}	2.5^{d}	26.7^{c}
1	20.3^{c}	1.2^{b}	36.0^{d}	3.8^{c}	81.7^{b}
1.5	**31.0^{a}**	**2.3^{a}**	**57.7^{a}**	**9.3^{a}**	**99.3^{a}**
2	26.3^{b}	2.2^{a}	51.3^{b}	4.8^{b}	85.0^{b}
2.5	16.7^{d}	1.1^{b}	42.3^{c}	3.8^{c}	80.0^{b}
3	8.0^{e}	0.9^{b}	31.7^{e}	3.2^{cd}	80.0^{b}

*Different letters in a column indicate significant differences at $p < 0.05$ by Duncan's multiple range tests.

The effects of different NPs on the growth of Ngoc Linh ginseng lateral roots *in vitro* are presented in Fig. 1, 2. In our combined analyses, the optimum for ginseng lateral root growth was nCu, and the lowest was nZn. NPs recognized the potential risk and benefits of nanotechnology in plant growth; and most studies showed that NPs could produce toxic effects above a certain concentration 111218. Our results also showed that nZnO from 0.5 mg/L (decease the lateral root number) and 2.5 mg/L (decrease percent of lateral root formation), nCu and nAg above 2.5 mg/L also inhibited root growth. Moreover, horizontal and vertical anatomical morphology of lateral roots on medium containing NPs showed that a thick epidermis, xylem, endodermis and root cap contained a lot of black points, especially, in nAg treatments (Fig. 1e, f, g), but controls don't have this phenomenon (Fig. 1d).

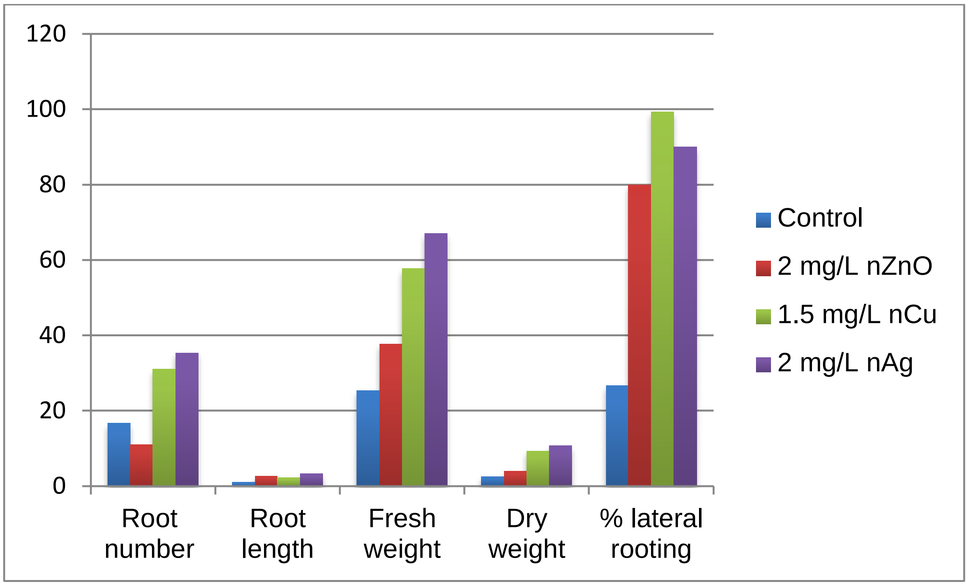

Fig. 2. Effect of metal nanoparticles on the growth of Ngoc Linh ginseng lateral roots

4 Conclusion

According to our results, the application of NPs was more effective in improving the Ngoc Linh ginseng lateral root growth compared with the control. This positive impact of nCu and nAg was more noticed than that of nZnO on the ginseng lateral root growth. The highest increase was observed with nCu at the concentration of 1.5 mg/L. Similar alterations in the anatomy, including black point appearance, phloem disorganization, and expansion of vascular bundles were observed in the root cultured in the medium supplemented with the metal nanoparticles.

Acknowledgements

This work was supported by "Effect of metal nanoparticles on the regeneration, growth and development of some valuable plants in Vietnam" Project by Vietnam Academy of Science and Technology under project No. VAST.TĐ.NANO.04/15–18.

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