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RESEARCH ARTICLE

Influence of metal and metal oxide nanoparticles on growth and total phenolic content accumulation of *Anoectochilus roxburghii* cultured *in vitro*

Huong Thi Trinh[1], Dang Hai Hoang[1] & Tuan Trong Tran[2*]

[1]Ho Chi Minh City University of Industry and Trade, HCM City 700 000, Vietnam
[2]Plant Cell Technology Department, Institute of Tropical Biology, Vietnam Academy of Science and Technology, HCM City 700 000, Vietnam

*Email: trantrongtuan.itb@gmail.com

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Abstract

The application of metal nanoparticles in agriculture and related fields, especially in plant cell tissue culture has increased interest in recent years due to its potential benefits. This study used gold nanoparticles (AuNPs), silver nanoparticles (AgNPs), copper nanoparticles (CuNPs) and magnetite nanoparticles (Fe_3O_4NPs) to evaluate their effect on the growth and total phenolic content (TPC) of *Anoectochilus roxburghii*. The results showed that Fe_3O_4NPs were the most positively influenced metal nanoparticles in increasing biomass and TPC of *A. roxburghii* among metal nanoparticles tested. After 8 weeks of culture, the dry weight (DW) and TPC of the plants cultured on the medium containing 5 ppm Fe_3O_4NPs were 39.07 mg and 13.0 mg gallic acid respectively per g of dry weight (mg GAE/g DW). Meanwhile, on the medium without Fe_3O_4NPs, they were 30.47 mg and 6.71 mg GAE/g DW respectively. This study proposed an effective approach to improve the growth and accumulation of TPC in *A. roxburghii*. Moreover, it suggests the potential application of metal nanoparticles in plant tissue culture and the production of bioactive compounds.

Keywords

Anoectochilus roxburghii; AgNPs; AuNPs; CuNPs; Fe_3O_4NPs; phenolic

Introduction

Anoectochilus roxburghii (Wall.) Lindl. (*A. roxburghii*), a perennial herb, also known as "Lan kim tuyến" in Viet Nam is a member of the Orchidaceae family. It is distributed in India, Nepal, Bhutan, China, Myanmar, Thailand, Laos, Cambodia, Malaysia, Indonesia and Vietnam. *A. roxburghii* contains secondary compounds such as polysaccharides, triterpenoids and phenolics and is used to treat diabetes, tumors, hyperlipidemia and hepatitis (1). *A. roxburghii* polysaccharides (ARPs), acts as one of the main components has multiple pharmacological activities including anti-oxidant, anti-inflammation, anti-hyperglycemia and anti-hyperlipidemia (2). Another study showed that ARPs lowered the pH value in the cecum and increased its content of probiotic bifidobacteria (3). Additionally, ARPs supplementation significantly decreased the relative abundance of several bacterial genera such as *Parabacteroides*, which may play regulatory roles in cognitive function (4). Dietary supplementation of

ARPs can protect mice against diet-induced obesity, fatty liver and insulin resistance (5). In a recent study, three new compounds, roxburic acid A and 2 flavone glycosides were isolated from the ethanol extract of fresh *A. roxburghii*. The free radical scavenging activity of these isolated compounds was assessed using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay and the results showed that kaempferol-7-O-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside and rutin (a known compound) exhibited potential antioxidant activity with IC_{50} values of 139 µg/mL and 22.5 µg/mL respectively (6). A recent study has revealed the potential anti-cancer effects of *A. roxburghi* (AR) on human acute T-cell (JURKAT), human multiple myeloma (MM1S, U266) and human acute monocytic leukemia (THP1) cell lines. The results of this study indicated that AR treatment decreased cell viability and induced apoptosis in each of these cancer cell lines (7). Compound *A. roxburghii* oral liquid may exhibit beneficial hepatoprotective effects by reducing inflammation, mitigating oxidative stress and modulating metabolites and their metabolic pathways (8). With its unique medicinal properties, this species has been continuously exploited for many years. In addition, with a limited number of individuals with slow growth and regeneration in the wild, the number has decreased significantly, with the risk of extinction (1). Therefore, it is essential to find some promising methods to facilitate plant growth and improve the productivity of bioactive metabolites to improve the overall quality of *A. roxburghii*.

Nowadays, along with the development of plant biotechnology, plant cell tissue culture is considered an optimal method to approach and resolve this issue. Several methods used to facilitate *A. roxburghii* biomass have been researched and applied today, such as the liquid shaking culture method and culture in a bioreactor. Besides, using exogenous elicitors is considered an effective strategy to increase the content of biologically active compounds in medicinal plants. More commonly used elicitors, such as methyl jasmonate, jasmonic acid, salicylic acid, chitosan and metal nanoparticles (NPs) are emerging as a new group of abiotic elicitors that are attracting researchers. The nano industry developed rapidly about a decade ago, but high production costs have impeded its further development (9, 10). Additionally, in most stages of producing NPs by physical and chemical methods, energy and hazardous reagents are required (mainly stabilizing and reducing agents) (11) that have unintended effects such as environmental pollution and potential health problems (12). Recently, nanotechnology has developed various methods for producing NPs to overcome these challenges. For example, the production cost of nano-silica using the sol-gel method might be expected to be around 1.3 € g^{-1} and this cost is lower than the conventional chemical and physical methods, indicating potentially high-cost competitiveness (13). Furthermore, the green synthesis method mainly uses microorganisms or plant extracts instead of industrial chemical agents that have been developed. Green synthesis is advantageous over traditional chemical synthesis because it is cost-effective, reduces pollution, enhances the safety of human health and is environmentally friendly (12). Metal nanoparticles have large surface areas, strong adsorption capabilities and high reduction potential, making them suitable for removing pollutants. For example, iron nanoparticles (FeNPs) synthesized from eucalyptus leaf extract have been applied to remove a mixture of Cu and Cr (VI) with efficiencies of 33.0 % and 58.9 % respectively (14) or zero-valent iron nanoparticles (NZVI) could remove 30.4 % of total phosphorus in wastewater (15). However, it should be noted that when used in agriculture, iron nanoparticles may reduce the phosphorus uptake by plants. Nanotechnology also harnesses agricultural waste and ecological balance. For example, *Eichhornia crassipes* (water hyacinth) is an invasive weed, using it to synthesize FeNPs could reduce its population in the environment. It would not only help reduce waste via waste utilization, but also limit serious ecological damage (16).

Recent advancements in nanotechnology and its application in agriculture are on the rise to improve crop production (17). Nanomaterials are utilized in creating biosensors, formulating nanopesticides and nanofertilizers, genetically transforming plants and animals to exhibit desirable traits and serving as nano plant-growth regulators. The incorporation of nano-based approaches into the agricultural system has the potential to boost economic growth by up to three trillion USD in recent years (18). They can be employed for the targeted delivery of various nutrients essential for promoting the growth, development and productivity of plants. NPs are applied at lower levels compared to chemical fertilizers. The refined use of NPs also mitigates the adverse effects of synthetic fertilizers on the environment. NPs also play a crucial role in protecting plants from diseases, pests and pathogens. NPs can also influence the modification of plant gene expression, leading to changes in biological and metabolic pathways controlled by associated genes, thereby impacting the growth and development of plants. The utilization of nano-enhanced solutions can enhance seed germination rates, provide plant protection and contribute to overall growth and development. This is primarily achievable through the judicious or controlled release of agrochemicals, consequently reducing nutrient losses caused by leaching, volatilization and other processes (19).

In plant biotechnology, many recent studies have used nanometals and metal oxide nanoparticles (NPs) to improve *in vitro* plant growth and biomass cultivation to collect bioactive compounds (20). AuNPs, AgNPs, CuNPs and Fe_3O_4NPs have been used in the *in vitro* cultivation of

medicinal plants and obtained promising results (21). Researchers studied the effects of copper and gold nanoparticles on the development of lettuce (22). *Araucaria excelsa* shoots cultured in MS medium supplemented with AgNPs had better growth than medium without AgNPs (23). Besides, metal nanoparticles promoted secondary metabolite accumulation. It was showed that phenolic acids, such as chlorogenic, coumaric, gallic and tannic acid, accumulated after *Prunella vulgaris* callus were exposed to Ag, Au and Ag/Au nanoparticles (24). CuO nanoparticles significantly improved the flavonoid, phenolics and tannin content along with antioxidant capacity in the roots of *Withania somnifera* (L.) Dunal (25).

Although nanometals have been studied in plant cell cultures, no publication has been reported on *A. roxburghii*. In addition, this species grows slowly in nature, and its population has decreased significantly, posing a risk of extinction. This highlights the necessity of applying plant cell culture technology combined with nanomaterials to facilitate plant growth and enhance the productivity of bioactive metabolites. In this study, we assessed the effects of nanometals on the growth and content of total phenolic compounds (TPC), a group of compounds that have been reported to be the main antioxidant components in many medicinal plants due to their ability to scavenge free radicals, prevent and treat many diseases related to oxidative stress (26). The aim is to determine the suitable culture medium for increasing the growth and accumulation of TPC of *A. roxburghii* cultured *in vitro*.

Materials and Methods

Plant material

A. roxburghii plants were grown on the Murashige and Skoog's medium (MS) (27) augmented with 30 g/L sucrose and 8.0 g/L agar. Every 10 weeks, these plants were subcultured in the same medium for preservation. Apical shoots with uniform sizes (height of 2.5 cm) were selected as explants in this study.

Experimental designs

The effect of various kinds and concentrations of metal nanoparticles on the growth and total phenolic content of *A. roxburghii*

A. roxburghii shoots (with a height of 2.5 cm) were cultured in the 250 mL flask containing 40 mL MS supplemented with 30 g/L sucrose, 8.0 g/L agar and individual nanometals (AuNPs, AgNPs, CuNPs and Fe_3O_4NPs) with various concentrations (1.0, 3.0, 5.0 and 7.0 ppm). Treatment without metal nanoparticles was used as the control. The pH of a medium was adjusted to 5.7–5.8 before autoclaving at 121 °C, 1 atm for 15 min. Each treatment was carried out on 5 flasks containing 3 shoot explants. After 8 weeks of culturing, the dry weight (g), the plant height (cm), SPAD index, total phenolic content (mg GAE/g DW) and total phenolic content/plantlet (mg GAE/plantlet) were observed.

The nanometals had a particle size of 5–20 nm and were provided by the Center for Radiation Technology Research and Development and Institute of Tropical Biology, Vietnam Academy of Science and Technology, Vietnam. SPAD index was measured by using a CL-01 Chlorophyll meter, (Hansatech Instrument Ltd, England). Total phenolic content (mg GAE/g DW) was quantified by the Folin-Ciocalteu method (28).

Total phenolic content/plantlet (mg GAE/plantlet) = Total phenolic content (mg GAE/g DW) × Dry weight of plantlet (g) (Eqn. 1)

Culture condition

The *A. roxburghii* plants were incubated in a 24 ± 2 °C, growth room with 2 white fluorescent lamps (36W each, Rang Dong, Viet Nam) illuminating each shelf at a light flux density of 35 ± 2 mol m^{-2} s^{-1}, a photoperiod of 12 h.

Statistical analysis

The experiments (each with three replications) were arranged in a completely randomized design. The data was statistically analyzed by using Statgraphics Centurion software at 95 % confidence level with LSD's test.

Results

The effect of gold nanoparticle concentrations on the growth and total phenolic content of A. roxburghii

After 8 weeks of cultivation, the growth indicators of *A. roxburghii* in the treatment using 1.0 ppm AuNPs were

Table 1. The effect of AuNPs concentrations on the growth and accumulation of TPC of *in vitro A. roxburghii* after 8 weeks of culture.

AuNP (ppm)	Plant height (cm)	DW/plantlet (mg)	SPAD index	TPC (mg GAE/g DW)	TPC/plantlet (mg GAE)
Control	3.17^{b}	31.19^{c}	9.81^{b}	6.55^{e}	0.204^{d}
1.0	3.49^{a}	40.90^{a}	10.99^{a}	7.09^{d}	0.290^{a}
3.0	3.20^{b}	37.48^{b}	9.62^{b}	7.62^{c}	0.286^{b}
5.0	3.11^{b}	31.76^{c}	9.25^{c}	8.96^{a}	0.285^{b}
7.0	2.84^{c}	26.81^{d}	7.24^{d}	7.82^{b}	0.210^{c}
p-value	*	*	*	*	*

*The different letters (a, b,...) in the same column indicated a statistically significant difference with a confidence level of 95 %. *p < 0.05 shows significant differences between groups

higher than in the other treatments, with the mean values of plant height, dry weight and the SPAD index being 3.49 cm, 40.90 mg and 10.99 respectively. However, these growth indicators decreased when the AuNPs concentration was continuously increased from 3 to 7 ppm. The plant's lowest plant height, dry weight and SPAD index were cultured in the medium supplemented with 7.0 ppm AuNPs (Table 1). Morphological observations showed that the plants in the treatments supplemented with 1.0 and 3.0 ppm AuNPs had larger leaves and were healthier than the remaining treatments (Fig. 1a).

The TPC in all treatments using AuNPs was higher than the control. The maximum TPC was obtained in the medium containing 5.0 ppm AuNPs (8.96 mg GAE/g DW) (Table 1). It can be seen that the concentration of AuNPs suitable for the growth and the phenolic accumulation was different. The TPC per plantlet, reflecting the yield of total phenolic obtained in cultivating *A. roxburghii* biomass was selected as the suitable indicator for defining AuNPs concentration compatible with the growth and TPC of *A. roxburghii*. Based on this indicator, the medium augmented with 1.0 ppm AuNP was suitable for both the growth and the TPC of *A. roxburghii* cultured *in vitro*.

The effect of silver nanoparticle concentrations on the growth and total phenolic content of A. roxburghii

Similar to AuNP, the highest growth index was reached in the treatment using 1.0 ppm AgNPs, with an average plant height of 3.84 cm, dry weight of 39.67 mg and SPAD of 9.42. The growth of *A. roxburghii* was inhibited when the shoots were cultured on a medium supplemented with 7.0 ppm AgNPs, with the dry weight and SPAD index obtained lower than the control (Table 2). Regarding morphology, the plants in the treatments with 1.0 and 3.0 ppm supplementation had significantly larger plant height and stem diameter than the remaining treatments.

The TPC obtained in the treatment using AgNPs was higher than that of the control. In particular, the TPC was highest in the medium supplemented with 3.0 ppm AgNPs (10.68 mg/g DW) (Table 2). Similar to the results observed in the medium supplemented with AuNPs, this experiment also showed that the optimal concentration of AgNPs for growth and TPC accumulation is different. Therefore, the selection of AgNP concentration was also based on the TPC/plantlet indicator. The results indicated that the optimal concentration of AgNPs to be added to the *A. roxburghii* culture medium is 3.0 ppm.

The effect of copper nanoparticle concentrations on the growth and accumulation of total phenolic content of in vitro A. roxburghii

When adding CuNPs to the culture medium, the results obtained after 8 weeks of culture showed that the growth parameters of *A. roxburghii* (DW, SPAD index) were lower than those of the control. In contrast, the TPC obtained in plants grown on a medium supplemented with CuNPs was higher than that of the control (Table 3, Fig. 1). Thus, CuNPs inhibited the growth of plants while promoting TPC accumulation of *A. roxburghii*. The highest TPC was collected in the 3.0 ppm CuNPs treatment and the highest TPC/plantlet content was also recorded in this treatment. Therefore, CuNPs with a concentration of 3.0 ppm were suitable for the growth and TPC of *A. roxburghii* (Table 3).

The effect of Fe_3O_4 nanoparticle concentrations on the growth and total phenolic content of A. roxburghii

The height of the plants cultivated in the medium without and with 1.0 ppm Fe_3O_4NPs was the highest (3.22 cm and 3.17 cm respectively), and there was no statistically

Table 2. The effect of AgNPs concentrations on the growth and accumulation of TPC of *in vitro A. roxburghii* after 8 weeks of culture.

AgNP (ppm)	Plant height (cm)	DW/plantlet (mg)	SPAD index	TPC (mg GAE/g DW)	TPC/plantlet (mg GAE)
Control	3.13^{c}	30.33^{d}	9.21^{ab}	6.97^{c}	0.211^{d}
1.0	3.84^{a}	39.67^{a}	9.42^{a}	7.48^{c}	0.297^{bc}
3.0	3.71^{a}	36.14^{b}	8.72^{bc}	10.68^{a}	0.386^{a}
5.0	3.47^{b}	33.57^{c}	8.57^{c}	9.60^{b}	0.322^{b}
7.0	3.15^{c}	27.95^{e}	8.48^{c}	9.98^{ab}	0.279^{c}
p-value	*	*	*	*	*

*The different letters (a, b,...) in the same column indicated a statistically significant difference with a confidence level of 95 %. *p < 0.05 shows significant differences between groups.

Table 3. The effect of CuNPs concentrations on the growth and accumulation of TPC of *in vitro A. roxburghii* after 8 weeks of culture.

CuNP (ppm)	Plant height (cm)	DW/plantlet (mg)	SPAD Index	TPC (mg GAE/g DW)	TPC/plantlet (mg GAE)
Control	3.11^{a}	30.62^{a}	9.65^{a}	6.96^{e}	0.213^{e}
1.0	2.98^{ab}	29.67^{b}	9.04^{b}	7.49^{d}	0.222^{d}
3.0	3.02^{ab}	29.23^{b}	8.09^{c}	8.44^{a}	0.247^{a}
5.0	3.02^{ab}	29.07^{b}	7.67^{d}	8.32^{b}	0.242^{b}
7.0	2.90^{b}	29.00^{b}	7.50^{d}	7.76^{c}	0.225^{c}
p-value	*	*	*	*	*

*The different letters (a, b,...) in the same column indicated a statistically significant difference with a confidence level of 95 %. *p < 0.05 shows significant differences between groups.

Fig. 1. Effects of metal nanoparticles on the growth and TPC of *in vitro A. roxburghii* plantlets after 8 weeks of culture. **a.** in medium with AuNPs; **b.** in medium with AgNPs, **c.** in medium with Fe_3O_4NPs; **d.** in medium with CuNPs. From left to right in each figure corresponds to the concentration of each type of metal nanoparticle: control, 1, 3, 5, 7 ppm.

Table 4. The effect of Fe_3O_4NPs concentrations on the growth and accumulation of TPC of *in vitro A. roxburghii* after 8 weeks of culture.

Fe_3O_4NP (ppm)	Plant height (cm)	DW/plantlet (mg)	SPAD index	TPC (mg GAE/g DW)	TPC/plantlet (mg GAE)
Control	3.22[a]	30.47[c]	9.89[d]	6.71[e]	0.204[e]
1.0	3.17[a]	32.00[c]	10.54[c]	6.97[d]	0.223[d]
3.0	3.03[b]	37.33[b]	11.10[c]	10.06[c]	0.376[c]
5.0	3.02[b]	39.07[a]	14.74[a]	13.00[a]	0.508[a]
7.0	3.05[b]	37.13[b]	13.70[b]	12.76[b]	0.474[b]
p-value	*	*	*	*	*

*The different letters (a, b,...) in the same column indicated a statistically significant difference with a confidence level of 95 %. *p < 0.05 shows significant differences between groups.

significant difference between the 2 treatments. As the concentration of Fe_3O_4NPs increases from 3.0 ppm to 7.0 ppm, the plant height obtained tends to decrease. However, the plants have better leaf development in these treatments than in the control (Table 4, Fig. 1c). This suggests that Fe_3O_4NPs do not increase plant height but rather stimulate the development of leaves and the total chlorophyll content in leaves, leading to increased biomass accumulation. Therefore, the dry weight of plants obtained in the treatments using 3.0–7.0 ppm Fe_3O_4 NPs was higher than that of the control and the treatment with 1.0 ppm Fe_3O_4 NPs. In the treatment using 5.0 ppm Fe_3O_4NPs, the dry weight of the plant and the TPC were the highest (39.07 mg and 13.0 mg GAE/g DW respectively). The TPC per plantlet indicator was also the highest in the treatment using the concentration of 5.0 ppm Fe_3O_4NPs (Table 4). Therefore, 5.0 ppm Fe_3O_4NPs is the optimal concentration for the growth and accumulation of TPC of *A. roxburghii* cultured *in vitro*.

The effects of metal nanoparticles on the growth and total phenolic content of in vitro A. roxburghii

In this study, we also compared the effects of different metal nanoparticles on the growth and TPC of *A. roxburghii* cultured *in vitro*. The shoot tips with an average height of 4.0 cm were cultured in a medium supplemented with metal nanoparticles at optimal concentrations for growth and TPC accumulation from the above experiments (1.0 pm AuNPs, 3.0 ppm CuNPs, 3.0 ppm AgNPs and 5.0 ppm Fe_3O_4NPs). The medium without metal nanoparticles was the control treatment. The recorded results show that the dry weight was the highest on the culture medium supplemented with 3.0 ppm AgNPs, but plant height and chlorophyll content were highest in the medium using 5.0 ppm Fe_3O_4NPs (Fig. 2). Morphological observations showed that the leaf area of these plants cultured on the medium supplemented with AgNPs is larger than the other media (Fig. 3). Therefore, the dry weight of *A. roxburghii* seedlings obtained on the medium supplemented with AgNPs is the highest.

The *A. roxburghii* were cultured on the medium supplemented with 5.0 ppm Fe_3O_4NPs with the highest TPC content. The results showed that the phenolic content per plantlet obtained in the control medium was lower than in the media supplemented with metal nanoparticles.

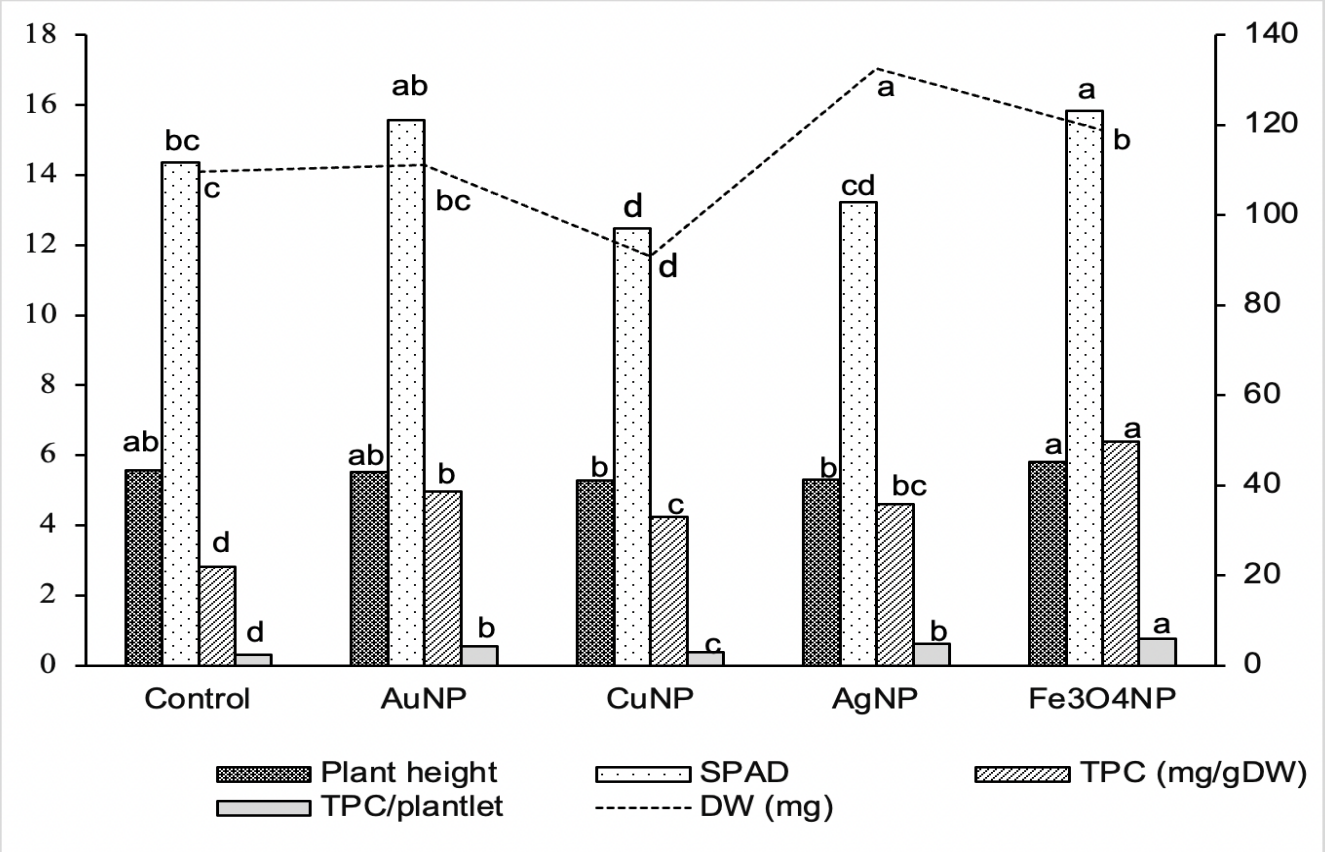

Fig. 2. The effects of metal nanoparticles on the growth and TPC of *in vitro A. roxburghii* plantlet after 8 weeks of culture.

Fig. 3. Effect of different types of metal nanoparticles on the growth of *in vitro A. roxburghii* plants after 8 weeks of culture. C: Control, Au: 1.0 ppm

Of the four metal nanoparticles surveyed, CuNPs yielded lower results than the others. In the medium using Fe_3O_4NPs, the dry weight obtained was not the highest. Still, the TPC obtained in this medium was the highest, resulting in the highest phenolic content per plantlet. Compared to the control, the medium supplemented with 5.0 ppm Fe_3O_4NPs increased the phenolic content per plantlet by 2.46 times (Fig. 2 and 3).

Discussion

Some studies reported plant responses to AuNPs, including positive and negative effects on plant growth and development (29). In our study, AuNPs with various concentrations have different effects on the *A. roxburghii* growth and accumulation of TPC. In the growth phase, adding AuNPs at a low concentration (1.0 ppm) to the medium increased plantlet height, dry weight and total chlorophyll content. However, the growth index of *A. roxburghii* decreased when the concentration of AuNPs increased higher than 1.0 ppm. This result was similar to a previous study on *Lavandula angustifolia* Mill., in which the lowest concentration of AuNPs (1.0 mg/L) in the medium enhanced the growth of this plant and AuNPs at high concentrations from 20–50 mg/L inhibited the growth of lavender plants (30). According to a study, AuNPs increase endogenous gibberellic acid content, leading to the shoot's elongation (31). In our experiment, AuNPs at concentrations of 3.0–5.0 ppm did not promote plant growth, but these concentrations of AuNPs stimulated TPC accumulation of *A. roxburghii* plants. The TPC in the plants increased with increasing AuNPs concentration. The TPC reached the highest level after 8 weeks of culture at a concentration of 5.0 ppm AuNPs. Similar to our results, another study also reported that AuNPs alone or combined with CuNPs enhanced biomass accumulation and the production of secondary metabolites in the adventitious root of *Stevia rebaudiana* (Berts) (32). *Ex vitro* 6-year-old ginseng sprouts treated with AuNPs increased the content of ginsenosides Rg_1, Re, Rf and Rb_1 (33).

The previous studies also reported the role of AgNPs in

the growth and development of higher plants. For example, AgNPs improved or inhibited the growth of wheat and *Eruca sativa* plantlets, enhanced the development of the roots as well as activating genes related to cell growth, metabolism and hormone signalling pathways, photosynthesis (34, 35). In our study, plant height, dry weight and total chlorophyll content of *A. roxburghii* cultured in a medium supplemented with 1.0 ppm AgNPs were the highest. However, when the AgNPs concentration was increased (higher than 1.0 ppm), these indicators gradually decreased (Table 2, Fig. 1b). Some previous studies have shown that AgNPs at high concentrations can disrupt chlorophyll synthesis, reducing chlorophyll content in leaves. This affects plants' photosynthesis, leading to plant growth inhibition (36). In the results of our experiment, compared to the control treatment, the chlorophyll content was equal to (1.0 ppm AgNPs) or lower (3.0–7.0 ppm AgNPs), which limited the increased biomass of *A. roxburghii* plants when adding AgNPs with increasing concentrations to the medium. Our result is similar to another study, the fresh and dry weight of *Spirodela polyrhiza* decreased significantly with the increase in AgNPs concentration (37). A study revealed that the plant height and mass of *Brassica* in a medium supplemented with AgNPs were lower than in the control (38). However, this result is contrary to another study, where AgNPs treatment improved the plant height of *Borago officinalis* plants (39). Researcher also reported that banana shoots grew well on the medium supplemented with AgNPs with concentrations of 3-15 mg/L and grew best on medium supplemented with 12 mg/L AgNPs (40). This shows that the positive or negative effects of AgNPs on plant growth and development depend on the concentration of AgNPs and various types of explants. Besides AuNPs, AgNPs is an abiotic elicitor used to promote the accumulation of secondary compounds in plants. The TPC obtained from *A. roxburghii* plants in treatments using AgNPs was higher than in the control. Similar to previous research, the authors also reported that suspension cells cultured in a medium supplemented with 5.0 mg/L AgNPs had higher phenolic content than the control and the remaining treatments (41). Similarly, the phenolic content in *Prunella vulgaris* callus also improved significantly in a medium supplemented with AgNPs (24). A study showed that AgNPs enhanced the accumulation of phenolic content in vanilla (*Vanilla planifolia*) (42).

In recent years, CuNPs have also attracted the attention of plant researchers. Various studies have been conducted to evaluate the impact of CuNPs on different plant species. Authors also studied the effects of CuNPs on the growth of lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*) (43). Their study showed that adding CuNPs at a concentration of 1.0 ppm increased plant species' fresh and dry weight. Another group of authors also studied the effects of CuNPs on wheat plants. The study reported that adding CuNPs at a concentration of 1.0 ppm improved the drought tolerance of wheat plants (44). Similarly, authors studied the effects of CuNPs on *Stevia rebaudiana*. Their study showed that 1.0 ppm CuNPs increased the content of steviol glycosides in *S. rebaudiana* (45). Copper is one of eight essential microelements for plants and is required for many enzymatic activities, chlorophyll biosynthesis and seed formation. Copper deficiency can lead to increased susceptibility to various diseases, which can cause a significant yield decrease. However, using copper at high concentrations will be toxic to plants. In the experiment, *Phaseolus radiatus* and *Triticum aestivum* plant height were negatively correlated with CuNPs concentration (46). These results are similar to our study; plant height, dry weight and chlorophyll content of *A. roxburghii* plants in the treatment using CuNPs were all lower than in the control. According to researchers, the addition of CuNPs to the culture medium also increased the content of hydrogen peroxide and lipid peroxidation in the roots, decreased the chlorophyll content, significantly increased the proline concentration and increased reactive oxygen species (ROS) in the roots resulting in reduced plant growth (47). A study reported that inhibition of growth and stress-induced activity in plants led to an increase in total phenol and flavonoid content (48). Several studies also showed that Cu promoted increased secondary compound content. For example, it stimulated the accumulation of aromatic amino acids in *Cucumis sativus*, *Zea mays* and *T. aestivum* L. (49, 50). In our study, the increase in TPC was opposite to the growth indicators; the TPC in the treatments using CuNPs was higher than the control.

Iron, a microelement essential for plant growth and development, is an important component of cytochromes, chlorophyll and many enzymes and plays a critical role in DNA synthesis, respiration and photosynthesis (51). In *in vitro* culture media, iron is often provided as chelated iron. Chelated iron is a form of iron bound to another molecule, which helps keep iron in a soluble form and makes it easier for plants to absorb. Plants absorb iron mainly through their roots and then transfer it to their leaves. However, some biological barriers, such as cell walls, cell membranes and Casparian strips can prevent the absorption and transport of iron in plants. Iron oxide nanoparticles, with tiny particle sizes (5–20 nm), can overcome these barriers so that iron can be easily absorbed and transported in plants. Current literature revealed that the uptake, translocation and accumulation of NPs depend on various factors, including plant species, plant age and growing environment. They are also influenced by the physiochemical properties of the NPs, such as type, size, shape, chemical composition, functionalization and stability of the nanoparticles in solution (52). Several mechanisms for the uptake of NPs by plant cells have been suggested, including binding to carrier proteins, through aquaporins, ion channels, endocytosis, the creation of new pores or interaction with organic chemicals in the surrounding

media (52). NPs may form complexes with membrane transporters or root exudates, facilitating their subsequent transport into plants (53). Once the NPs have entered the cell membrane, they can be transported apoplastically or symplastically and plasmodesmata may serve as conduits for intercellular NPs movement (52). Another aspect that needs attention is the location and manner in which absorbed nanoparticles are stored within plants. Available literature indicates that NPs are found in plant cells and tissues such as vacuoles, cytoplasmic strands (54), the surface of root cell organelles (55) and stems (56). In the present study, we determined the iron content in *A. roxburghii* plants using the flame atomic absorption spectrometry (F-AAS) method. The results revealed that iron concentration in the explants was found to be on an average 5.3 times higher in the plants exposed to 5 ppm Fe_3O_4NPs than in the control. This result suggests that Fe_3O_4NPs were uptaken and accumulated, leading to an increase in iron content in the plant. Increasing iron content improved the chlorophyll content, thereby stimulating photosynthetic reactions, resulting in enhanced dry weight of *A. roxburghii* plants (Table 4). Similar to our results, iron oxide NPs have been observed to enhance the dry weight of soybean pods and leaves (57).

On the other hand, iron is a microelement, so the need for iron content in plants is not high. Therefore, the growth of *A. roxburghii* plants was inhibited at a concentration of 7.0 ppm Fe_3O_4NPs in our study (Table 4). According to a study, cell viability decreased at high concentrations of magnetite nanoparticles (58). Excessive iron content can disturb physiological functions and stimulate the generation of ROS, leading to damage and death in plant cells (59). The cause of oxidative stress in plants is that Fe_3O_4NPs absorbed on the root surface and root cells disrupt metabolic activities (e.g., respiration) and lead to the loss of local stabilization of the cell wall and/or the lipid bilayer system on root cell membranes (52). The aggregation of the NPs, caused by the precipitation of nanomaterial at high concentrations (60), was also identified as another factor contributing to cell damage. This aggregation resulted in the blockage of the apoplastic pathway, which is a probable route for the uptake of these NPs in the plant tissues (52). For example, zero-valent Fe NPs completely inhibited the germination of ryegrass, flax, and barley at very high concentrations (2000 and 5000 mg/L) (61). Likewise, authors reported that low concentrations of magnetite nanoparticles stimulated the growth of young popcorn plants, while high concentrations caused an inhibitory effect (62). Thus, metal nanoparticles can cause positive or negative effects on plant growth and development. Therefore, it is necessary to determine the appropriate concentration and type of metal nanoparticles for plant growth to improve the culture process's efficiency.

When comparing the different types of metal nanomaterials in this study, it is found that the *A. roxburghii* plant height obtained in the culture medium using 5.0 ppm Fe_3O_4NPs is much higher than the control and the remaining media. Authors stated that nano-iron oxide promotes growth by increasing plant cell size. Iron is a component of the cytochrome system of the electron transport chain (63). It can transfer electrons to O_2 to form H_2O, and it also forms H_2O_2 and generates the extraordinary reactive OH radical (64). In plants, these OH radicals can act as a loosening agent by degrading pectin polysaccharides in the cell walls (64, 65). The cell wall functions as the skeleton of the cytoplasm; therefore, loosening the cell wall is essential for growth and development, especially elongation in plants. Therefore, in the medium supplemented with iron nanoparticles, cells can grow faster than those in the remaining media, leading to the highest plant height. These results again demonstrate the potential value of applying metal nanomaterials in plant cell culture.

Conclusion

The effects of various types and concentrations of nanomaterials on the growth and TPC of *A. roxburghii* are different. To our knowledge, this is the first study using metal nanoparticles (AuNPs, AgNPs, CuNPs, Fe_3O_4NPs) in the culture medium of *A. roxburghii* to increase biomass and TPC accumulation. Expressly, the medium supplemented with 5.0 ppm Fe_3O_4NPs increased both growth and TPC of this plant, compared with the medium without Fe_3O_4NPs. This led to an increase in the biomass cultivation efficiency of medicinal plants through an increase in the phenolic content per plantlet by 2.46 times. The results of this study not only contribute to improving the efficiency of *A. roxburghii* biomass cultivation but also the TPC. It also improves the current scarcity of medicinal resources derived from *A. roxburghii* in nature. Furthermore, this study enriches the literature on the role of metal nanoparticles in the growth and metabolism of secondary compounds in *A. roxburghii* and suggests new ideas for research on medicinal plants and the development of metabolic pathways. However, the mechanisms of action of metal nanoparticles on plant biomass and TPC accumulation still need to be better understood, so more research is needed on plant growth when exposed to nanoparticles. This information will be helpful in applications in improving crop yields.

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Authors' contributions

HTT and TTT designed the experiment. HTT and DHH carried out the experiment and HTT and TTT performed growth and physiological analysis of plants. TTT and HTT performed statistical analysis. TTT and HTT wrote and reviewed the manuscript. All authors read and approved the final version.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

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