

SILVER NANOPARTICLES ENHANCED EFFICIENCY OF EXPLANT SURFACE DISINFECTION AND SOMATIC EMBRYOGENESIS IN *BEGONIA TUBEROUS* VIA THIN CELL LAYER CULTURE

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SUMMARY

In vitro culture establishment is one of the most important stages in micropropagation. The disinfectant effectiveness depends on the type of surface disinfectant, concentration and the time treatment. In this initial study, silver nanoparticles (AgNPs) were used as a disinfectant for petioles, flower stalks and stems of *Begonia tuberosus*. In addition, thin cell layer culture (TCL) technique has been applied for the purpose of somatic embryogenesis. The results showed that AgNPs were effective in eliminating infectious microorganisms on *B. tuberosus* explants; which were identified included 4 species of fungi (*Fusarium* sp., *Aspergillus aculeatus*, *Trichoderma* sp. and *Penicillium* sp.) and 1 species of bacteria (*Pseudomonas* sp.). At concentrations of 200 ppm and 300 ppm, AgNPs were not only effective in disinfection but also increased the induction rate of somatic embryogenesis in flower stalk TCL explants (approximately 40.00%); a similar effect was observed in stem TCL explants at the same concentration. Meanwhile, for petiole TCL explants, the induction rate of somatic embryogenesis was optimal when using AgNPs at a concentration of 100 - 300 ppm to disinfected the explant. In contrast, at high (400 ppm) or low (50 ppm) concentrations of AgNPs did not play a disinfecting role and stimulated somatic embryogenesis. In addition, explants derived from AgNPs sterilization did not show any abnormalities in somatic embryogenesis with shapes such as globular, heart, torpedo, and cotyledon. AgNPs showed double efficacy in sterilization of explants and improved efficiency of somatic embryogenesis from TCL petioles, flower stalks and stems explants; thus increasing the efficiency micropropagation of *B. tuberosus*.

Keywords: *Begonia*, disinfection, silver nanoparticles, somatic embryogenesis.

INTRODUCTION

Establishment of *in vitro* cultures is the first step to play an important role in micropropagation. However, this stage is challenged by contamination or disinfectant that can cause explant necrosis and is not safe for human health. There are many reasons for this, one of which is the operation of the sample disinfection process (Abdi *et al.*, 2008). Type,

concentration, and time of unsuitable cultures were the main reasons leading to failure in the initial sample stage. The common disinfectants used in micropropagation such as HgCl₂, Ca(ClO)₂, antibiotics, bromine water, etc. are known to be highly corrosive; thus, they provide a high microbial removal efficiency. However, these common disinfectants are also responsible for necrosis or even explant death (Ines *et al.*, 2013). In addition, most of the

substances used in the disinfection of cultures currently have negative impacts on human health (WHO, 2000). Finding a new type of disinfectant that is safe for health, effective in sterilizing the sample and stimulating the implant is extremely important.

The antimicrobial effect of silver nanoparticles (AgNPs) has been noticed for a long time and nowadays. AgNPs with small size (less than 20 nm) have been applied in biomedical field and medicine. There are several studies on the effects of AgNPs on plants such as germination rate of some plant species (Rezvani *et al.*, 2012), physiology and morphology of plants (Syu *et al.*, 2014).

Begonia is a model plant for many studies of plant physiology under *in vitro* condition such as *in vitro* flowering (Ringe, Nitsch, 1968), epidermal cells cultured *in vitro* (Chlyah, Van, 1975), changes in the starch content during organogenesis in *in vitro* cultured stem explants (Mangat *et al.*, 1990) and genetic diversity (Aswathy, Murugan, 2019). Micropropagation of *Begonia* has achieved some successes such as

shoot regeneration (Espino *et al.*, 2004; Sara *et al.*, 2012; Rowe, Gallone, 2016); micropropagation protocol for commercial purposes (Nhut *et al.*, 2005, 2010) and plant regeneration from petal samples (Rosas *et al.*, 2018). However, the previous studies just only describing the process of shoot regeneration, there is no study on somatic embryogenesis of *Begonia tuberosus*.

In this study, we used AgNPs instead of traditional disinfectant (HgCl_2 and $\text{Ca}(\text{ClO})_2$) to evaluate the ability to explant surface disinfection as well as evaluation the somatic embryogenesis of *B. tuberosus*, which is a high economic value of potted plant in Da Lat, Lam Dong.

MATERIALS AND METHODS

Plant material

The petiole (P), flower stalk (F) and stem (S) explants of *B. tuberosus* (3-month-old) were used as initial materials. The explants were collected and cut into transverse thin cell layer (tTCL) with 1 mm in thick (Fig. 1).

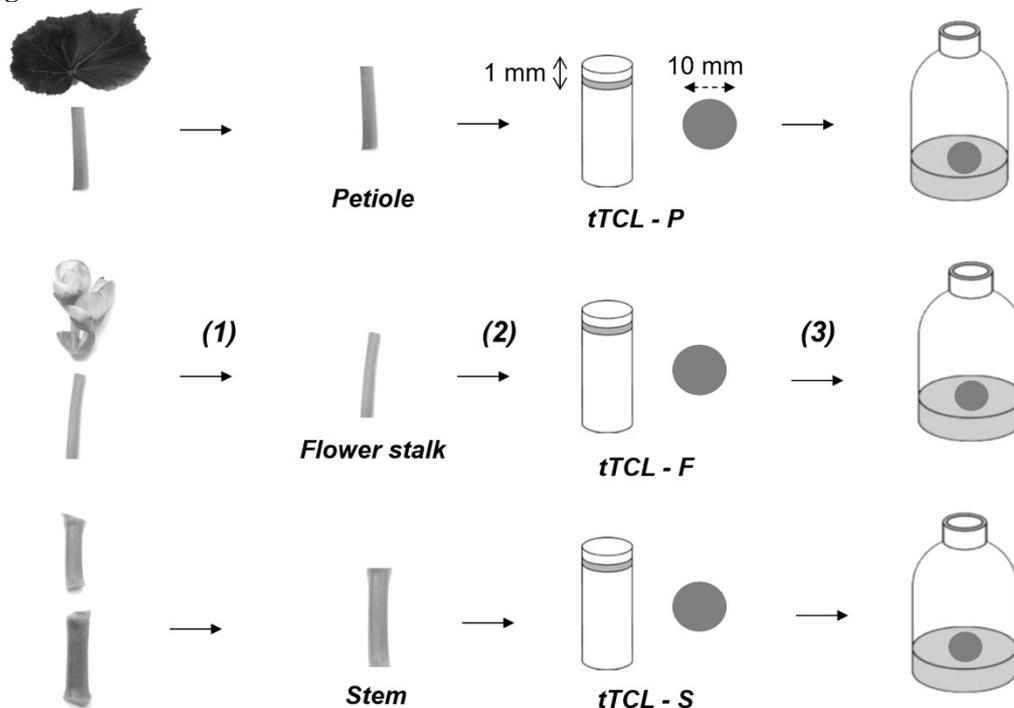


Figure 1. Diagram describes the different explants were cut into transverse thin cell layer (t-TCL).

Culture medium and condition

The explants were cultured on Murashige and Skoog (1962) (MS) medium supplemented with plant growth regulators depends on the experiment. The medium was adjusted to pH 5.8 then autoclaved at 121°C, 1 atm for 30 min.

The explants were cultured in culture room at the temperature of $25 \pm 2^\circ\text{C}$, average humidity of 55 - 60% under fluorescent lamp with photoperiod of 16 h per day and light intensity of $45 \mu\text{mol}/\text{m}^2/\text{s}$.

Silver nanoparticles solution

AgNPs with size of less than 20 nm was used as follows: AgNO_3 : 750 - 1000 ppm, β -chitosan 250 - 300 ppm, NaBH_4 : 200 ppm, mole ratio $\text{NaBH}_4/\text{AgNO}_3$: $\frac{1}{4}$ and a drip rate of NaBH_4 is 10 - 12 drops per minute (Chau *et al.*, 2008). AgNPs solution was prepared to 5 concentrations (50 ppm, 100 ppm, 200 ppm, 300 ppm and 400 ppm).

Effect of AgNPs on explant surface disinfection and embryogenic callus formation

The explants were sterilized with AgNPs solution at different concentrations (50, 100, 200, 300 and 400 ppm) in 6 min as an alternative bactericidal agent for the commonly used disinfectant such as 0.1% HgCl_2 in 6 min or 60 g/L $\text{Ca}(\text{ClO})_2$ in 10 min.

The tTCL - P, tTCL - F and tTCL - S explants were cultured on MS medium added to 0.2 mg/L NAA, 0.1 mg/L TDZ, 30 g/L sucrose; and 8 g/L agar (Nhut *et al.*, 2005) to evaluation the embryogenic callus formation after 28 days of culture.

Isolating infected microorganisms

The culture media containing one-week explants (tTCL - P, tTCL - F and tTCL - S) sterilized with tap water were recorded and washed with 8.5% physiological saline. It transplanted on potato dextrose agar (PDA) and water agar (WA) then incubated at 28°C in dark.

PDA was prepared by 24 g potato dextrose broth (Difco, USA), 15 g bacto agar (Difco, USA), and filled to 1000 mL by distilled water. WA was prepared by 15 g bacto agar (Difco, USA) and filled to 1000 mL by distilled water.

Microorganisms were obtained by stereo microscope after each 24 h until one week. Each colony was isolated and examined morphological characteristics by microscope 1000 \times magnification (Proctor, 1977).

Somatic embryogenesis

Twenty-eight-day-old embryogenic callus derived from tTCL - P, tTCL - F and tTCL - S explants were cut into 0.5×0.5 cm in size and cultured on $\frac{1}{2}$ MS medium supplemented with 0.2 mg/L TDZ, 0.2 mg/L NAA, 30 g/L sucrose and 8 g/L agar (Nhut *et al.*, 2005) to evaluation the somatic embryogenesis after 42 days of culture.

Statistical analysis

Each treatment was repeated 3 times with 15 explants/treatment. Data were processed and analyzed by Microsoft Excel 2013 and SPSS 18.0 software according to Duncan test with $P < 0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

Explant surface disinfection with AgNPs

In this study, the explants were only surface washed by tap water to eliminate surface-binding agents. After that, the explants (tTCL - P, tTCL - F and tTCL - S) were cultured on the embryogenic callus formation medium. The recorded results showed that, after 4 - 7 days of culture, 100% of the explants were contaminated with microorganisms. Through the screening process, 7 groups of morphological types of microbial contamination were recorded (Fig. 2). All of the leaf explants washed by sterilized distilled water were contaminated with microorganisms after 4 - 7 days of culture. In this study, the results were identified 4 fungi species (*Fusarium* sp., *Aspergillus aculeatus*, *Trichoderma* sp., and *Penicillium* sp.) and a bacterial species

(*Pseudomonas* sp.) in the media containing one-week explants sterilized with tap water (Fig. 3).

The survival rate of the t-TCL disinfected with various disinfectants (AgNPs, HgCl₂ and Ca(ClO)₂) was recorded in Table 1, 2, and 3.

The results showed that using 50 - 300 ppm AgNPs or HgCl₂ (0.1%) gave the highest surface sterilization effect of tTCL – F explants (the survival rate from 88.87% - 93.33%); meanwhile, Ca(ClO)₂-sterilizing samples showed the lowest disinfection efficiency surface (the survival rate was 77.80%) after 28

days of culture (Table 1).

Similar in t-TCL – S explants, 50 ppm to 300 ppm AgNPs also gave the highest survival rate (77.80% - 86.67%). The traditional disinfectants in this treatment have a high survival rate with Ca(ClO)₂ (84.47%) and HgCl₂ (77.80%) (Table 3).

For t-TCL – P explants, the results showed that using 100 ppm - 300 ppm AgNPs for sample survival rate from 75.53% - 80%, higher than that of HgCl₂ (71.13%) and Ca (ClO)₂ (62.20%) (Table 2).

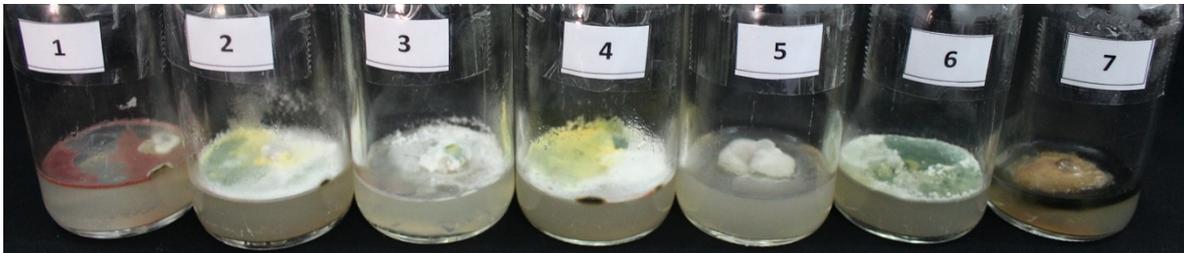


Figure 2. Infectious microorganisms during the sample sterilization period after 1 week of culture.

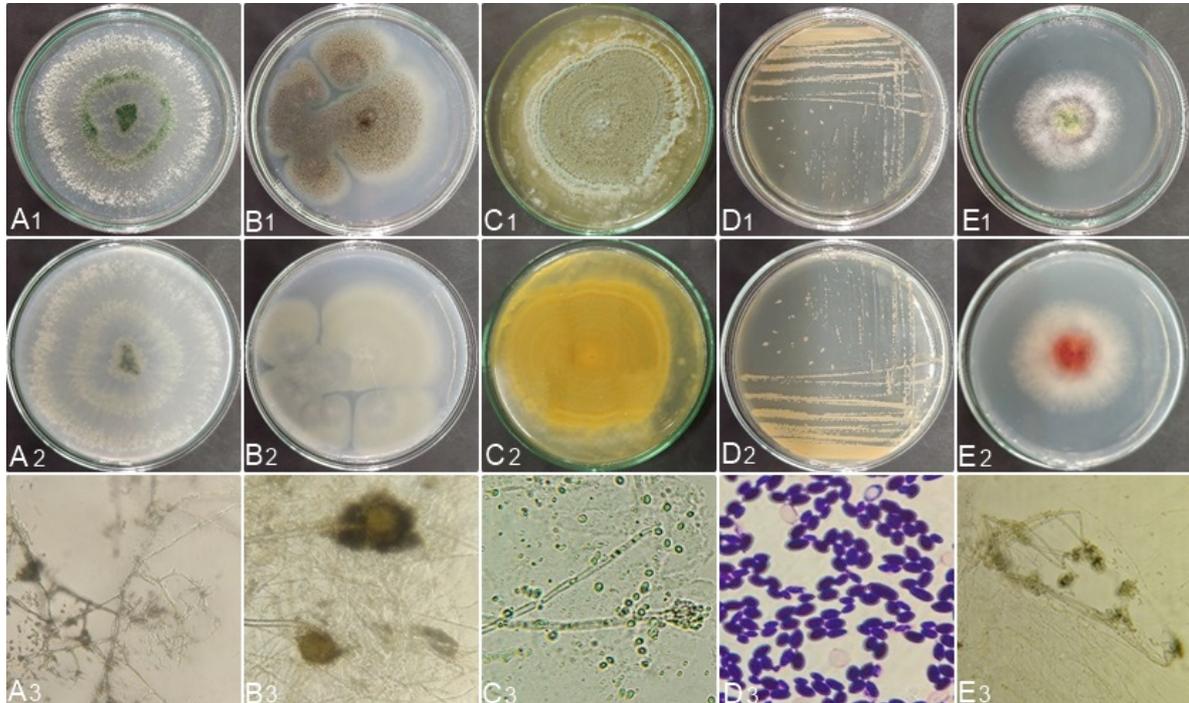


Figure 3. The morphological types of microorganisms formed on explant surface disinfection by sterilized distilled water after 1 week of culture. **A:** *Trichoderma* sp.; **B:** *Fusarium* sp.; **C:** *Pseudomonas* sp.; **D:** *Penicillium* sp.; **E:** *Aspergillus* sp.; **1:** Upper side of culture plates; **2:** Lower side of culture plates; **3:** Mycelium.

In general, concentrations of AgNPs of 50 ppm - 300 ppm gave high disinfection efficiency for all three types of explants from the flower stalks, petioles and stems. Those AgNPs concentrations showed equal and higher disinfection efficiency compared with sterilizing the explants with 0.1% Ca(ClO)₂ and 60 g/L HgCl₂ after 28 days of culture. However,

when the concentration of AgNPs increased to 400 ppm, many explants were not contaminated with microorganisms but the samples browned to black and necrosis. Hence, the survival rates of all types of explants from, tTCL – F (73.33%), tTCL – P (62.20%) and tTCL – S (73.33%) were low in high concentrations of AgNPs (Tables 1, 2 and 3).

Table 1. The survival percentage of tTCL – F explants derived from AgNPs, HgCl₂ and Ca(ClO)₂ sterilization.

Disinfectants	Concentration	The percentage of survival (%)				
		3 rd day	7 th day	14 th day	21 st day	28 th day
AgNPs	50 ppm	100.00a	95.53a	93.33a	91.13a	88.87ab
	100 ppm	100.00a	97.80a	97.80a	95.53a	91.13a
	200 ppm	100.00a	95.53a	93.33a	93.33a	93.33a
	300 ppm	100.00a	100.00a	97.80a	93.33a	93.33a
	400 ppm	100.00a	82.20b	77.80b	75.53b	73.33c
HgCl ₂	0.1%	100.00a	95.53a	95.53a	95.53a	93.33a
Ca(ClO) ₂	60 g/L	100.00a	82.20b	82.20b	80.00b	77.80bc

Note: *Different letters shown in the same column represent significant differences at $p < 0.05$ in Duncan's test.

Table 2. The survival percentage of t-TCL-P explants derived from AgNPs, HgCl₂ and Ca(ClO)₂ sterilization.

Disinfectants	Concentration	The percentage of survival (%)				
		3 rd day	7 th day	14 th day	21 st day	28 th day
AgNPs	50 ppm	100.00a	77.80c	75.53c	68.87de	60.00c
	100 ppm	100.00a	95.53a	82.20ab	77.80abcd	75.53ab
	200 ppm	100.00a	88.87ab	82.20ab	80.00abc	75.53ab
	300 ppm	100.00a	88.87ab	84.47ab	82.20ab	80.00a
	400 ppm	100.00a	77.80c	75.53bc	71.13cde	62.20c
HgCl ₂	0.1%	100.00a	95.53a	86.67a	84.47a	71.13b
Ca(ClO) ₂	60 g/L	100.00a	82.20bc	75.53bc	73.33bcd	62.20c

Note: *Different letters shown in the same column represent significant differences at $p < 0.05$ in Duncan's test.

Table 3. The survival percentage of t-TCL-S explants derived from AgNPs, HgCl₂ and Ca(ClO)₂ sterilization.

Disinfectants	Concentration	The percentage of survival (%)				
		3 rd day	7 th day	14 th day	21 st day	28 th day
AgNPs	50 ppm	100.00a	84.47bc	80.00bc	77.80bc	77.80ab
	100 ppm	100.00a	91.13ab	86.67ab	86.67ab	84.47ab
	200 ppm	100.00a	91.13ab	88.87a	88.87a	86.67a
	300 ppm	100.00a	91.13ab	88.87a	88.87a	86.67a
	400 ppm	100.00a	80.00c	77.80c	75.53c	73.33b
HgCl ₂	0.1%	100.00a	88.87ab	84.47abc	77.80bc	77.80ab
Ca(ClO) ₂	60 g/L	100.00a	95.53a	91.13a	86.67ab	84.47ab

Note: *Different letters shown in the same column represent significant differences at $p < 0.05$ in Duncan's test.

Embryogenic callus formation

The effect of various disinfectants (AgNPs, HgCl₂ and Ca(ClO)₂) on the ability to explants induction was recorded after 28 days of culture (Table 4).

In general, all treatments induced embryogenic callus (Table 4 and Fig. 5). Using 200 ppm and 300 ppm AgNPs to sterilize all explants gave the highest percentage of embryogenic callus formation.

For tTCL – P explants, 100 ppm to 300 ppm AgNPs gives the highest proportion of embryogenic callus formation (35.53% - 37.8%), higher than that of 0.1% HgCl₂ and 60 g/L Ca(ClO)₂ (17.8% and 22.20%, respectively) (Table 4 and Fig. 4).

The concentration of AgNPs increased from 50 ppm - 200 ppm, showed an increase in the proportion of embryogenic callus formation in the tTCL – S and tTCL – F explants; the highest was 40% in both cases. Increasing the concentration of AgNPs from 200 ppm to 300 ppm, the results showed a negligible change in the proportion of embryogenic callus formation in both these types of explants (42.20% and 35.53%, respectively). However, at the 400 ppm AgNPs, the proportion of embryogenic callus

formation was not high (20.00%) in flower stalk explants (Table 4 and Fig. 4).

From the results noted above, it is shown that AgNPs is effective for disinfection of samples and explant induction. However, each type of explants responds differently to the appropriate concentration of AgNPs. The effectiveness of AgNPs depends on the combination of chemistry, size, surface coverage, chemical interactions, concentrations as well as the plants used (Syu *et al.*, 2014). The application of AgNPs in plant tissue culture to prevent microbial contamination was first reported by Abdi *et al.* (2008). The authors' research was conducted on *Valeriana officinalis* L. under greenhouse conditions to reduce the likelihood of *Xanthomonas* sp. Research on resistance to many microorganisms of AgNPs in culture is still very limited, so this is a prerequisite study to add more data on the microorganism resistance of AgNPs.

Tung *et al.* (2018) used AgNPs in micoponic culture, the results showed that AgNPs plays a role in increasing plant growth as well as reducing a number of microorganisms (eight bacterial species and three fungal species) in the micoponic medium.

Table 4. The effect of AgNPs, HgCl₂ and Ca(ClO)₂ on the embryogenic callus formation after 28 days of culture.

Disinfectants	Concentration	tTCL - P	tTCL - S	tTCL - F
AgNPs	50 ppm	22.20bc	13.33e	20.00bc
	100 ppm	37.80a	22.20cd	24.47b
	200 ppm	37.80a	40.00a	40.00a
	300 ppm	35.53a	42.20a	35.53a
	400 ppm	28.87b	28.87bc	20.00bc
HgCl ₂	0.1%	17.80c	20.00de	13.33c
Ca(ClO) ₂	60 g/L	22.20bc	31.13b	22.20bc

Note: *Different letters shown in the same column represent significant differences at $p < 0.05$ in Duncan's test.

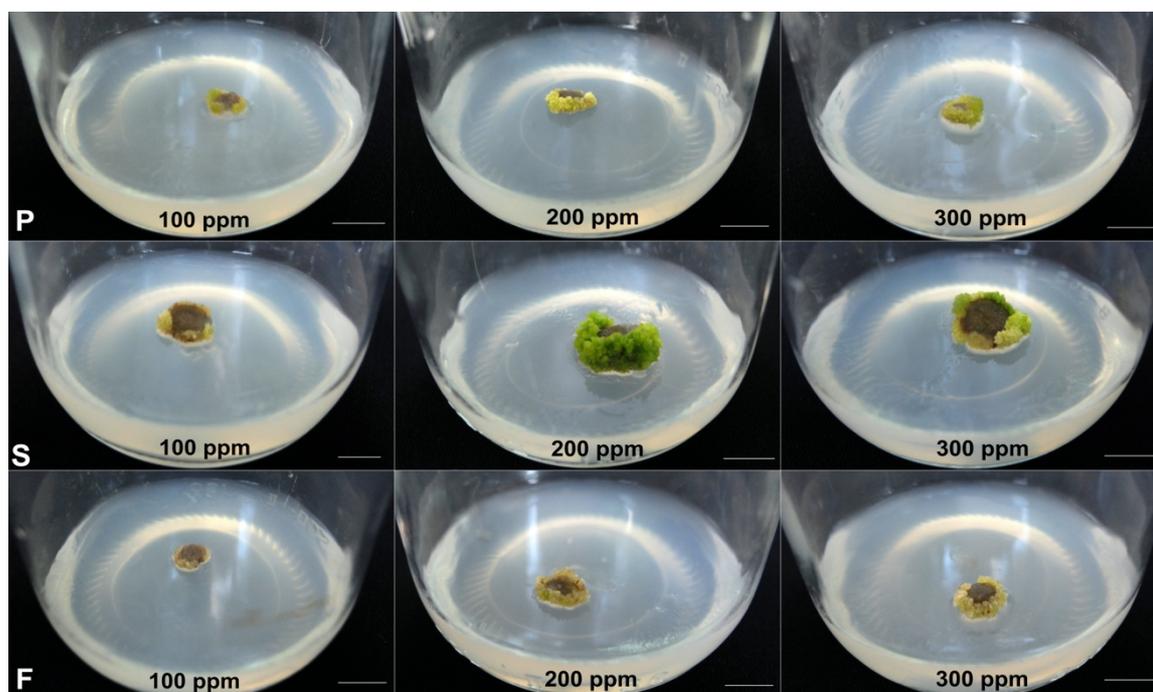


Figure 4. The effect of AgNPs and HgCl₂ on somatic embryogenesis of different explants after 28 days of culture. **P:** Peptiole; **S:** Stem; **F:** Flower stalk (Bars: 1 cm).

Disinfection of *ex vitro* leaves, flower stalks and stems with HgCl₂ disinfectant, the recorded results show that the explant induction only reach about 30 - 35% (Nhut *et al.*, 2005). Meanwhile, the results of this study show that AgNPs has higher disinfection efficiency (40 - 42%) at 200 - 300 ppm AgNPs in all 3 different

types of explant sources. Recently, research by Khiem and Hau (2018) showed that disinfection of leaf with Ca(ClO)₂ for 10 min gave a survival rate of 24.3% which is much lower compared to those with AgNPs (> 90%).

AgNPs has been shown to be effective in disinfecting cultures on several crops such as

African violet (Nhut *et al.*, 2018), passion fruit (Hieu *et al.*, 2019), seaweed (Mo *et al.*, 2020), strawberry (Tung *et al.*, 2021a), chrysanthemum (Tung *et al.*, 2021b). In this study, sterilizing explants with AgNPs has shown a higher effect on the survival rate as well as the explant

induction compared to those with traditional disinfectants. Through this investigated, the recorded results provide a new direction in plant micropropagation, which is to optimize the *ex vitro* explant sterilization stage by using AgNPs as a disinfecting agent.

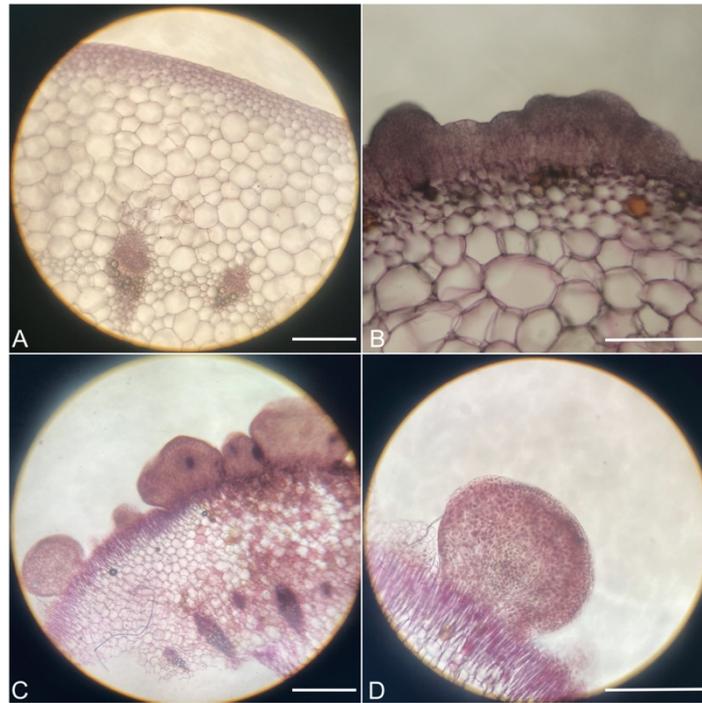


Figure 5. The morphology of embryogenic callus derived from AgNPs treatment. **A:** Original explant at day 0, **B:** Embryogenic callus at day 14th; **C, D:** Globular embryos at day 28th (Bars: 1 mm).

Somatic embryogenesis

The different shapes of somatic embryos derived from AgNPs and HgCl₂ treatment was recorded after 42 days of culture (Table 5 and Fig. 6). After 1 week of culture, all treatments formed somatic embryos (100%). However, there were differences in number of somatic embryos at different shapes (Table 5).

The total number of embryos of embryogenic callus derived from the tTCL – P, tTCL – F and tTCL – S sterilized with AgNPs was higher than those sterilized with HgCl₂ (Table 5). Then, internode and flower stalk-derived explants sterilized with AgNPs were highest the total number of embryos (34.33 and

36.33 embryos; respectively) as well as cotyledon shape (12.33 and 14.33 embryos, respectively) (Table 5).

This is the first study to describe somatic embryogenesis with full stages such as globular, heart, torpedo and cotyledon. Research by Nhut *et al.* (2005, 2010) only described the process of shoot regeneration from different explant sources. The results of this study are significance in demonstrating the morphogenesis of the explants via the somatic embryogenesis pathway as well as the role of AgNPs in increasing the efficiency of embryogenesis as well as the short-time of somatic embryogenesis with explant sources sterilized by common disinfectant.

Table 5. The somatic embryogenesis shapes of AgNPs derived explants after 42 days of culture.

Explant	Treatment	The percentage of somatic embryogenesis (%)	Total**	Somatic embryo shapes			
				Globular	Heart	Torpedo	Cotyledon
P	HgCl ₂	100a*	29.33bc	8.67b	7.67b	6.33c	6.67c
	AgNPs	100a	32.00b	8.33b	5.00d	7.67ab	9.00b
S	HgCl ₂	100a	30.67bc	10.33a	7.00bc	6.67bc	7.00c
	AgNPs	100a	34.33ab	6.33d	5.00d	10.67a	12.33a
F	HgCl ₂	100a	29.33bc	7.67bc	9.67a	6.33c	5.67cd
	AgNPs	100a	36.33a	4.33e	9.67a	8.00ab	14.33a

Note: *Different letters shown in the same column represent significant differences at $p < 0.05$ in Duncan's test. **The total number of embryos was the sum of the number of embryos at the all shapes (Globular, Heart, Torpedo and Dicotyledon)

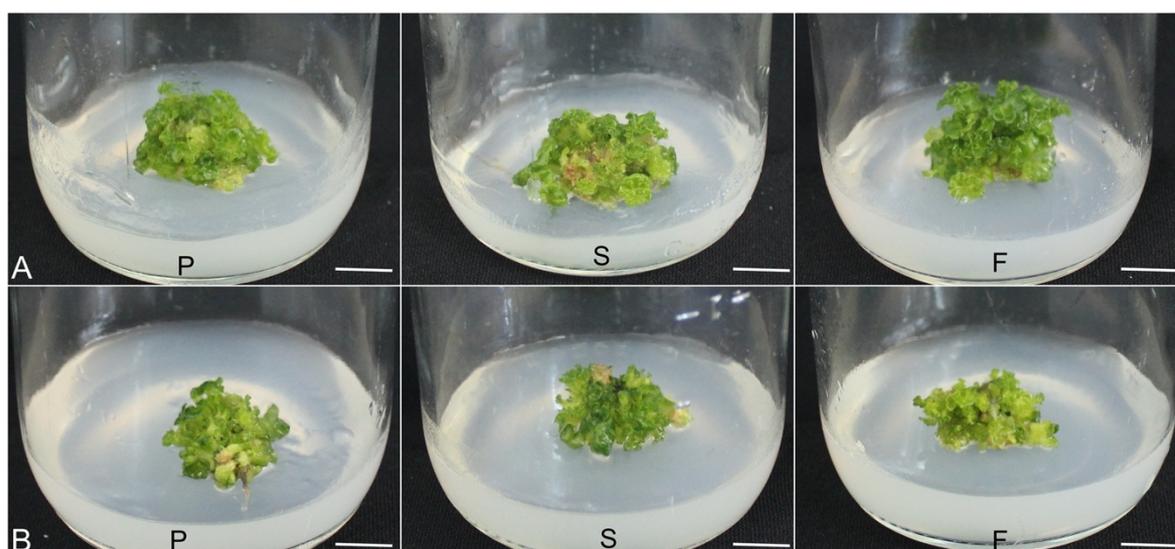


Figure 6. The effect of AgNPs and HgCl₂ on somatic embryogenesis of different explants after 42 days of culture. **A:** AgNPs; **B:** HgCl₂; **P:** Peptiole; **S:** Stem; **F:** Flower stalk (Bars: 1 cm).

CONCLUSION

In this study, AgNPs could be replaced the HgCl₂ and Ca(ClO)₂ in explant surface disinfection. The optimal embryogenic callus formation rate of tTCL – F and tTCL – P was 200 – 300 ppm AgNPs-sterilized; meanwhile, those of tTCL – S were 100 – 300 ppm AgNPs-sterilized. Moreover, AgNPs was effective in enhancing the frequency of somatic

embryogenesis with full embryos shapes (globular, heart, torpedo and cotyledon) as compared with Ca(ClO)₂ and HgCl₂.

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NANO BẠC NÂNG CAO HIỆU QUẢ KHỬ TRÙNG MẪU CÂY VÀ PHÁT SINH PHÔI SOMA CÂY THU HẢI ĐƯỜNG (*BEGONIA TUBEROUS*) THÔNG QUẢ NUÔI CÂY LỚP MỎNG TẾ BÀO

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TÓM TẮT

Thiết lập mẫu cây *in vitro* là một trong những giai đoạn quan trọng nhất trong vi nhân giống. Hiệu quả khử trùng thì phụ thuộc vào loại chất khử trùng bề mặt, nồng độ và thời gian xử lý. Trong nghiên cứu bước đầu này, nano bạc (AgNPs) đã được sử dụng như một chất khử trùng mẫu cây cuống lá, phát hoa và đoạn thân của cây Thu hải đường (*Begonia tuberosus*). Bên cạnh đó, kỹ thuật nuôi cấy lớp mỏng tế bào (TCL) đã được ứng dụng cho mục đích phát sinh phôi soma. Kết quả cho thấy AgNPs có hiệu quả trong việc loại trừ các vi sinh vật gây nhiễm trên các mẫu *B. tuberosus* và đã xác định được 4 loài nấm (*Fusarium* sp., *Aspergillus aculeatus*, *Trichoderma* sp. và *Penicillium* sp.) và 1 loài vi khuẩn (*Pseudomonas* sp.). Ở nồng độ 200 ppm và 300 ppm, AgNPs không chỉ hiệu quả trong việc khử trùng mà còn tăng tỷ lệ cảm ứng phát sinh phôi soma từ mẫu cây TCL cuống hoa (xấp xỉ 40,00%); hiệu quả tương tự đã được ghi nhận trên mẫu TCL đoạn thân ở cùng nồng độ. Trong khi đó, đối với mẫu cây TCL cuống lá thì tỷ lệ cảm ứng sinh phôi soma đã đạt tối ưu khi sử dụng AgNPs ở nồng độ từ 100 - 300 ppm để khử trùng mẫu cây. Ngược lại, ở nồng độ cao (400 ppm) hay thấp (50 ppm) thì AgNPs không thể hiện vai trò khử trùng và kích thích sự tái sinh mẫu cây. Bên cạnh đó, các mẫu có nguồn gốc từ khử trùng AgNPs thì không có bất kỳ sự khác thường nào trong quá trình phát sinh phôi soma với các dạng như hình cầu, hình tim, thủy lôi và hai lá mầm. AgNPs cho hiệu quả kép trong việc khử trùng mẫu cây và cải thiện hiệu quả phát sinh phôi soma từ các mẫu cây TCL cuống lá, phát hoa và đoạn thân; do đó tăng hiệu quả vi nhân giống *B. tuberosus*.

Từ khóa: Nano bạc, khử trùng, Thu hải đường, phát sinh phôi soma.