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# Enhanced shoot and plantlet quality of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar on medium containing silver and cobalt nanoparticles

Hoang Thanh Tung [a], Phan Le Ha Nguyen [a, b], Tran Van Lich [a], Ha Thi My Ngan [c], Do Manh Cuong [a], Vu Quoc Luan [a], Hoang Dac Khai [a], Nguyen Thi Nhu Mai [a], Bui Van The Vinh [c, *], Duong Tan Nhut [a, *]

[a] *Tay Nguyen Institute for Scientific Research, VAST, Dalat, Viet Nam*
[b] *Graduate University of Science and Technology, VAST, Hanoi, Viet Nam*
[c] *HUTECH University, Ho Chi Minh, Viet Nam*

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ABSTRACT

The effects of silver nanoparticles (AgNPs) and cobalt nanoparticles (CoNPs) in Murashige and Skoog (MS) medium were investigated with regard to shoot multiplication, *in vitro* rooting, acclimatization, growth and flowering of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar. The results showed that AgNPs had effects on the shoot multiplication stage, while CoNPs had effects on the *in vitro* rooting, acclimatization, growth and flowering stages. Single shoots grown on MS medium supplemented with 2 mg $L^{-1}$ AgNPs recorded optimal shoot multiplication efficiency, with shoots taller than 2 cm in height as well as reduced vitrification and yellowing of the leaf after 4 weeks of culture. In addition, the 2.5 cm shoots cultured on MS medium with 0.0465 mg $L^{-1}$ CoNPs being used instead of $CoCl_2.6H_2O$ gave rooting two days earlier and improved *in vitro* rooting. Moreover, reduction in vitrification, yellowing of the leaf and browning of the explant were observed in the rooting stage. There were reductions in ethylene accumulation and cellulase and pectinase enzyme activities, while the antioxidant activity increased in the rooting stage after 4 weeks, and this all enhanced the survival rate of plantlets in the acclimatization at the nursery stage.

## 1. Introduction

There are more than 80 countries in the world that produce and export gerbera plants, which rank among the top 10 most consumed flowers in the world (Ahmed et al., 2018). The output of cut gerbera was estimated at about 36 million USD in 2016, corresponding to 100 to 120 million cuttings per year (Deng and Bhattarai, 2018). Gerbera can be propagated by seeding or by separating shoots from the mother plant (Winarto and Yufdy, 2017). In addition, the gerbera micropropagation process has been developed almost completely, with stages such as original *in vitro* material and callus induction (Minerva and Kumar, 2013; Rahman, 2016; Winarto and Yufdy, 2017), shoot regeneration and multiplication (Rahman, 2016; Winarto and Yufdy, 2017), *in vitro* rooting and subsequent growth (Bhatia et al., 2012; Minerva and Kumar, 2013; Winarto and Yufdy, 2017). Cardoso and Teixeira da Silva (2013) detailed gerbera micropropagation on the main culture media used in different stages. During the shoot regeneration and multiplication stages, the culture media used most in studies is MS medium supplemented with different types of cytokinins such as BA, Kinetin or TDZ alone or in combination with auxins (IBA or NAA) in low concentrations (Kanwar and Kumar, 2008; Gantait et al., 2011; Hasbullah et al., 2011; Cardoso and Teixeira da Silva, 2012, etc.). However, using cytokinins at high concentrations in gerbera micropropagation often results in vitrification, stomatal deformation, curling and yellowing of leaves (Kumar et al., 2004).

In addition, there have been many studies on *in vitro* gerbera rooting with the addition of 6-Benzylaminopurine (BAP) combined with Indole-3-acetic acid (IAA) in culture medium (Kumar et al., 2004) or 1-Naphthaleneacetic Acid (Hussein et al., 2008) or Indole-3-butyric acid (IBA) (Bhatia et al., 2012; Cardoso and Teixeira da Silva, 2012) or pre-

*Abbreviation:* ACC, Acetyl-CoA carboxylase; AgNPs, Silver nanoparticles; APX, Ascorbate peroxidase; BA, Benzyl adenine; CAT, Catalase; Co, Cobalt; CoNPs, Cobalt nanoparticles; IBA, Indole-3-butyric acid; MSR, Rooting medium; MSS, Shoot multiplication medium; PGRs, Plant growth regulators; SOD, Superoxide dismutase

* Corresponding authors.
*E-mail addresses:* bvt.vinh@hutech.edu.vn (B.V.T. Vinh), duongtannhut@gmail.com (D.T. Nhut).

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treating shoots with IBA (Minerva and Kumar, 2013), as well as studies on factors affecting rooting such as genotype - culture medium and culture system (Cardoso and Teixeira da Silva, 2013). Some studies have worked to overcome the yellowing or curling of the leaf and vitrification in gerbera plantlets, such as with the addition of $AgNO_3$ or activated charcoal to the culture medium. Moreover, *in vitro* rooting has been found to cause the development of thin and poor roots, leading to reduced acclimatization and subsequent growth in the greenhouse (Winarto and Yufdy, 2017).

In recent years, the interactions between nano-materials and plants have attracted the attention of many scientists around the world, and studies on the effects of metal nanoparticles on plants have been carried out (Singh et al., 2021; Fiol et al., 2021). The researchers showed both positive and negative effects of nanomaterials on plant growth and development, depending on the composition, structure, nanoparticle concentration, and plant species (Kim et al., 2017). In micropropagation, a number of studies have investigated the positive effects of metal nanoparticles on plants cultured *in vitro* with regard to germination (Rai-Kalal and Jajoo, 2021; Khalaki et al., 2021), growth (Zhou et al., 2021; Azmat et al., 2022), and secondary compounds (Rivero-Montejo et al., 2021; Goga et al., 2021), etc. To date, there is a very large bibliography on the effects of using nanoparticles on the quality of plant material (Jakubowska and Ruzik, 2021; Kulus and Tymoszuk, 2021), but studies of their effects with regard to causing abnormal features on *in vitro* plantlets during micropropagation remain limited.

Silver nanoparticles (AgNPs) are used as disinfectants for *in vitro* media (Tung et al., 2021a), explant surface sterilization of seaweed (Mo et al., 2020), chrysanthemums and strawberries (Tung et al., 2021a, 2021b), or added to *in vitro* media to increase plantlet growth in *Anthurium* (Tung et al., 2021c) and strawberries (Tung et al., 2021b), as well as being used to reduce microbial contamination in the medium of microponic systems (Tung et al. 2018). In addition, cobalt nanoparticles (CoNPs) have been shown to reduce yellowing of the leaf and defoliation in roses (Ngan et al., 2020a). Moreover, the effects of metal nanoparticles supplemented or replaced with some metal salts in the culture media have been shown to prevent some abnormal phenomena, and improved *in vitro* rooting and acclimatization (Tung et al., 2021b; Cuong et al., 2021; Khai et al., 2022). They also changed ethylene production and enzyme activity, and improved shoot and plantlet quality in the culture system (Ngan et al., 2020b; Tung et al., 2021b).

Therefore, this study investigated the effects of AgNPs and CoNPs on the growth, development, and improvement of the shoot and plantlet quality of gerbera. Through this study, we evaluated the effects of using CoNPs instead of $CoCl_2.6H_2O$ or supplementing them with AgNPs on culture media in the shoot multiplication and *in vitro* rooting stages. Moreover, the abnormal phenomenon (vitrification, yellowing of the leaf, browning of the explant), ethylene accumulation, hydrolytic and oxidative enzyme activities were also investigated.

## 2. Materials and methods

### 2.1. Plant material

The plant material used in this study was 4-week-old shoots of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar that were subcultured several times on MS medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs) at the Tay Nguyen Institute for Scientific Research (VAST, Dalat, Vietnam).

### 2.2. Media culture and nanoparticle solution

MS medium was supplemented with 1.0 mg $L^{-1}$ BA, 0.1 mg $L^{-1}$ IBA, 30 g $L^{-1}$ sucrose and 8 g $L^{-1}$ agar (Viet Xo Vegetable and Fruit JSC., Haiphong, Vietnam) for the shoot multiplication - MSS medium (Bhatia et al., 2012); while MS medium was supplemented with 1.0 mg $L^{-1}$ IBA, 30 g $L^{-1}$ sucrose and 8 g $L^{-1}$ agar for the *in vitro* rooting - MSR medium (Bhatia et al., 2012) as the control media.

The AgNPs and CoNPs solutions used in the experiments were provided by the Institute of Environmental Technology (VAST, Hanoi, Vietnam). AgNPs are smaller than 20 nm in size with a concentration of 1000 mg $L^{-1}$ established by Chau et al. (2008), and CoNPs range in size from 20 to 60 nm at a concentration of 1000 mg $L^{-1}$, as established by Ngo et al. (2014).

AgNPs were added to MSS and MSR media at different concentrations; meanwhile, 0.025 mg $L^{-1}$ $CoCl_2.6H_2O$ was removed from the MSS and MSR media and CoNPs added with different concentrations (0.0155; 0.031; 0.0465; 0.062; 0.124 mg $L^{-1}$ - CoNPs content corresponding to 0, ¼, ½, ¾, 1, and 2 times the molar concentration of cobalt (Co) in MS medium). Forty mL media (pH = 5.8) were poured in a 250 mL culture bottle before autoclaving at 121°C and 15 psi for 30 min.

### 2.3. Culture condition

*In vitro*: The explants were grown in a culture room with a temperature of about 25 ± 2°C, relative humidity of 55 - 60% (outside humidity of the culture vessel), placed under a fluorescent light with a photoperiod of 12 h/day and light intensity of 45 $\mu mol.m^{-2}.s^{-1}$.

*Ex vitro*: Plantlets were grown in a greenhouse at the Tay Nguyen Institute for Scientific Research (Dalat, Lamdong, Vietnam) with a daytime temperature of 25 ± 2°C at daytime and night temperature of 15 ± 2°C, average humidity of about 75 - 80% with natural light, and 40% shading with a black net (Hoa Phat Dat Co., Ho Chi Minh, Vietnam), while the soil pH was 6.5.

### 2.4. AgNPs or CoNPs in shoot multiplication stage

The shoots (1 cm in height) cultured on MSS medium were supplemented with AgNPs (1, 2, 3, 5, 7 mg $L^{-1}$) or CoNPs (0; 0.0155; 0.031; 0.0465; 0.062; 0.124 mg $L^{-1}$). The control experiment was shoots cultured on MSS medium without AgNPs or CoNPs. For collecting data, the number of shoots (total number of shoots, shoots more than 2 cm in height), leaf size [leaf length and width (cm)], SPAD value (obtained using a Konica Minolta Chlorophyll Meter Spad-502 Plus SPAD 502, Japan), fresh and dry weight (mg), dry mass ratio and abnormal phenomena (vitrification, leaf yellowing) were recorded after 4 weeks of culture.

### 2.5. AgNPs or CoNPs in rooting stage

The shoots (2.5 cm in height) cultured on MSR medium were supplemented with AgNPs (1, 2, 3, 5, 7, 9 mg $L^{-1}$) or CoNPs (0; 0.0155; 0.031; 0.0465; 0.062; 0.124 mg $L^{-1}$). The control experiment was shoots cultured on MSR medium without AgNPs or CoNPs. For collecting data, the plantlet height (cm), number of roots per plantlet, root length (cm), number of leaves per plantlet, leaf size [leaf length and width (cm)], SPAD value, fresh and dry weight (mg) and abnormal phenomena (vitrification, yellowing of the leaf, and browning of the explant) were recorded after 4 weeks of culture.

### 2.6. Acclimatization and subsequent growth of plantlets in the greenhouse

Ninety plantlets from each treatment (derived from AgNPs or CoNPs-plantlets and control-plantlets) were collected, carefully washed and planted in foam blisters with soil substrate containing Canadian Sphagnum Peat Moss, Bark, Vermiculite, Dolomite Lime, Long-Lasting Wetting Agent, RESiLIENCE (Metro- Mix 350, Marysville, Ohio, USA), and placed in *ex vitro* condition. After 2 weeks, the plantlets were transplanted into plastic pots (10 cm in diameter and 20 cm in height) with small holes at the bottom. With regard to the data on plantlet growth

and development in the greenhouse after 2 weeks, survival rate (%), plantlet height (cm), number of roots per plant, root length (cm), number of leaves, leaf size [leaf length and width (cm)], fresh and dry weight of plantlets (mg), and SPAD value were recorded. Moreover, the remaining plants (45 plants) were examined after 12 weeks with regard to plantlet height (cm), number of leaves, plantlet fresh and dry weight (mg), flower bud formation and flowering (weeks), flower diameter (cm) and peduncle (cm).

### 2.7. Ethylene in the culture bottle

Ethylene (1 mL) from a 250 mL culture bottle culture capped with nylon film and covered with parafilm was collected directly with a syringe and manually injected into the chromatograph with a leak-free syringe. The ethylene gas was then quantified by gas chromatography. A stainless steel column (3 m × 1.5 mm) filled with Porapack R (adsorbent) with a particle size of 80–100 Mesh was used. The detection temperatures of the column, injector, and ionizing flame were 60, 90, and 90 °C, respectively. Electrometer sensitivity 1 × 10-12 Am/V. Nitrogen gas ($N_2$) was used as the carrier gas (55 $cm^3$/min) (Cristescu et al., 2012).

### 2.8. Antioxidant enzyme activity in plantlets

The fresh explants (300 mg) were ground with liquid nitrogen and the powder homogenized by ultrasonic scattering in 2 mL of 0.1 M phosphate buffer (pH = 7.4) containing 0.1 mM ethylene diamine tetra acetic acid. Explants were centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was then collected and stored under cold conditions to determine the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX).

SOD activity was analyzed according to the method of Marklund and Marklund (1974). The SOD in the explant catalysed the decomposition of peroxide (-O-O-), preventing self-oxidation of pyrogallol. The inhibition rate reflects the activity of the SOD in the explant. The SOD activity was calculated based on the inhibition rate of self-oxidation of 0.25 to 4 mM pyrogallol solution in 2 mM HCl. The self-oxidation rate of pyrogallol was calculated by the formula: $dA/dt = (ODt\text{-}ODt_0)/t$ min (*where, ODt: OD value at t = 5 min; $ODt_0$: OD value at t = 0 min*). Percent inhibition (%) = (dA/dt blank - dA/dt explant) / (dA/dt blank × 100). Enzyme unit (U) = (% inhibition/50) × dilution ratio. One unit of enzyme activity (U/g) was the 50% inhibition of pyrogallol self-oxidation, as determined by the change in absorbance at 320 nm.

CAT activity in the sample was determined by reacting the explant with 100 µL of 65 mM $H_2O_2$ for 2 min, the remaining $H_2O_2$ after the reaction was combined with 100 µL of ammonium molybdate ($NH_4Mo_7O_{24}$) to form a complex yellow sediment that absorbs at 405 nm. One unit of catalase activity (U/g) is equivalent to 1 µmol of $H_2O_2$ hydrolyzed in 1 min according to the method of Goth et al. (1991).

APX activity was determined according to the method of Nakano and Asada (1981). The APX enzyme in the explant oxidizes and reduces the maximum absorption of ascorbate at 290 nm. The APX activity unit (U) was calculated by recording the maximum absorbance of ascorbate (290 nm) in the presence of 0.5 mM $H_2O_2$ for 3 min. The amount of oxidized ascorbate was calculated directly with an absorption coefficient of 2.8 mM/cm.

### 2.9. Hydrolytic enzyme activity in plantlets

This method is based on the hydrolysis of carboxymethyl cellulose substrate by cellulase enzyme at pH = 5 and 40°C. The amount of reducing sugar produced gives a color reaction with the DNS reagent. The color produced after the reaction was determined by the colorimetric method on a spectrophotometer operated at 540 nm. One unit of cellulase enzyme activity was calculated as the number of mg of glucose produced by 1 mL (or 1 g of the preparation) at 40°C for 15 min (Zhang et al. 2009). Spectral density at 540 nm based on the glucose standard curve was used to calculate the concentration of reducing sugar produced: H (UI $mL^{-1}$) = CVL / 180.vt (where: C: concentration of glucose produced (µg $mL^{-1}$), V: total reaction volume (mL), L: dilution, v: volume of enzyme preparation added to the reaction (mL), t: incubation time).

The enzyme reacts with pectin substrate, the product formed is D-galacturonic acid with color reaction with DNS reagent, with the optical density measured at 575 nm. One unit of pectinase activity is the number of µmol of D-galacturonic acid produced by 1 mL of enzyme solution at 37°C for 60 min (Vatanparast et al., 2014). The concentration of reducing sugars produced by the formula: H (UI/mL) = CVL / vt (where: C: concentration of D-galacturonic acid (µmol $mL^{-1}$), L: dilution, V: total reaction volume reaction (mL), v: volume of enzyme added to the reaction (mL), t: incubation time).

### 2.10. In vitro root anatomical morphology

Root morphology in the *in vitro* rooting experiment was dissected, stained, and observed under a light microscope (Peterson et al., 2008). The roots were cut transversely using a razor blade into slices about 1–2 µm in size and bleached with 10% Javel water for 15 min, followed by washing with distilled water 3 times, then it was rinsed with 45% acetic acid for 10 min and washed with distilled water another 3 times. The explants were stained with carmine (Sigma-Aldrich, USA) for 3 min and washed with distilled water 3 times. Finally, the samples were observed and photographed under at optical microscope (Keynce Corporation, Japan) with a × 10 objective lens.

### 2.11. Data analysis

Each treatment was repeated 3 times with 30 culture bottles/treatment (nursery experiment with 90 plantlets/treatment). All data after collection corresponding to each indicator were processed using Microsoft Excel 2016 and SPSS 20.0 software according to LSD and Duncan's test with $p < 0.05$ (Duncan, 1955).

## 3. Results

### 3.1. Observation of abnormal phenomena in shoot multiplication and in vitro rooting stages

During the shoot multiplication and *in vitro* rooting stages, abnormal phenomena such as vitrification (Fig. 1A), yellowing of the leaf (Fig. 1B), and browning of the explant (Fig. 1C) were observed after 4 weeks of culture. The results showed that the vitrification (23.33% in the shoot multiplication stage and 27.67% in the *in vitro* rooting stage) and yellowing of the leaf (23.33% in the shoot multiplication stage and 20.00% in the *in vitro* rooting stage) were more common than browning of the explant during both shoot multiplication and *in vitro* rooting stages (Figs. 1 and 2).

### 3.2. AgNPs and CoNPs enhanced shoot multiplication and reduced abnormal phenomena

The results showed that the MSS medium supplemented with AgNPs or CoNPs affected the shoot multiplication and reduced vitrification and yellowing of leaf after 4 weeks of culture (Tables 1 and 2, Fig. 2A). The highest number of shoots obtained from shoots cultured on MSS medium supplemented with 1–2 mg $L^{-1}$ AgNPs was higher than that seen with other concentrations of AgNPs and the control experiment (without AgNPs). However, the addition of 2 mg $L^{-1}$ AgNPs to the MSS medium caused increases in the number of shoots more than 2 cm in height (11.67 shoots), shoot height (4.3 cm), leaf width (0.97 cm),


**Fig. 1.** Abnormal phenomena of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar in shoot multiplication and *in vitro* rooting stages. *Bars: 1 cm* **A:** Vitrification **B:** Yellowing of leaf **C:** Browning of explant.

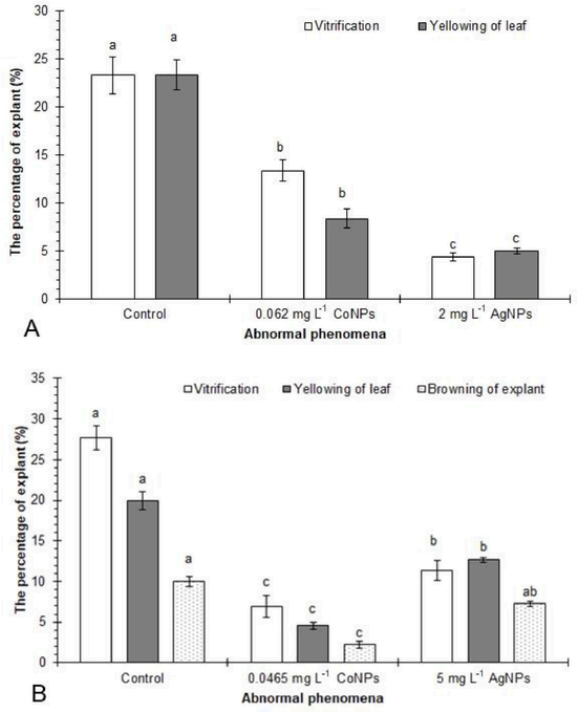


**Fig. 2.** Reducing abnormal phenomena of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar shoots (A) and plantlets (B) on medium containing AgNPs or CoNPs after 4 weeks of culture.

shoot cluster fresh weight (1256.67 mg), shoot cluster dry weight (148.70 mg) and dry mass ratio (11.83%) compared to the results found for shoots cultured on MSS medium supplemented with 1 mg $L^{-1}$ AgNPs (Table 1). Meanwhile, shoots cultured on MSS medium with CoNPs recorded a higher number of shoots larger than 2 cm, with 6.67 shoots, while the shoot growth characteristics (shoot height, leaf size, SPAD) were lower than those found with 2 mg $L^{-1}$ AgNPs (Tables 1 and 2). The dry mass ratio of shoots cultured on MSS medium with CoNPs or supplemented with 2 mg $L^{-1}$ AgNPs was higher than that of the control (Tables 1 and 2).

### *3.3. AgNPs and CoNPs enhanced in vitro rooting, reduced abnormal phenomena and changed ethylene gas content and enzyme activity*

*In vitro* rooting of gerbera shoots reached 100% in all treatments supplemented with AgNPs or CoNPs and control (without AgNPs and CoNPs) after 1 week of culture. However, the *in vitro* rooting (5.33 days) of shoots cultured on MSR medium supplemented with 5 mg $L^{-1}$ AgNPs or 0.0465 mg $L^{-1}$ CoNPs occurred 2 days earlier than for those on the control medium (7.33 days). A recent study by Tung et al. (2021b) on strawberry plants also showed that AgNPs were able to induce *in vitro* rooting about 3 days earlier in comparison with the control (without AgNPs). In addition, 5 mg $L^{-1}$ AgNPs or 0.0465 mg $L^{-1}$ CoNPs effectively reduced vitrification, yellowing of the leaf and browning of explant as compared with the control (without AgNPs or CoNPs), in which 0465 mg $L^{-1}$ CoNPs effectively reduced vitrification, yellowing of the leaf and browning of explant by 5 times, and 5 mg $L^{-1}$ AgNPs reduced these by 2 times compared to the control (Fig. 2B).

In this study, AgNPs and CoNPs at optimal concentrations not only increased the *in vitro* rooting efficiency and antioxidant activity (SOD, CAT and APX), but also reduced abnormal phenomena, ethylene gas accumulation and hydrolytic enzyme activity (cellulase and pectinase) after 4 weeks of culture (Tables 3, 4 and 5, Fig. 3A-B). However, at the *in vitro* rooting stage the opposite results were obtained compared to the shoot multiplication stage, in which the *in vitro* rooting efficiency of 0.0465 mg $L^{-1}$ CoNPs treatment was higher than that with 5 mg $L^{-1}$ AgNPs. The plantlets grown in the 0.0465 mg $L^{-1}$ CoNPs treatment saw improvements with regard to plantlet height, number of roots per plantlet, number of leaves per plantlet, plantlet fresh and dry weight, along with reductions in abnormal phenomena (vitrification, yellowing of the leaf and browning of the explant), ethylene gas accumulation and oxidative activity (SOD, CAT and APX) were reduced (Tables 4 and 5, Figs. 3B and 4A–E) .

The results also showed that MSR medium with the addition of 0.0465 mg $L^{-1}$ CoNPs was optimal for overcoming vitrification, yellowing of the leaf, browning of the explant of gerbera plantlets after 4 weeks of culture. In addition, the photosynthetic efficiency was significantly improved, as reflected in the higher SPAD value (55.77) compared with the other treatments (Table 4). The plantlet had dark-green leaves and reduced yellowing of the leaf and browning of the explant. In addition, the activities of hydrolytic enzymes (pectinase and cellu-

**Table 1**
Shoot multiplication of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar on medium containing AgNPs after 4 weeks of culture.

| AgNPs (mg $L^{-1}$) | No. of shoots | | Shoot height (cm) | Leaf size (cm) | | SPAD value | Shoot cluster | | Dry mass ratio (%) |
|---|---|---|---|---|---|---|---|---|---|
| | Total | > 2 cm in height | | Length | Width | | Fresh weight (mg) | Dry weight (mg) | |
| 0 | 5.67 ± 0.33d* | 4.00 ± 0.00d | 2.50 ± 0.57c | 0.73 ± 0.07d | 0.50 ± 0.06b | 23.89 ± 0.80c | 453.33 ± 14.53e | 42.80 ± 1.53e | 9.44 ± 0.33c |
| 1.0 | 13.33 ± 0.33a | 9.82 ± 0.33b | 3.17 ± 0.33b | 1.33 ± 0.06a | 0.67 ± 0.03b | 38.13 ± 1.10a | 1153.33 ± 21.77c | 118.67 ± 4.27c | 10.29 ± 0.41b |
| 2.0 | 13.67 ± 0.33a | 11.67 ± 0.33a | 4.30 ± 0.33a | 1.10 ± 0.06bc | 0.53 ± 0.03b | 38.43 ± 0.53a | 1256.67 ± 13.33ab | 148.70 ± 2.64a | 11.83 ± 0.24a |
| 3.0 | 11.33 ± 0.33b | 10.00 ± 0.58b | 4.40 ± 0.67a | 1.23 ± 0.07ab | 0.97 ± 0.09a | 34.97 ± 0.52ab | 1416.67 ± 20.28a | 157.87 ± 1.82a | 11.14 ± 0.38ab |
| 5.0 | 11.33 ± 0.58b | 9.67 ± 0.67b | 4.27 ± 0.33a | 1.07 ± 0.07bc | 0.57 ± 0.07b | 32.67 ± 0.29b | 1243.33 ± 37.12abc | 134.03 ± 2.49b | 10.78 ± 0.46ab |
| 7.0 | 8.33 ± 0.33c | 6.33 ± 0.33c | 3.03 ± 0.18c | 1.00 ± 0.03c | 0.60 ± 0.03b | 30.27 ± 0.73b | 946.67 ± 27.56d | 106.20 ± 3.23d | 11.22 ± 0.39ab |

* Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).

**Table 2**
Shoot multiplication of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar on medium containing CoNPs after 4 weeks of culture.

| CoNPs (mg $L^{-1}$) | No. of shoots | | Shoot height (cm) | Leaf size (cm) | | SPAD value | Shoot cluster | | Dry mass ratio (%) |
|---|---|---|---|---|---|---|---|---|---|
| | Total | > 2 cm in height | | Length | Width | | Fresh weight (mg) | Dry weight (mg) | |
| Control ** | 5.67 ± 0.33d* | 4.00 ± 0.00ab | 2.50 ± 0.06b | 0.73 ± 0.07ab | 0.50 ± 0.03ab | 22.57 ± 0.80c | 453.33 ± 14.53d | 42.80 ± 1.53d | 9.44 ± 0.25bc |
| 0 | 6.00 ± 0.58cd | 0 ± 0.00e | 1.80 ± 0.06d | 0.60 ± 0.06bc | 0.43 ± 0.03bc | 25.37 ± 1.62b | 303.33 ± 17.64e | 24.63 ± 1.50e | 8.12 ± 0.34cd |
| 0.0155 | 11.00 ± 0.58a | 1.67 ± 0.33d | 2.17 ± 0.09c | 0.53 ± 0.03c | 0.33 ± 0.00c | 26.29 ± 0.46b | 693.33 ± 8.82c | 60.80 ± 0.74c | 8.77 ± 0.21bc |
| 0.031 | 7.33 ± 0.33bc | 2.67 ± 0.33c | 2.67 ± 0.09b | 0.73 ± 0.03ab | 0.60 ± 0.06a | 25.20 ± 0.17b | 853.33 ± 33.83a | 87.13 ± 4.51a | 10.21 ± 0.47ab |
| 0.0465 | 8.00 ± 0.00b | 3.33 ± 0.33bc | 3.1 ± 0.06a | 0.80 ± 0.03a | 0.60 ± 0.03a | 29.70 ± 0.32a | 886.67 ± 8.82b | 91.67 ± 6.24b | 11.34 ± 0.42a |
| 0.062 | 8.67 ± 0.58b | 6.67 ± 0.33a | 3.30 ± 0.06a | 0.77 ± 0.063a | 0.47 ± 0.03b | 29.33 ± 0.52a | 863.33 ± 33.83c | 88.07 ± 4.01c | 10.20 ± 0.34ab |
| 0.124 | 8.67 ± 0.41b | 2.67 ± 0.33c | 2.53 ± 0.09b | 0.57 ± 0.03c | 0.33 ± 0.02c | 23.33 ± 0.61bc | 690.00 ± 39.71c | 54.00 ± 3.36c | 9.28 ± 0.41bcd |

* Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).
** Shoot cultured on MSS medium without CoNPs

**Table 3**
*In vitro* rooting of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar shoots on medium containing AgNPs after 4 weeks of culture.

| AgNPs (mg $L^{-1}$) | Plantlet height (cm) | No. of roots/ plantlet | Root length (cm) | No. of leaves/ plantlet | Leaf size (cm) | | SPAD value | Plantlet weight (mg) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Length | Width | | Fresh | Dry |
| 0 | 5.83 ± 0.03a* | 7.33 ± 0.33a | 4.33 ± 0.09b | 6.33 ± 0.33a | 1.20 ± 0.06de | 0.90 ± 0.06d | 43.27 ± 0.50bc | 396.67 ± 29.63cd | 32.93 ± 2.93cd |
| 1.0 | 5.63 ± 0.17ab | 6.33 ± 0.33a | 3.50 ± 0.06d | 5.67 ± 0.33a | 2.47 ± 0.03a | 1.73 ± 0.03a | 40.70 ± 0.57c | 466.67 ± 27.28bc | 36.93 ± 1.25bc |
| 3.0 | 5.63 ± 0.09ab | 6.67 ± 0.33a | 3.70 ± 0.06c | 6.33 ± 0.33a | 1.87 ± 0.09b | 1.50 ± 0.06b | 47.73 ± 0.49ab | 536.67 ± 24.04ab | 38.47 ± 0.73b |
| 5.0 | 5.23 ± 0.17b | 6.67 ± 0.33a | 4.77 ± 0.03a | 5.67 ± 0.33a | 1.50 ± 0.06c | 1.17c ± 0.03 | 50.47 ± 3.53a | 626.67 ± 29.06a | 46.50 ± 2.00a |
| 7.0 | 4.33 ± 0.09c | 5.67 ± 0.33b | 4.23 ± 0.03b | 6.33 ± 0.33a | 1.33 ± 0.03cd | 1.07 ± 0.03c | 34.70 ± 0.50d | 456.67 ± 31.80bc | 39.47 ± 0.86b |
| 9.0 | 3.36 ± 0.15d | 4.33 ± 0.33b | 2.80 ± 0.05e | 5.33 ± 0.33a | 1.13 ± 0.03e | 0.870.003d | 29.10 ± 0.25e | 316.67 ± 39.30d | 27.57 ± 1.53d |

* Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)

**Table 4**
*In vitro* rooting of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar shoots on medium containing CoNPs after 4 weeks of culture.

| CoNPs (mg $L^{-1}$) | Plantlet height (cm) | No. of roots/ plantlet | Root length (cm) | No. of leaves/ plantlet | Leaf length (cm) | Leaf width (cm) | SPAD value | Plantlet weight (mg) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Fresh | Dry |
| Control ** | 5.83d ± 0.03* | 7.33 ± 0.33e | 4.33 ± 0.09a | 6.33 ± 0.33b | 1.20 ± 0.06e | 0.90 ± 0.03d | 43.27 ± 0.50c | 396.67 ± 29.63f | 32.67 ± 2.94e |
| 0 | 6.03 ± 0.12d | 13.33 ± 0.33c | 2.37 ± 0.07e | 6.67 ± 0.33ab | 1.50 ± 0.063d | 0.87 ± 0.06d | 41.40 ± 0.47cd | 596.67 ± 29.06e | 77.67 ± 0.29d |
| 0.0155 | 6.37 ± 0.09c | 20.33 ± 0.33b | 2.60 ± 0.06d | 6.67 ± 0.88ab | 1.47 ± 0.07d | 1.00 ± 0.07cd | 46.77 ± 0.54b | 1123.33 ± 20.28d | 131.13 ± 2.87c |
| 0.031 | 7.23 ± 0.03a | 20.33 ± 67b | 3.13 ± 0.03c | 6.33 ± 0.33ab | 1.87 ± 0.03c | 1.13 ± 0.09bc | 48.33 ± 0.58b | 1630.00 ± 32.15b | 172.73 ± 3.33a |
| 0.0465 | 7.40 ± 0.06a | 22.33 ± 0.67a | 3.83 ± 0.07b | 6.33 ± 0.33ab | 2.97 ± 0.09a | 1.37 ± 0.06a | 55.77 ± 0.23a | 1743.33 ± 17.64a | 180.77 ± 0.87a |
| 0.062 | 7.37 ± 0.07a | 14.33 ± 0.88c | 3.70 ± 0.06b | 6.00 ± 0.58ab | 2.37 ± 0.12b | 1.20 ± 0.09abc | 47.80 ± 0.21b | 1230.00 ± 26.46c | 150.00 ± 3.16b |
| 0.124 | 6.97 ± 0.09b | 11.67 ± 0.33d | 2.73 ± 0.07d | 5.00 ± 1.15c | 1.67 ± 0.09cd | 1.27 ± 0.04ab | 41.90 ± 0.78cd | 1130.00 ± 37.46d | 138.40 ± 4.74c |

* Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).
** Shoot cultured on MSS medium without CoNPs

**Table 5**
Antioxidant and hydrolytic enzyme activity of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar derived plantlets after 4 weeks of culture.

| Treatment | | Antioxidant enzyme activity (U $g^{-1}$) | | | Hydrolytic enzyme activity (UI $mL^{-1}$) | |
|---|---|---|---|---|---|---|
| AgNPs (mg $L^{-1}$) | CoNPs (mg $L^{-1}$) | SOD | CAT | APX | Cellulase | Pectinase |
| 0 | 0 | 21.24 ± 0.25d* | 206.77 ± 5.31c | 0.26 ± 0.02c | 1.40 ± 0.05a | 0.55b ± 0.07 |
| 5 | - | 40.47 ± 0.61b | 306.35 ± 4.95b | 0.52 ± 0.04b | 0.24 ± 0.02d | 0.21 ± 0.03c |
| 9 | - | 32.27 ± 0.74c | 216.91 ± 3.17c | 0.18 ± 0.02d | 0.95 ± 0.05bc | 0.84 ± 0.06a |
| - | 0.0465 | 50.36 ± 1.04a | 348.92 ± 8.10a | 0.72 ± 0.05a | 0.15 ± 0.02e | 0.10 ± 0.01d |
| - | 0.124 | 42.69 ± 0.53b | 293.50 ± 7.24b | 0.52 ± 0.06b | 1.13 ± 0.03b | 0.67 ± 0.05b |

* *Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).*

lase) of the plantlets grown with the AgNPs and CoNPs treatments were also significantly different after 4 weeks of culture (Table 5). In the MSR medium with 0.0465 mg $L^{-1}$ CoNPs, cellulase enzyme activity decreased 9 times and pectinase enzyme activity decreased 5 times; meanwhile, antioxidant enzyme activity (APX, CAT and SOD) increased 1.5 to 3 times as compared to that seen in the control. In addition, shoots cultured on MSR medium supplemented with 5 mg $L^{-1}$ AgNPs and 0.0465 mg $L^{-1}$ CoNPs recorded a significantly reduced ethylene content (1.5-2 times) compared with the treatment without AgNPs and CoNPs.

### *3.4. Acclimatization, subsequent growth and flowering of plantlets in the greenhouse*

After 2 weeks of planting in the nursery, the results showed that the survival rate of plantlets (derived from 0.0465 mg $L^{-1}$ CoNPs) was higher than seen with 5 mg $L^{-1}$ AgNPs and the control (Table 6 and Fig. 5). Moreover, plantlet height, number of roots per plantlet, root length, number of leaves, leaf size, SPAD, plantlet fresh and dry weight on 5 mg $L^{-1}$ AgNPs treatment did not differ compared with the 0.0465 mg $L^{-1}$ CoNPs treatment, and were all better than with the control. In addition,

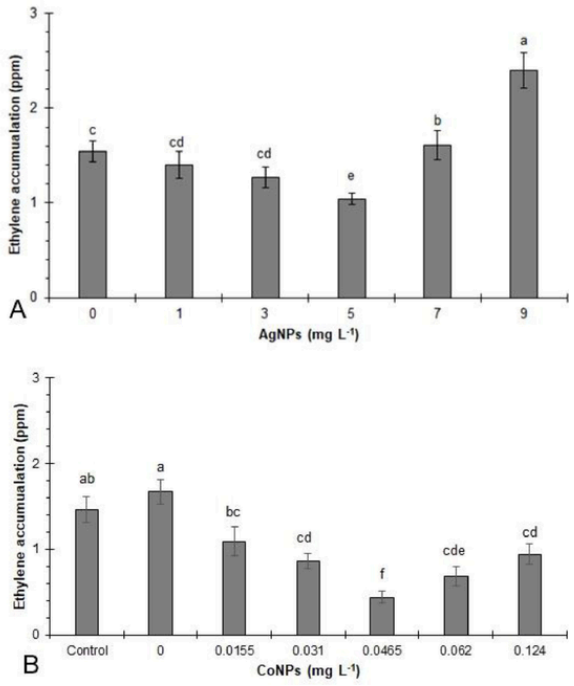


**Fig. 3.** Ethylene accumulation in culture bottle of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar plantlets on medium containing AgNPs (A) or CoNPs (B) after 4 weeks of culture.

the flower bud formation and flowering of 5 mg $L^{-1}$ AgNPs or 0.0465 mg $L^{-1}$ CoNPs-derived plantlets occurred about 1 week earlier than those of the control (Table 7). The growth of plantlets derived from 0.0465 mg $L^{-1}$ CoNPs at the flowering stage showed plantlet height (20.07 cm), fresh weight (25.24 g), dry weight (2.94 g), flower diameter (7.57 cm), and peduncle length (21.27 cm) were highest (Table 7 and Fig. 6).

## 4. Discussion

### *4.1. Abnormal phenomena in shoot multiplication and in vitro rooting stages*

Vitrification, a physiological and morphological disorder in plants cultured *in vitro* that causes the most serious problems in micropropagation, has been reported in a number of crops such as sunflowers (Mayor et al., 2003), *Scrophularia yoshimurae* (Lai et al., 2005), carnations (Kharrazi et al., 2011), *Turbinicarpus valdezianus* (García-Osuna et al., 2011), and roses (Ngan et al., 2020a). The cause of vitrification is a combination of many factors such as culture medium, type and concentration of PGRs in micropropagation (Kevers et al., 2004), relative humidity and ethylene gas accumulation (Lai et al., 2005), culture systems and gelling agents (Winarto et al., 2016).

*In vitro* explants are frequently affected by biotic and abiotic stresses that adversely affect growth, development, reproduction and survival rate when transferred to the nursery, thereby reducing crop yield. Besides the vitrification phenomenon, micropropagation of some plant species also suffers from yellowing of the leaf and defoliation (Gonzalez-Carranza et al., 2012). Micropropagation with culture conditions in closed vessels, high humidity and the heterotrophic vegetative form of cultured plants leads to the accumulation of ethylene gas,

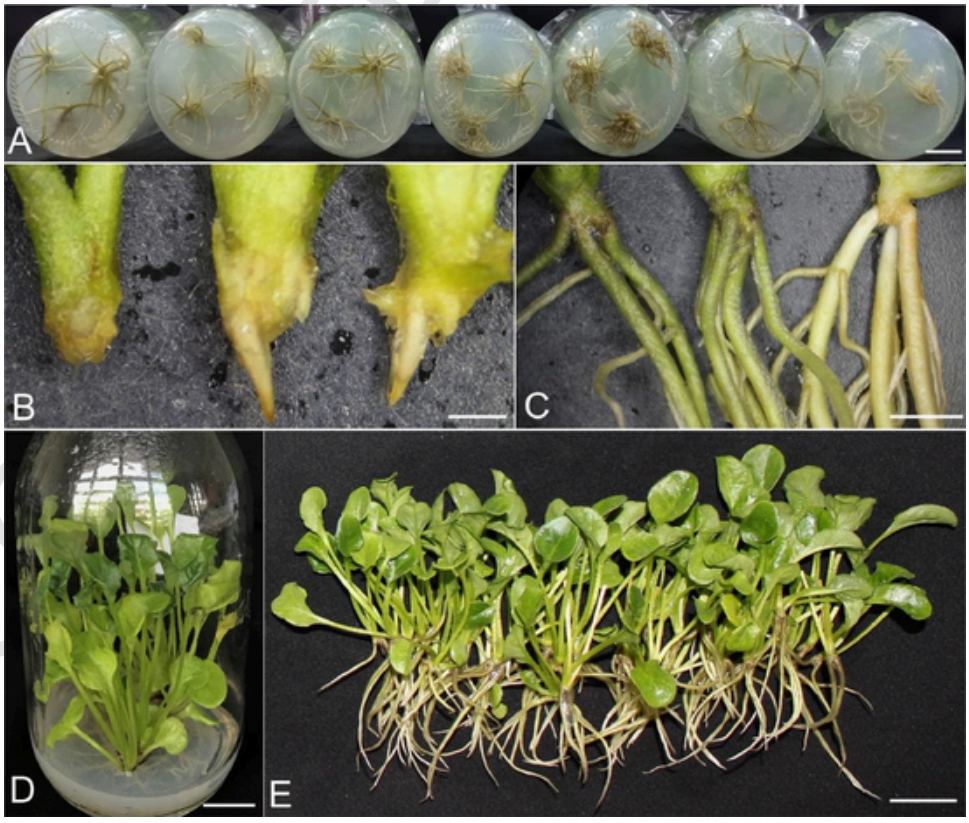


**Fig. 4.** *In vitro* rooting of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar shoots on medium containing CoNPs. **A:** Root of plantlets on rooting medium containing different concentrations of CoNPs ($CoCl_2.6H_2O$; 0; 0.155; 0.031; 0.0465; 0.062; 0.124 mg $L^{-1}$; left to right) after 4 weeks of culture. *Bars: 2 cm* **B, C:** *In vitro* rooting of plantlets (control; 5 mg $L^{-1}$ AgNPs; 0.465 mg $L^{-1}$ CoNPs; left to right) after 1 week and 4 weeks of culture. *Bars: 1 cm* **D, E:** Plantlets on medium containing 0.0465 mg $L^{-1}$ CoNPs after 4 weeks of culture. *Bars: 2 cm.*


**Fig. 5.** Acclimatization of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar plantlets after 2 weeks in the greenhouse. **A:** Plantlets planted in foam blisters with mix substrate (Control, 5 mg $L^{-1}$ AgNPs and 0.0465 mg $L^{-1}$ CoNPs, left to right). *Bars: 2 cm* **B:** Plantlets removed from the mix substrate (Control, 5 mg $L^{-1}$ AgNPs and 0.0465 mg $L^{-1}$ CoNPs, left to right). *Bars: 5 cm.*

which is one of the main factors that causes yellowing of the leaf (Agustí et al., 2009; Merelo et al., 2017; Ngan et al., 2020a). This seriously affects the growth, development and plantlet quality, reducing their acclimatization and survival rate at the nursery stage (Ferrante and Francini, 2006; Xu and Zhang, 2015).

Moreover, browning or necrosis of explants were also a problem that seriously affects micropropagated efficiency. In this study, browning becomes so severe that the explant turns brown/black, becomes necrotic, and eventually dies (Fig. 1C). This phenomenon has been observed in bananas due to the phenols formed from wound of explants depending on species, age of tissue or organ, nutrient medium and several other factors (Babaei et al., 2013; Jones and Saxena, 2013). The browning of explants depends on the cultured plant species, genotype, age of explants (older tissues show a higher chance of browning), season (more in winter and autumn), and medium (Bhojwani and Dantu, 2013).

Gerbera micropropagation is an effective method to replace traditional propagation methods in order to produce a large number of plantlets that are disease-free, homogenous, and seasonally independent (Cardoso and Teixeira da Silva, 2013). However, the culture conditions in the closed vessel, the high humidity in the culture vessel (> 95%), the composition of the culture media and the accumulation of gases (especially ethylene) have resulted in some undesirable phenomena when cultured *in vitro*. This seriously affects the growth, development and quality of plantlets, reducing their acclimatization at the nursery stage (Ferrante and Francini, 2006; Xu and Zhang, 2015). Therefore, it is necessary to carry out further research to optimize the culture process and find new sources of materials to improve the quality of plantlets.

### *4.2. AgNPs and CoNPs enhanced shoot multiplication and reduced abnormal phenomena*

AgNPs have been shown to be effective for shoot multiplication in several plant species, such as *Araucaria excelsa* (Sarmast et al., 2011), rose (Ngan et al., 2020a), strawberry (Tung et al., 2021b), and Vietnamese ginseng (Cuong et al., 2021). In addition, Bernard et al. (2015) have shown that AgNPs effectively form shoots with stem elongation and normal leaf development, while the shoots in the treatment without AgNPs were limited in stem length and showed leaf abnormalities such as leaf curl and deformity. Furthermore, AgNPs were found to be highly effective in inhibiting ethylene activity, enhancing growth and prolonging subculture, thereby reducing leaf defoliation and improving micropropagation efficiency (Aghdaei et al., 2012; Rezvani et al., 2012). The role of AgNPs on chlorophyll biosynthesis was also noted in *Vigna radiata* and *Vanilla planifolia*, which significantly increased leaf chlorophyll content and plant fresh weight (Saeideh and Rashid, 2014; Spinoso-Castillo et al., 2017) similar to AgNPs in this study.

For CoNPs, shoot multiplication efficiency has also been demonstrated in mint (Talankova-Sereda et al., 2016) and roses (Ngan et al., 2020a). The results of this study also showed that 2 mg $L^{-1}$ AgNPs or 0.062 mg $L^{-1}$ CoNPs effectively reduced vitrification and yellowing of the leaf as compared with the control (without AgNPs or CoNPs), in which 2 mg $L^{-1}$ AgNPs effectively reduced vitrification and yellowing of the leaf by 5 times, and 0.062 mg $L^{-1}$ CoNPs reduced these by 2 to 3 times compared to the control (Fig. 2A). Several studies have shown the role of CoNPs in promoting plantlet growth. The growth and yield indicators such as chlorophyll content, sugar, starch, amino acids, protein content, macro and micro minerals were increased as compared to those in the control (Jaleel et al., 2009). In this study, the leaves (without CoNPs treatment) became yellow, while the leaves remained green and did not appear with abnormal morphology (CoNPs treatment), which proves that CoNPs inhibited ethylene biosynthesis, and slowed down the senescence of shoot clusters (Saha and Gupta, 2018). This result shows that CoNPs with small size should be easily absorbed, due to their large surface area, and therefore have a greater impact efficiency than when used in the salt complex form (Shah and Belozerova, 2009).

To date, very few studies have documented abnormal phenomena in the shoot multiplication stage. This study showed that AgNPs increased shoot multiplication efficiency via the number of shoots, shoot quality and reduced vitrification and yellowing of the leaf, and more specifically that the addition of 2 mg $L^{-1}$ AgNPs to the medium was optimal for the shoot multiplication stage.

### *4.3. AgNPs and CoNPs enhanced in vitro rooting, reduced abnormal phenomena and changed ethylene and enzyme activity*

Recent studies show that cobalt has an important role in inhibiting the formation and activity of ethylene. Co inhibited ethylene biosynthesis via inhibition of Acetyl-CoA carboxylase (ACC) and thus conversion to ethylene was limited (Thao et al., 2015). In *Swertia chirata* with an increasing CoNPs concentration in the culture medium, all the parameters of shoot regeneration and suppression of ACC to ethylene conversion were significantly increased (Saha and Gupta, 2018). In addition, polyamines (PA) play an important role in cell growth and division, and are involved in the physiology of flowering, metabolite synthesis, and response to viral infection. PA biosynthesis is closely related to ethylene biosynthesis, because both compete for the same S-adenosyl-L-methionine (SAM) precursor. AgNPs is a potent inhibitor of ethylene activity, and therefore increased arginine decarboxylase activity leads to increased endogenous PA in tissue cultures and induces *in vitro* flowering (Evans and Malmberg, 1989).

Moreover, Jaleel et al. (2009) showed that Co increased the chlorophyll content of leaves, and that this varied depending on the genotype and response pattern to ethylene in *Zea mays* plants. Photosynthetic

**Table 6**
Acclimatization of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar plantlets after 2 weeks planted in the greenhouse.

| Treatment | Survival rate (%) | Plantlet height (cm) | No. of roots/ plantlet | Root length (cm) | No. of leaves | Leaf size (cm) | | SPAD value | Plantlet weight (mg) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Length | Width | | Fresh | Dry |
| Control | 83.33 ± 3.52b* | 3.70 ± 0.40c | 4.67 ± 1.15b | 7.50 ± 0.68b | 6.67 ± 0.33b | 1.87 ± 0.17c | 1.63 ± 0.18c | 47.40 ± 2.40b | 2191.77 ± 33.83b | 342.40 ± 5.68c |
| 0.0465 **mg L$^{-1}$** CoNPs | 100.00 ± 0.00a | 5.20 ± 0.32a | 5.33 ± 0.88a | 8.90 ± 0.85a | 7.67 ± 0.58a | 2.47 ± 0.23a | 1.97 ± 0.23ab | 58.60 ± 1.45a | 2458.50 ± 29.82a | 363.07 ± 3.93a |
| 5 **mg L$^{-1}$** AgNPs | 88.00 ± 2.31b | 4.97 ± 0.49ab | 5.00 ± 0.67ab | 8.50 ± 0.26ab | 7.33 ± 0.33ab | 2.23 ± 0.09ab | 2.20 ± 0.03a | 52.70 ± 1.62ab | 2361.00 ± 34.00ab | 297.07 ± 1.10b |

* Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).

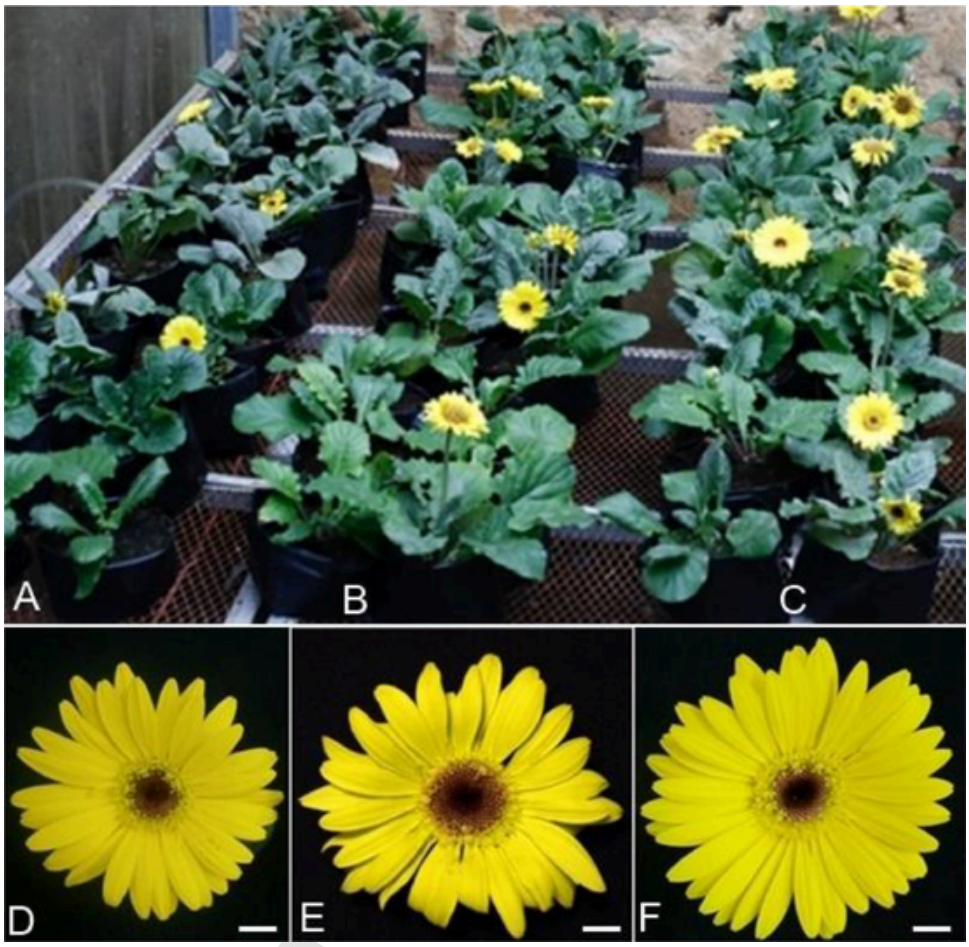


**Fig. 6.** The flowering of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar plantlets after 12 weeks in the greenhouse. *Bars: 2 cm* **A, D:** Flowering stage of plantlet derived from control (without AgNPs and CoNPs) **B, E:** Flowering stage of plantlet derived from 5 mg $L^{-1}$ AgNPs treatment **C, F:** Flowering stage of plantlet derived from 0.0465 mg $L^{-1}$ CoNPs treatment.

**Table 7**
The flowering of *Gerbera* (*Gerbera jamesonii* Revolution Yellow) cultivar plantlets after 12 weeks planted in the greenhouse.

| Treatment | Plantlet height (cm) | No. of leaves/ plantlet | Plantlet weight (g) | | Flowering time (week) | | Flower diameter (cm) | Peduncle length (cm) |
|---|---|---|---|---|---|---|---|---|
| | | | Fresh | Dry | Flower bub formation | Flowering | | |
| Control | 15.60 ± 0.38c* | 11.67 ± 67ab | 14.17 ± 0.21c | 1.24 ± 0.35c | 10.73 ± 0.15a | 12.17 ± 0.13a | 6.20 ± 0.06c | 16.40 ± 0.15c |
| 0.0465 **mg $L^{-1}$** CoNPs | 20.07 ± 0.32a | 13.33 ± 0.88a | 25.24 ± 0.37a | 2.94 ± 0.54a | 9.97 ± 0.15b | 11.17 ± 0.15b | 7.57 ± 0.09a | 21.47 ± 0.27a |
| 5 **mg $L^{-1}$** AgNPs | 17.70 ± 0.37b | 13.00 ± 0.58a | 22.59 ± 0.33b | 2.77 ± 0.40b | 10.27 ± 0.12ab | 11.67 ± 0.15ab | 7.23 ± 0.17b | 19.33 ± 0.14b |

* Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).

pigments such as chlorophyll a, chlorophyll b and carotenoids were increased at low Co concentrations (50 mg $Kg^{-1}$), while a higher Co concentration decreased these parameters because Co inhibited the activity of the photosynthetic enzymes involved in chlorophyll synthesis, such as 5-aminolevulinic acid and protoporphyrin. This shows that the size and concentration of the nanoparticles used are also factors that affect the efficiency of nutrient absorption and metabolism in plants. Co at low concentrations helps maintain the growth of peanuts (Basu et al., 2006) and cherries (Sarropoulou et al., 2016), promotes plantlet growth in terms of the height and number of leaves per plantlet, as well as fresh and dry weight of leaves and roots, and also enhanced the photosynthesis, respiration, metabolism and nutrient absorption of plantlets (Aziz et al., 2007).

In micropropagation, yellowing of the leaf and defoliation are abnormal, undesirable physiological phenomena that are often related to the effects of ethylene and hydrolytic enzyme activity. Yellowing of the leaf reduces the plantlet quality, along with the acclimatization and survival rate of plantlets transferred into nursery conditions (Ferrante and Francini, 2006). MacDonald et al. (2011) showed that ethylene increased cellulase activity in *Abies balsamea*. When the branches were exposed to endogenous ethylene, cellulase activity increased 8-fold compared to the control, while it increased 12-fold when exposed to exogenous ethylene. Similar results have been reported in other species, such as cotton and beans (Mishra et al., 2008).

Besides, biotic and abiotic stress can increase ROS production and promote plant defense responses. SOD, CAT, and APX are three essential antioxidant enzymes that play important roles as protective factors. At low concentrations, endogenous ROS is a signal to apoptosis, activates transcription factors, and regulates the expression of coding genes for antioxidant enzymes (Radville et al., 2011). However, at higher lev-

els ROS can cause protein denaturation, lipid oxidation, decreased photosynthesis, and even cell death (Ahmad et al., 2008). Plants then have a mechanism to effectively regulate ROS content by increasing the activity of antioxidant enzymes such as APX, CAT, and SOD to minimize ROS toxicity (Zhu-Salzman et al., 2004). Several studies have shown that treatment with most types of nanoparticles induces oxidative stress manifested by changes in ROS content and root and plant structure such as AgNPs in *Oryza sativa* (Nair and Chung, 2014), and CoNPs in some crops (Iqbal et al., 2019), etc. In this study, 0.0465 mg $L^{-1}$ CoNPs increased the *in vitro* rooting efficiency and antioxidant activity (SOD, CAT and APX).

In summary, AgNPs and CoNPs reduced the accumulation of ethylene gas in the culture bottle compared with common metal salts, thereby reducing abnormal phenomena. CoNPs reduced the activity of cellulase and pectinase enzymes, which are important enzymes with regard to inducing yellowing of the leaf in plantlets cultured *in vitro*.

### *4.4. Acclimatization, subsequent growth and flowering of plantlets in the greenhouse*

The results in this study showed that AgNPs and CoNPs improved the survival and acclimatization of plantlets cultured *in vitro* at nursery conditions. In particular, 0.0465 mg $L^{-1}$ CoNPs added to the culture medium helped improve the quality of plantlets cultured *in vitro*, and increased the acclimatization, growth and development of plantlets in ex vitro conditions better than seen with 5 mg $L^{-1}$ AgNPs and the control. Responses to Co have been studied in some plants, and found to depend on the CoNPs concentrations (Jaleel et al., 2009; Shah et al., 2013; Prazak, 2014). The results of this study once again demonstrated the role of CoNPs in increased *in vitro* growth, which increased acclimatization, subsequent growth and flowering in the greenhouse.

## 5. Conclusion

The results showed that 2 mg $L^{-1}$ AgNPs in culture media was effective in enhancing the shoot multiplication stage, while 0.0465 mg $L^{-1}$ CoNPs used instead of $CoCl_2.6H_2O$ was effective with regard to the *in vitro* rooting as well as reduction in vitrification, yellowing of the leaf and browning of the explant, and their acclimatization, growth and flowering stages. In addition, AgNPs and CoNPs reduced the ethylene accumulation, cellulase and pectinase enzyme activities and increased antioxidant activities.

## Compliance with ethical standards

***Ethical approval*:** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of Interest**: The authors declare no competing interests.

## CRediT authorship contribution statement

**Hoang Thanh Tung :** Funding acquisition, Writing – original draft. **Phan Le Ha Nguyen :** Investigation, Methodology, Visualization. **Tran Van Lich :** Investigation, Methodology, Visualization. **Ha Thi My Ngan :** Investigation, Methodology, Visualization. **Do Manh Cuong :** Investigation, Methodology, Visualization. **Vu Quoc Luan :** Investigation, Methodology, Visualization. **Hoang Dac Khai :** Investigation, Methodology, Visualization. **Nguyen Thi Nhu Mai :** Investigation, Methodology, Visualization. **Bui Van The Vinh :** Conceptualization, Visualization. **Duong Tan Nhut :** Conceptualization, Visualization.

## Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

## Data Availability

The data that has been used is confidential.

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