

***IN VITRO* REGENERATION OF *Renanthera imschootiana* Rolfe
FROM PROTOCORM-LIKE BODY**

H'Yon Nie Bing, Tran Thai Vinh, Vu Kim Cong, Dang Thi Tham, Nong Van Duy*

Tay Nguyen Institute for Scientific Research, VAST, Vietnam

Received 19 May 2022; accepted 22 May 2023

ABSTRACT

Renanthera imschootiana Rolfe is an extremely rare and endangered tropical epiphytic orchid. Studies on *in vitro* culture of *R. imschootiana* were conducted in order to conserve and increase the genetic pool of this wild orchid species. In this study, the effect of plant growth regulators (BA and NAA), ripe bananas in combination with potato, humic acid, and silver nanoparticles (AgNPs) were investigated to find a suitable condition for *in vitro* plant regeneration from *R. imschootiana* protocorm-like body (PLB). The results showed that after 45 days of culture, MS medium supplemented with 100 g ripe banana/liter and 100 g potato/liter was suitable for regenerating shoots from the protocorm-like body (PLB) (17.56 shoots/PLBs and 100.00% of shoot-regenerating PLBs); MS medium supplemented with 2.0 mg/L BA, 0.3 mg/L NAA and 4 ppm AgNPs was the most suitable for the growth of shoots after 60 days of culture (10.00 leaves/shoot and shoot length of 4.22 cm). The *in vitro* shoots were transferred to half-strength MS supplemented with humic acid and AgNPs to investigate the root formation of *R. imschootiana*. After 60 days of culture, the best rooting was obtained at 2.0 mg/L humic acid and 6 ppm AgNPs (8.33 roots/shoot; root length of 4.00 cm and 100.00% root formation). The findings suggest that the *in vitro* micropropagation from *R. imschootiana* protocorm-like body provides a useful alternative tool for the conservation of this endangered species.

Keywords: Conservation, epiphytic orchid, humic acid, plant growth regulators, silver nanoparticles.

Citation: H'Yon Nie Bing, Tran Thai Vinh, Vu Kim Cong, Dang Thi Tham, Nong Van Duy, 2023. *In vitro* regeneration of *Renanthera imschootiana* Rolfe from protocorm-like body. *Academia Journal of Biology*, 45(2): 47–59. <https://doi.org/10.15625/2615-9023/17155>

*Corresponding author email: vanduyvshtn@gmail.com

©2023 Vietnam Academy of Science and Technology (VAST)

INTRODUCTION

Renanthera imschootiana Rolfe is a rare orchid species, whose natural origin is from some primaeval forests featured with an altitude of 1,000–1,500 m in China, India, Myanmar, and Vietnam (Chen et al., 2009). The plant belongs to the group of single stems without pseudobulbs; inflorescence axillary, to 1 m, usually branched, many-flowered; peduncle and rachis slender; floral bracts broadly ovate, apex obtuse (Chen et al., 2009). In nature, this orchid is narrowly distributed in mountainous areas in Highland and Northwestern Vietnam (Ho, 1992). It is considered to be an endangered species and listed in Appendix I of the regulations formulated by the Committee for International Trade in Endangered Species Wild Fauna and Flora (CITES, 2021).

In vitro propagation by plant tissue culture is considered the most effective method for the rapid multiplication and conservation of many rare orchid species. Plant tissue culture media can be manipulated by phytohormones such as auxin, organic extracts, and other classes to influence *in vitro* growth and development. Auxin plays a focal turn in the formation of adventitious roots (Haissig & Davis, 1994; Guan et al., 2019), and the interdependent physiological stages of the rooting process are supported by changes in concentrations of endogenous auxin (Heloir et al., 1996). Besides auxin, organic extracts are known to be natural sources of carbon, containing a wide range of vitamins, natural hormones, proteins and minerals. When culturing orchids, the culture medium is supplemented with organic extracts such as coconut water, banana, potato, corn, papaya (Islam et al., 2003; Aktar et al., 2008; Gnasekaran et al., 2010).

Humic acids (also known as the black gold of agriculture) are the most important organic constituents of the soil (humus), defined as the organic matter in the soil, a mixture of partially and totally humified substances. Humic acid plays a vital role in the plant tissue culture as a growth hormone for *in vitro* propagation of many plant

seedlings (Dhanapal & Sathish, 2014). Humic acid has a direct effect on plant cell membranes, which increases their permeability and makes mineral elements move back and forth through the membrane, resulting in increased transport of various mineral nutrients to sites of metabolic need (Robert, 2007). They are used to help increase the germination rate and support the development of roots (Gallant, 2004). In particular, humic acid has antibacterial activity by inhibiting the growth of bacteria and fungi, thereby reducing mycotoxins (Islam et al., 2005). To date, there have been several studies on the investigation of the effect of humic acid on *in vitro* propagation of plants, for example, effects of auxins and humic acids on *in vitro* rooting of strawberry (*Fragaria x ananassa* Duch.) (Rzepka-Plevnes et al., 2011), the influence of humic acid (HA) on histological development, antioxidant enzyme changes and endogenous hormone levels during adventitious root formation in evergreen azalea microshoots (Elmongy et al., 2018) but only one for the orchid species *Paphiopedilum x dalatense* (Vinh et al., 2021).

In recent years, many studies on the effects of metal nanoparticles on plants have been conducted (Kole et al., 2016; Sarmast & Salehi, 2016; Sanzari et al., 2019). Among metal nanoparticles, silver nanoparticles (AgNPs) are widely used in the field of plant tissue culture. Many previous studies have proved the vital effect of AgNPs on plants. AgNPs had a remarkable ability to improve the growth rate and yield of wheat crops *ex vitro*. Specifically, when wheat plants were grown in clay pots supplemented with AgNPs at concentrations of 25 ppm and 50 ppm, fresh weight, dry weight and chlorophyll content were recorded higher than the control treatments and the grain yield was the highest (Razzaq et al., 2016). Ngan et al. (2019) conducted a study on the effect of AgNPs in *Gerbera jamesonii in vitro* and found that AgNPs have played an important role in reducing hyper-hydration and enhancing plantlet quality. In detail, on culture media

supplemented with a concentration of 2 ppm AgNPs, an increase in shoot multiplication and shoot quality was observed; whereas, hyper-hydration was observed to be significantly reduced, and the addition of 5 ppm silver nanoparticle solution to the root induction medium was the most optimal. In addition, when it comes to acclimation, the survival rate of young plantlets was witnessed to be high, which contributed to a source of high quality plantlets. Sanzari et al. (2019) have published an overview of nanomaterials used in plant science and accurately described

the main interactions in terms of uptake, mobilization mechanisms and biological effects. Ali et al. (2019) also showed that the addition of different concentrations of AgNPs increased callus induction, development and biomass when AgNPs were combined with plant growth regulators supplemented on MS medium in *in vitro* culture of *Caralluma tuberculata*. Besides, Zia et al. (2020) noted that AgNPs effectively enhanced the *in vitro* shoot multiplication and plantlet proliferation of *Carnation cultivars cv. Noblessa, cv. Antigua* and *cv. Mariposa*.



Figure 1. Tree and flower of *Renanthera imschootiana* Rolfe

In the genus *Renanthera*, several micropropagation protocols have been successfully developed such as *Renanthera ammani* (*Vanda* Josephine Van Brero x *Renanthera storiei*) (Goh & Tan, 1982), *R. imschootiana* (Seeni & Latha, 1992; Lin et al., 2008; Wu et al., 2014; Dan et al., 2018), *R. imschootiana* x *Vanda coerulea* (Rajkumar & Sharma, 2009), and *R. imschootiana* x *R. monachica* (Wu et al., 2012). However, there is still no study on the effects of AgNPs on the

micropropagation of *R. imschootiana* in general, as well as on improving plantlet quality in particular.

This research aims to investigate the effect of plant growth regulators (BA, NAA), ripe bananas in combination with potato, humic acid, and silver nanoparticles (AgNPs) for *in vitro* plant regeneration from *R. imschootiana* protocorm-like body. The best condition was found to shorten culture time.

MATERIALS AND METHODS

Materials

Protocorm-like body (PLB) (about 0.3 cm long induced from *R. imschootiana* seed was used for experiments. The materials are currently available in the laboratory of the Department of Plant Resources, Tay Nguyen Institute for Scientific Research, VAST.

Methods

Medium and cultural condition

The explants were cultured on Murashige and Skoog (1962) (MS) medium supplemented with plant growth regulators, banana extract, banana extract in combination with potato extract, humic acid and AgNPs with concentrations depending on the experiment. The organic extract was prepared as follows: remove ripe bananas' thin outer skin and grind them finely; wash and boil potatoes with the skin, then use boiled water to grind them finely. The medium was adjusted to pH 5.8 and then autoclaved at 121 °C, 1 atm for 25 min.

Condition of culture room: The explants were cultured in a culture room at a temperature of 25 ± 2 °C, average humidity of 55–60%, under the fluorescent lamp with a photoperiod of 16 hours per day and light intensity of $35 \mu\text{mol}/\text{m}^2/\text{s}$.

Silver nanoparticle solution

AgNPs with a size of less than 20 nm were used as follows: AgNO_3 750–1,000 ppm, β -chitosan 250–300 ppm, NaBH_4 200 ppm, mole ratio $\text{NaBH}_4/\text{AgNO}_3$ of 1/4, and a drip rate of NaBH_4 of 10–12 drops per minute (Chau et al., 2008). A nanoparticle solution was provided by the Institute of Environmental Technology, VAST.

Experimental set-up

Effect of organic extracts on shoot regeneration from PLB

PLB was separated into blocks about 0.3 cm in size and cultured on a medium containing MS supplemented with different concentrations of ripe banana extract (0, 50,

100, 150 g)/liter medium and in another experiment with MS supplemented with banana extract (0, 50, 100, 150 g)/liter medium in combination with potato extract at 100 g/liter medium. The results on the number of shoots and the rate of shoot regeneration will be collected after 45 days of culture.

Effect of BA and NAA on the growth of shoots

The shoots with a height of 0.6 cm were cultured on MS medium supplemented with different concentration of BA (0; 1.0; 1.5; 2.0 mg/L) in combination with NAA (0.3; 0.5 mg/L). The effects of culture media on the growth and development of shoots were evaluated by the number of leaves/shoots, the average length of shoots (cm) and shoot morphology after 60 days of culture.

Effect of AgNPs on the growth of shoots

The best shoots with a height of 3.0 cm from the above experiment were cultured on MS medium supplemented with optimal BA and NAA treatment in the above experiment combined with different concentrations of AgNPs (0; 2.0; 4.0; 6.0 ppm). The effects of culture media on the growth and development of shoots were evaluated by the number of leaves/shoots, the average length of shoots (cm) and shoot morphology after 60 days of culture.

Effect of humic acid on in vitro root regeneration of shoots

For the root induction experiments, the *in vitro* shoots with a height of about 4.0 cm were cultured on 1/2 MS medium supplemented with different concentrations of humic acid (0; 0.5; 1.0; 1.5; 2.0; 2.5 mg/L). The number of roots, length of roots, and rooting percentage were determined after 60 days.

Effect of AgNPs on in vitro root regeneration of shoots

For the root induction experiments, the *in vitro* shoots of about 4 cm were cultured on 1/2 MS culture medium with the optimal humic acid concentration in the above experiment and were then combined with different AgNPs concentrations (0; 2.0; 4.0; 6.0; 8.0 ppm). The

number of roots, length of roots, and rooting percentage were determined after 60 days.

Statistical analysis

The designed experiments were repeated three times following Complete Randomize Design. Each treatment comprised five vessels (V = 250 ml) with 30 ml culture solution, with 3 shoots per vessel. The data of treatment effects were analyzed using the ANOVA test, and a comparison between mean values of treatments made by Duncan's test (Duncan, 1955). All statistical analyses were performed using SPSS 22.0 software.

RESULTS AND DISCUSSION

Effect of organic additives on shoot regeneration from PLB

The ability to regenerate shoots of *R. imschootiana* after 45 culture days was shown in Table 1.

After 45 days of culture, different supplements had different effects on shoot number and shoot regeneration rate. In the

experiment without the appearance of mashes, the number of shoots was only 7.27 per PLB, whereas, in the medium supplemented with ripe banana and potato mash, the number of shoots/PLBs increased from 8.85 to 17.56. When individually adding ripe banana to the culture medium, the culture medium supplemented with 100 g ripe banana/liter was noticed to bring the best effect with 13.96 shoots/explant and 88.33% of shoot regeneration. Van et al. (1975) showed that the addition of blended banana to culture medium stimulates the orchid growth because it helps balance the pH medium. However, when the ripe banana content was increased to 150 g/L, the monitoring indicators tended to decrease (10.04 shoots/PLB and 79.00% of shoot regeneration samples). When the ripe banana content in the culture medium is too high, it inhibits the formation and growth of shoots, possibly due to the high osmotic pressure of the medium, and prevents the absorption of water as well as substances necessary for the growth of shoot development.

Table 1. Effect of organic additives on shoot regeneration from PLB of *Renanthera imschootiana*

Ripe banana (g/liter medium)	Potato (g/liter medium)	Average no. of shoots/explant	Regenerating shoot percentage (%)
0	0	7.27 [*]	50.33
50	0	8.85 ^c	76.00
100	0	13.96 ^c	88.33
150	0	10.04 ^d	79.00
50	100	15.45 ^b	77.00
100	100	17.56 ^a	100.00
150	100	15.18 ^b	86.33

Note: *: In each column, the mean values with a different letter (a, b, c,...) indicated significant difference between treatments with $p < 0.05$ by Duncan's test.

The medium supplemented with banana and potato mash had higher regenerating shoot percentages and numbers of shoots than the control medium. According to Islam et al. (2000), bananas, potatoes, and taros contain niacin and some vitamins, which have a stimulant effect on the germination and growth of orchids. In the culture medium supplemented with ripe banana and potato

mash, there was a marked increase in the monitoring parameters compared with the other treatments. The highest number of shoots/PLB of *R. imschootiana* was observed at 100 g ripe banana/liter medium and 100 g potato/liter medium, which was 17.56. A maximum rate of shoot regeneration (100.00%) was also found in this medium. When increasing the ripe banana content to 150 g/L combined with

100 g potato/liter medium, it inhibits the formation of shoots. To date, there has been no publication on the combined addition of banana and potato to the shoot regeneration medium on orchids of the genus *Renanthera*. This may be a new direction for further studies to regenerate shoots from PLB in orchids of this genus. The results of the high regeneration rate and the number of shoots in this study are consistent with the results of the study on *Paphiopedilum x dalatense* (Vinh et al., 2021). In a study of *R. imschootiana* shoot multiplication derived from protocorm, MS medium supplemented with 1.5 mg/L KIN and 15% CW was observed to be the most suitable,

specifically, shoot multiplication rate reached 15.33 shoots/explant after 12 weeks of culture (Dan et al., 2018). Thus, compared with the conditions established by Dan et al. (2018), shoot multiplication on the investigated media in this study was better, which may indicate a positive role of organic additives (ripe banana and potato mash) in the shoot multiplication of this orchid.

Effect of BA and NAA on the growth of shoots

After 60 culture days, the shoot growth and development results of *R. imschootiana* were summarised in Table 2.

Table 2. Effect of BA and NAA on the growth and development of *Renanthera imschootiana* shoots

BA (mg/L)	NAA (mg/L)	The number of leaves/shoot	The average length of shoots (cm)	Shoot morphology
0	0	2.67 ^c	0.867 ^{f*}	Small shoots, weak shoots
1.0	0.3	5.67 ^b	1.65 ^e	Small shoots, weak shoots
1.5	0.3	6.00 ^b	2.93 ^b	Small shoots, dark green leaves
2.0	0.3	7.67 ^a	3.50 ^a	Big shoots, dark green leaves
1.0	0.5	7.33 ^a	2.87 ^b	Small shoots, dark green leaves
1.5	0.5	6.33 ^b	2.75 ^c	Small shoots, light green leaves
2.0	0.5	5.33 ^b	1.75 ^d	Small shoots, light green leaves

Note: *: In each column, the mean values with a different letter (a, b, c...) indicated significant difference between treatments with $p < 0.05$ by Duncan's test.

The results in Table 2 show that the concentration of BA in combination with NAA influenced the average number of leaves produced per shoot as well as the mean length of the shoots. With the same concentration of 0.3 mg/L NAA, shoot growth increased as the concentration of BA increased. The highest number of leaves/shoot and shoot lengths observed at the concentration of BA 2.0 mg/L combined with 0.3 mg/L NAA was 7.67 leaves/shoot and 3.5 cm, respectively, in this experiment, it has shown to have big shoots and dark green leaves (Table 2 & Fig. 2c2). When the MS medium was supplemented with 0.5 mg/L NAA combined with BA (1.0; 1.5; 2.0 mg/L), the growth of shoots decreased gradually, and the number of leaves/shoot decreased from 7.33 leaves/shoot to 5.33 leaves/shoot and the average length of

shoots decreased from 2.87 cm to 1.75 cm, also with small shoots and light green leaves. The presence of BA at appropriate concentrations in the culture medium has been shown to have a positive effect on shoot growth in many orchid species (Hossain et al., 2010). Results are also supported by the findings of Roy & Banerjee (2002), who reported that BA enhances shoot multiplication more actively than kinetin. In this study, the combination of BA with NAA showed better growth and development of shoot than the studies adding only BA, which is a special point in our study. Meanwhile, Wu et al. (2014) concluded that Hyponex N016 medium supplemented with 0.5 mg/L NAA, 1 g/L peptone, 150 g/L BH and 20% CW was the most suitable for the growth of *in vitro* *R. imschootiana* plants, with a height of 3.63 cm after 8 weeks of culture.



Figure 2. *In vitro* regeneration of *Renanthera imschootiana* from PLB. (a) PLB; (b) Shoot cluster formed from PLB; (c) Effect of BA and NAA on the growth and development of shoots in treatments (Control; 0.3 mg/L NAA combined with 2.0 mg/L BA; 0.5 mg/L NAA combined with 1.0 mg/L BA); (d) Effect of AgNPs on the growth and development of shoots; (e) Effect of humic acid on root regeneration; (f) Effect of AgNPs on root regeneration

Effect of AgNPs on the growth of shoots

After 60 days of culture, the effects of different concentrations of AgNPs in the

culture medium containing 2.0 mg/L BA in combination with 0.3 mg/L NAA on the growth and development of *R. imschootiana* shoots were shown in Table 3.

Table 3. Effect of AgNPs on the growth and development of *Renanthera imschootiana* shoots

AgNPs (ppm)	The number of leaves/shoot	The average length of shoots (cm)	Shoot morphology
0	7.67 ^b	3.50 ^{c*}	Big shoots, dark green leaves
2.0	9.33 ^a	3.70 ^b	Big shoots, dark green leaves
4.0	10.00 ^a	4.22 ^a	Big and strong shoots, dark green leaves
6.0	8.33 ^b	3.54 ^c	Small shoots, yellow leaves

Note: *: In each column, the mean values with a different letter (a, b, c,...) indicated significant difference between treatments with $p < 0.05$ by Duncan's test.

After 60 culture days, the results showed that at all investigated concentrations of AgNPs, the number of leaves per shoot and shoot height were higher than those of the control. In the medium without AgNPs addition, the number of leaves per shoot was only 7.67 and the shoot height was 3.50 cm (Table 3 & Fig. 2d1). When the medium was supplemented with 2.0 ppm AgNPs, the monitoring parameters increased to 9.33 leaves/shoot and 3.70 cm, respectively (Table 3 & Fig. 2d2). Especially when the AgNPs concentration was increased to 4.0 ppm, the number of leaves per shoot, as well as the average length of shoots, reached the highest (10.00 leaves/shoot and 4.22 cm) (Table 3 & Fig. 2d3). However, when continuing to increase the concentration of AgNPs to 6.0 ppm, the two monitoring parameters of the experiment (the number of leaves per shoot as well as the shoot height) tended to decrease gradually, the shoots were weak and the leaves yellowed (Table 3 & Fig. 2d4). The high AgNPs concentration causes reduced growth of *R. imschootiana*, which can be explained by heavy metal poisoning (silver) in cells leading to phenol polymerization due to peroxidase enzyme activity (Backor et al., 2009). Cuong et al. (2018) used AgNPs on *Fragaria x ananassa*, showing that AgNPs not only increased the number of shoots but the shoots also had larger petioles, enlarged leaves, and darker green color than the control treatment. Huong & Gioi (2021) also reported

that a medium supplemented with 4.0 ppm AgNPs was appropriate for the shoot multiplication and development of shoots of *Gomphrena globose* L.

There have been many studies on the effects of AgNPs in many plant species, but for orchids, there are very few studies. Gioi & Huong (2019) used AgNPs for *Phalaenopsis* orchids and showed that the optimal medium for the formation of shoots from the PLB of phalaenopsis orchid was a culture medium containing 4.0 ppm AgNPs. Thus, our study demonstrated that AgNPs promoted shoot growth of *R. imschootiana* and the promotive effects of growth by AgNPs are beneficial to plant propagation.

Effect of humic acid on root regeneration of shoots *in vitro*

Root regeneration of shoots is the final step in the *in vitro* propagation process. At the end of this period, complete plants (with roots, stems and leaves) will be obtained for the training phase of plants in the nursery.

In this study, the data on the number of roots, length of roots, and rooting percentage after 60 days of culture were recorded in Table 4.

All the treatments produced roots with varying root numbers and lengths. In the control treatment where 1/2 MS medium was not supplemented with humic acid, rooting percentage, number of roots and root length

were lower than in the medium added with humic acid, which showed that the addition of humic acid to the culture medium had a positive effect on the root formation of *R. imschootiana*. When the concentration of humic acid was increased from 0.5 mg/L to 1.5 mg/L, all three monitoring parameters of the experiment increased, the number of roots per shoot increased from 2.22 to 3.78, the average root length from 0.85 cm to 1.52 cm and the rate of root regeneration from 62.67 to 82.00% (Table 4). In particular, the treatment of medium added with 2.0 mg/L humic acid was the best response, which produced 5.33 roots/shoot with a root length of 2.07 cm and a rooting rate of 99.00% (Table 4 & Fig. 2e5). However, when the concentration of humic acid increased to 2.5 mg/L, the number of roots/shoots, root

length, and rooting rate decreased by 4.22 roots/shoot, 1.62 cm, and 80.67%, respectively (Table 4 & Fig. 2e6).

A few studies used humic acid in orchid culture. For *R. imschootiana*, when using humic acid, the quality of seedlings is good with dark green leaves, and strong root, and this will be a new direction in the propagation process of the genus *Renanthera* in the next research rounds. Vinh et al. (2021) in the *Paphiopedilum x dalatense* propagation study indicated that the growth and development of roots were maximum at 2.0 mg/L humic acid, which is consistent with our results. In addition, Mohamed et al. (2017) studied the propagation of evergreen *Azalea* and obtained the optimal rooting medium results when adding 1.0 mg/L of humic acid.

Table 4. Effect of humic acid on root regeneration of shoots *in vitro*

Humic acid (mg/L)	No. of roots/shoot	Root length (cm)	Rooting percentage (%)
0	0.67 ^d	0.38 ^{e*}	49.33
0.5	2.22 ^{cd}	0.85 ^d	62.67
1.0	3.22 ^c	1.20 ^c	72.00
1.5	3.78 ^{bc}	1.52 ^b	82.00
2.0	5.33 ^a	2.07 ^a	99.00
2.5	4.22 ^b	1.62 ^b	80.67

Note: *: In each column, the mean values with a different letter (a, b, c,...) indicated significant difference between treatments with $p < 0.05$ by Duncan's test.

Effect of AgNPs on root regeneration of *in vitro* shoots

The experiment was continued with the culture medium containing 2.0 mg/L of humic acid combined with AgNPs at different concentrations (0; 2.0; 4.0; 6.0; 8.0 ppm). The *in vitro* root regeneration ability of shoots after 60 days of culture is presented in Table 5.

The results obtained in Table 5 showed that AgNPs had a positive effect on the rooting ability of shoot *R. imschootiana*, the experiment supplemented with AgNPs gave a higher number of roots/shoot and root length compared to experiments without the addition of AgNPs. As for the number of roots, in the control treatment, only 5.33

roots/shoot were obtained, while this indicator in the treatment with AgNPs addition ranged from 6.67 roots/shoot (addition treatment 2.0 ppm AgNPs) to 8.33 roots/shoot (addition treatment 6.0 ppm AgNPs) (Table 5 & Fig. 2f). Regarding the root length criterion, in the control treatment, the average root length was 2.07 cm, while this indicator in the AgNPs additional treatments ranged from 2.53 cm (addition treatment 2.0 ppm AgNPs) to 4.00 cm (addition treatment 6.0 ppm AgNPs) (Table 5 & Fig. 2f). Among these treatments, the treatment with 6.0 ppm AgNPs showed the best results in terms of growth parameters (8.33 roots/shoot, root length 4.00 cm and 100.00% root formation) (Table 5 &

Fig. 2f4). It is observed that the seedlings are healthy with strong green leaves. They have many thick and uniform roots, which will be a source of quality *in vitro* seedlings in the future. When AgNPs concentration increased to 8.0 ppm, root formation was inhibited and the number of roots decreased (Table 5 & Fig. 2f5). The positive effect on rooting in this case may be due to the additive effect of humic acid (2.0 mg/L) combined with AgNPs (6.0 ppm).

AgNPs have an important role in plant tissue culture. It not only enhances the growth and development of *Brassica juncea*, bean (*Phaseolus vulgaris* L.), and corn (*Zea mays* L.) (Salama, 2012) but also accelerates root formation in *Crocus sativus* (Rezvani et al., 2012). In our research, AgNPs was proved to be superior in the *in vitro* culture system for *R. imschootiana*. In the future, this may be an interesting method to use in propagation in orchids.

Table 5. Effect of AgNPs on root regeneration of shoots *in vitro*

AgNPs (ppm)	No. of roots/shoot	Root length (cm)	Rooting percentage (%)
0	5.33 ^c	2.07 ^{e*}	99.00
2.0	6.67 ^b	2.53 ^d	98.67
4.0	7.33 ^b	3.17 ^c	99.00
6.0	8.33 ^a	4.00 ^a	100.00
8.0	7.00 ^b	3.85 ^b	96.67

Note: *: In each column, the mean values with a different letter (a, b, c,...) indicated significant difference between treatments with $p < 0.05$ by Duncan's test.

CONCLUSION

In vitro micropropagation of *R. imschootiana* Rolfe, the most suitable medium for regenerating shoots from Protocorm-like body was MS medium supplemented with 100 g ripe banana/liter medium and 100 g potato/liter medium. For subculture, suitable growth of shoots was obtained on MS medium supplemented with 2.0 mg/L BA, 0.3 mg/L NAA and 4.0 ppm AgNPs. The medium used for root formation was 1/2 MS supplemented with 2.0 mg/L humic acid and 6.0 ppm AgNPs.

Acknowledgements: The authors would like to thank the Tay Nguyen Institute for Scientific Research and the Institute of Environmental Technology, VAST for supporting this study.

REFERENCES

- Aktar S., Nasiruddin K. M., Hossain K., 2008. Effects of different media and organic additives interaction on *in vitro* regeneration of *Dendrobium* orchid. *Journal of Agriculture and Rural Development*, 6: 69–74. <https://doi.org/10.3329/jard.v6i1.1659>
- Ali A., Mohammad S., Khan M. A., Raja N. I., Arif M., Kamil A., Mashwani Z. U. R., 2019. Silver nanoparticles elicited *in vitro* callus cultures for accumulation of biomass and secondary metabolites in *Caralluma tuberculata*. *Artificial Cells, Nanomedicine, and Biotechnology*, 47(1): 715–724. <https://doi.org/10.1080/21691401.2019.1577884>
- Backor M., Kovik J., Dzubaj A., Bakorova M., 2009. Physiological comparison of copper toxicity in the lichens *Peltigera rufescens* (Weis) Humb. and *Cladonia arbuscula* subsp. *mitis* (Sandst.) Ruoss. *Plant Growth Regulation*, 58: 279–286. <https://doi.org/10.1007/s10725-009-9376-x>
- Elmongya M. S., Zhoua H., Caoa Y., Liua B., Xiaa Y., 2018. The effect of humic acid on endogenous hormone levels and antioxidant enzyme activity during *in vitro* rooting of evergreen azalea. *Scientia Horticulturae*, 227: 234–243. <https://doi.org/10.1016/j.scienta.2017.09.027>

- Huong B. T. T., Gioi D. H., 2021. Study on *in vitro* application of silver nanoparticles in leaf propagation of Globe amaranth (*Gomphrena globosa* L.). *Vietnam Journal of Agricultural Sciences*, 19(3): 363–369.
- Chau H. N., Bang L. A., Buu N. Q., Dung T. T. N., Ha H. T., Quang D. V., 2008. Some results in manufacturing of nanosilver and investigation of its application for disinfection. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 9(2): 241–248.
- Chen S., Liu Z., Zhu G., Lang K., Tsi Z., Luo Y., 2009. *Flora of China*, Beijing: Science Press.
- CITES. <http://www.cites.org/eng/app/appendices.php>. Appendices I, II and III. accessed: 22/11/2021.
- Dhanapal S., Sathish S. D., 2013. Humic acids and its role in plant tissue culture at low nutrient level. *JAIR*, 2(6): 338–340.
- Dhanapal S., Sathish S. D., 2014. Enhanced *in vitro* propagation of *Musa accuminata* induced by humic acid from coal extract as compared with commercially available humic acid products. *IJIRSET*, 3(7): 300–307. <https://doi.org/10.15623/ijret.2014.0307051>
- Cuong D. M., Phuong T. T. B., Nhut D. T., 2018. Effect of nanosilver on callus induction and shoot regeneration ability from leaf explants of strawberry (*fragaria x ananassa*) cultured *in vitro*. *HUJOS: Natural Science*, 127(1C): 61–70. <https://doi.org/10.26459/hueuni-jns.v127i1C.4893>
- Gioi D. H., Huong B. T. T., 2019. Study on use of silver nanoparticles in phalaenopsis orchid (*Phalaenopsis sp.*) tissue culture. *Forestry science and technology journal*, 1: 19–24.
- Duncan D. B., 1955. Multiple range and multiple F tests. *Biometrics*, 11(1): 1–42.
- Gallant A., 2004. Biostimulants: What they are and how they work. *Turf & Rec*: 1–4.
- Gnasekaran P., Xavier R., Sinniah U. R., Subramaniam S., 2010. A study on the use of organic additives on the protocorm-like bodies (PLBS) growth of *Phalaenopsis violacea* orchid. *J Phytol.*, 2: 29–33.
- Goh C. J., Tan H., 1982. Clonal propagation from leaf explants in *Renanthera* orchid hybrid. *Orchid Rev.*, 90: 295–296.
- Guan L., Tayengwa R., Cheng Z. M., Peer W. A., Murphy A. S., Zhao M., 2019. Auxin regulates adventitious root formation in tomato cuttings. *BMC Plant Biol.*, 19(1): 1–16. <https://doi.org/10.1186/s12870-019-2002-9>
- Ngan H. T. M., Trinh T. D. H., Cuong D. M., Tung H. T., Linh N. T. N., Hien V. T., Nguyen P. L. H., Luan V. Q., Le B. V., Nhut D. T., 2019. Limitation of hyperdricity and enhanced survival rate of *Gerbera jamesonii* plantlets cultured *in vitro* on medium supplemented with silver nanoparticles. *Journal of Biotechnology*, 17(1): 115–124.
- Haissig B. E., Davis T. D., 1994. A historical evaluation of adventitious rooting research to 1993. In: *Biology of Root Formation*. Davis T.D. and B.E., Haissig editors. New York: Plenum Press: 275–331.
- Heloir M. C., Kevers C., Hausman J. F., Gaspar T., 1996. Changes in the concentrations of auxins and polyamines during rooting of *in vitro*-propagated walnut shoots. *Tree Physiology*, 16(5): 515–519. <https://doi.org/10.1093/treephys/16.5.515>
- Hossain M., Sharma M., Da Silva J. A. T., Pathak P., 2010. Seed germination and tissue culture of *Cymbidium giganteum* Wall. Ex Lindl. *Scientia Horticulturae*, 123 (4): 479–487. <https://doi.org/10.1016/j.scienta.2009.10.009>
- Islam K. M. S., Schuhmacher A., Gropp J. M., 2005. Humic acid substances in animal agriculture. *Pakistan Journal of Nutrition*, 4(3): 126–134. <http://dx.doi.org/10.3923/pjn.2005.126.134>

- Islam M. O., Matsui S., Ichihashi S., 2000. Effect of complex organic additives on seed germination and carotenoid content in *Cattleya* seedlings. *Lindleyana*, 15(2): 81–88.
- Islam O., Rahman A., Matsui S., Prophan A., 2003. Effects of complex organic extracts on callus growth and PLB regeneration through embryogenesis in the *Doritaenopsis* Orchid. *Jpn. Agric. Res. Q.*, 37: 229–235. <http://dx.doi.org/10.6090/jarq.37.229>
- Kole C., Kumar D. S., Khodakovskaya M. V., 2016. Plant nanotechnology: principles and practices. Springer, Switzerland.
- Lin D.N., Chen Z.L., Duan J., Wu K.L., Zeng S.J., 2008. Seed germination *in vitro* of *Renanthera imschootiana* Rolfe. *Journal of Tropical and Subtropical Botany*, 16(1): 83–88. <https://doi.org/10.3969/j.issn.1005-3395.2008.1.013>
- Mohamed S. E., Hong Z., Yan C., Bing L., Yiping X., 2017. The effect of humic acid on endogenous hormone levels and antioxidant enzyme activity during *in vitro* rooting of evergreen azalea. *Scientia Horticulturae*, 227: 234–243. <https://doi.org/10.1016/j.scienta.2017.09.027>
- Murashige T., Skoog F., 1962. Arevised medium for rapid growth and bio assays with tobacco tissue cultures. *Plant Physiology*, 15: 473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Ho P. H., 1992. An illustrated flora of Vietnam, Vol. 3. Tre Publishing House, pp. 967–968.
- Rajkumar K., Sharma G. J., 2009. Intergeneric hybrid of two rare and endangered orchids, *Renanthera imschootiana* Rolfe and *Vanda coerulea* Griff. ex L. (Orchidaceae): Synthesis and characterization. *Euphytica*, 165: 247–256. <http://dx.doi.org/10.1007/s10681-008-9755-9>
- Razzaq A., Ammara R., Jhanzab H. M., Mahmood T., Hafeez A., Hussain S., 2016. A novel nanomaterial to enhance growth and yield of Wheat. *Journal of Nanoscience and Nanotechnology*, 2(1): 55–58.
- Rezvani N., Sorooshzadeh A., Farhadi N., 2012. Effect of nano-silver on growth of saffron in flooding stress. *World Acad Sci Eng Technol*, 1: 517–522.
- Robert E. P., 2007. Organic matter, humus, humates, humic acid, fulvic acid and humin. Emeritus Associate Professor Texas A & M University.
- Roy J., Banerjee N., 2002. Rhizome and shoot development during *in vitro* propagation of *Geodorum densiflorum* (Lam.) Schltr. *Scientia Horticulturae*, 94: 181–192. [https://doi.org/10.1016/S0304-4238\(01\)00373-9](https://doi.org/10.1016/S0304-4238(01)00373-9)
- Rzepka-Plevnes D., Kulpa D., Gołębiowska D. and Porwolik D., 2011. Effects of auxins and humic acids on *in vitro* rooting of strawberry (*Fragaria x ananassa* Duch.). *Journal of Food, Agriculture and Environment*, 9 (3&4): 592–595.
- Salachna P., Byczyńska A., Zawadzińska A., Piechocki R., Mizielińska M., 2019. Stimulatory effect of silver nanoparticles on the growth and flowering of otted oriental Lilies. *Agronomy*, 9(10): 1–14. <https://doi.org/10.3390/agronomy9100610>
- Salama H. M. H., 2012. Effects of silver nanoparticles in some crop plants, Common bean (*Phaseolus vulgaris* L.) and Corn (*Zea mays* L.). *International Research Journal of Biotechnology*, 3(10): 190–197.
- Sanzari I., Leone A., Ambrosone A., 2019. Nanotechnology in plant science: to make a long story short. *Frontiers in Bioengineering and Biotechnology*, 7: 1–12. <https://doi.org/10.3389/fbioe.2019.00120>
- Sarmast M. K., Salehi H., 2016. Silver nanoparticles: an influential element in plant nanobiotechnology. *Mol Biotechnol*, 58: 441–449. <https://doi.org/10.1007/s12033-016-9943-0>

- Seeni S., Latha P. G., 1992. Foliar regeneration of the endangered Red Vanda, *Renanthera imschootiana* Rolfe. *Plant Cell, Tissue and Organ Culture*, 29(3): 167–172.
- Dan T. Q., Ly N. M., Tuan V. C., 2018. *In vitro* propagation of a precious orchid species *Renanthera imschootiana* Rolfe research. *Journal of Science and Technology, Da Nang University*, 3(124): 89–93.
- Vinh T. T., Bing H. N., Tham D. T., Hang N. T. T., Cong V. K., Duy N. V., 2021. Micropropagation of *paphiopedilum x dalatense*. *Journal of Biotechnology*, 19(1): 155–163. <https://doi.org/10.15625/1811-4989/14550>.
- Van S. J., Stewart J., 1975. Cytokinins in banana fruit. *Zeitschrift für Pflanzenphysiologie*, 76(3): 280–283. doi: 10.1016/S0044-328X(75)80024-9
- Wu K., Zeng S., Teixeira da Silva J. A., Chen Z., Zhang J., Yang Y., Duan J., 2012. Efficient regeneration of *Renanthera* Tom Thumb ‘Qilin’ from leaf explants. *Scientia Horticulturae*, 135: 194–201. <https://doi.org/10.1016/j.scienta.2011.11.028>
- Wu K., Zeng S., Lin D., Teixeira da Silva J. A., Bu Z., Zhang J., Duan J., 2014. *In vitro* propagation and reintroduction of the endangered *Renanthera imschootiana* Rolfe. *PLoS ONE*, 9(10): e110033. <https://doi.org/10.1371/journal.pone.0110033>
- Zia M., Yaqoob K., Mannan A., Nisa S., Raza G., Rehman R., 2020. Regeneration response of carnation cultivars in response of silver nanoparticles under *in vitro* conditions. *Vegetos*, 33(1): 11–20. <http://dx.doi.org/10.1007/s42535-019-00074-9>