EFFECT OF $AGNO_3$ NANOPARTICLES ON STERILIZATION AND ALKALOID PRODUCTION OF *CATHERENTHUS ROSEUS* TISSUE BY

Heba Shahin

FROM

Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City University, Sadat City, P.O. Box 79/22857, Egypt. Email: heba.shahin@ ebri.usc.edu .eg

Abstract

Bacterial contamination is a serious problem in plant tissue culture procedures.

An experiment has been conducted to evaluate the potential of nanosilver (NS) to remove bacterial contaminants from Catherenthus shoot tips and single nodes.

The experiments were performed as a totally randomized design in a faculty with four replications and each replicate with ten explants. Treatments involved NS in two steps (before and after surface sterilization along with control) at three concentrations (25, 50 and 100 mg 1^{-1}) at three times (30, 45 and 60 minutes). Plants were grown on MS medium supplemented with 5 mg $l^{-1}$of Kinetin (KIN) and 0.1 mgl^{-1} of naphthaline acetic acid (NAA). The results showed that the use of 100 mg l^{-1} NS solution after sterilization of the surface resulted in the highest percentage (89%) of disinfected explants. Nano silver solution did not affect the measured characters. According to the data obtained in the experience, it was concluded that NS had a good view to eliminate bacterial diseases in plant tissue culture procedures. Further research on other cultural species requires that in the tissue cultures of other cultures, to enhance the effectiveness of the NS in order to eliminate bacterial diseases.

Key words: nano silver, *Catherenthus roseus*, contamination, alkaloids

Introduction

A technique of plant tissue culture that enables rapid production of many genetically identical plants by small space, supplies and time **(Odutayo et al., 2004)**. The development of micropropagation techniques has been achieved to determine rapid multiplication. The elimination of exogenous and endogenous contaminating microorganisms is a high demand for tissue culture of all plants **(Buckley et al., 1994, Constantine 1986**). Different methods for elimination of contamination in vitro propagation have been developed because contamination is a major challenge for plant tissue culture. **(Barrett and Casselles, 1994, Herman, 1996)**. The contamination in in vitro plants by fungi, bacteria and yeast is one of the most serious problems in commercial and research laboratories for plant tissue. Contaminated plants can reduce multiplication and rotational speed or cost, effort and time. It is necessary to remove foreign impurities, including bacteria and fungi, from explanted substances, and it is very difficult to get sterile plant material completely free of contamination. (**Mihaljević, et al., 2013**)

Antiviral activity of silver ions is registered and interaction with -SH groups has been involved in the behavior **(Thurmann and Gerba 1989)**. Silver ions clearly have no way of operating. Within the microorganisms, they interact with a wide variety of

molecular processes that result in various effects of growth inhibition and loss of cell death. The mechanism depends on the concentration of silver ions present and the susceptibility of microbial species to silver.

Both the frequency and the extent of antimicrobial activity can be influenced by contact time and temperature. **(Dibrov et al., 2002)**. Use of antibiotics in media has a mutation risk or may exhibit in vitro inhibitory effects. Nanosilver has antimicrobial effects at low concentrations.

Plants are a rich source of various bioactive secondary metabolites, which play a significant role in the survival of plants in their respective environments. The addition of nano particles (NPs) to the plant in vitro culture medium may act as a nutrient source and an elicitor. (**kim et al., 2017**).

The current study was conducted to assess the potential for NS to eliminate fungal and bacterial contaminants in *Catherenthus roseus*, which showed high value due to the active ingredients with a high anticancer effect.

Material and Methods

Nano silver preparation

Synthesis of the solution of nanoparticles: the silver nano-particles were synthesized in aqueous phase with the addition of double distilled water. All chemicals were purchased from Sigma chemicals and were used as received. For agar particle compositions, 10^{-3} M solutions of $AgNO_3$ were reduced with 10^{-3} M $NaBH_4$ in doubly distilled water. Tween-20 was added as a surfactant to prevent particle aggregation. (**Mazumdar, 2014**)

Plant material, sterilization and preparation of explants.

Plant Materials

The experiment was conducted at plant biotechnology labs in Genetic engineering and research biotechnology Institute (GEBRI) labs during 2018.

In this study, we selected *Catherenses roseus* that collected from the field and has high bacterial and fungal contamination percentage in vitro. Explants included single nodes and apical shoots.

Explants were submerged in 70% ethanol for 0.5 min and after washing three times in distilled water they were submerged in 10 % commercial Clorox (containing 5.25% sodium hypochlorite) for 5 min.

Disinfection treatments with nano-silver

Nano-silver particles were used as disinfecting means after surface sterilization by ethanol and Clorox. Nano-silver treatments include two parts:

(1) Submerged explants in nano-silver solutions.

Explants were submerged in 0, 25, 50 and 100 mg/L nano-silver solutions at three times (30, 45 and 60 minutes). (**Abdi et al., 2008**).

(2) Different amount of nano-silver (0, 5, 25, 50, 75 and 100 mgL^{-1}) were added to media and then media were autoclaved. Four weeks after culture the percentage of infection and developed explants were recorded.

Culture media

The explants were cultured aseptically on basal solid **Murashige and Skoog** (1962) MS-medium with several treatments. The pH was adjusted to 5.7 with 1 N KOH or 1 N HCl before adding gelrite and prior to autoclaving at 121 ˚C (0.1 MPa) for 20 min. The cultures were kept in a growth chamber at 22 ± 1 ˚C, and a photoperiod of 16 h (30 µE m-2s-1, Philips TL 33 light).

The cultivation was done in 300 ml glass jars containing 50 ml of basal MS-medium. Plants were grown on MS medium supplemented with 5 mg $l^{-1}$of Kin and 0.1 mgl^{-1} of naphthaline acetic acid (NAA).

Modification of plant tissue culture media by nanomaterial

In this stage, we don't undergo sterilization with nano solutions and only 1% alcohol and 10% NaOCl then washed with distilled water. We added different amounts of nano silver to tissue culture media. Experiment involved NS with five rates (0, 5, 25, 50, 75 and 100 mg/l) in MS medium. Explants were cultured on MS medium and evaluate at four times (1, 2, 3 and 4 weeks). The rate of growth proliferation was studied.

Estimation of chlorophyll a, b and total chlorophyll.

A defined volume (3 ml) of the crushing plant was centrifuged at a rate of 8000 rpm for 10 minutes; then the granules of the plants were treated with 3 ml of ethyl alcohol and kept in a water bath for 30 minutes and then centrifuged again. The color of the granules should be white to ensure maximum pigment extraction. The absorbance of the combined extracts was recorded on a Unico UV-2000 spectrophotometer at 650, 665 and 452 nm.

Total alkaloid content

Total alkaloid content was estimated in dry leaf powder as described by **Misra and Gupta (2006).** Freshly harvested leaves were baked at 70 ° C for 72 hours and pulverized. Five hundred milligrams of fine leaf powder were extracted into 90% ethanol. The mixture was then filtered and concentrated to dry. The dried residue was redissolved in ethanol and diluted with the same volume of water, followed by addition of dilute HCl (3%). The mixture was extracted (3 times) by transferring it to a separatory funnel to which hexane was added. This mixture was stirred for 15-20 minutes. The lower water layer was decanted in a beaker and made basic by adding 3% ammonium hydroxide to pH 8.5 and cooled to 10 ° C. Then, the mixture was transferred back to a separatory funnel which added chloroform (three times). The contents were stirred for 15-20 minutes. The lower layer was discarded and the upper chloroform layer was decanted. The decanter was transferred to a pre-weighed dry porcelain plate and then evaporated to dryness. Total alkaloids expressed as a percentage of the alkaloid content in the dried leaves.

2.8. Statistical analysis

Each treatment consisted of 40-50 polypropylene jars (5 cm high) with 1 explant per jar. In the graphs, we show percentages and means ± SE. The Student *t*-test and the χ2-test were used to estimate the significance of differences with respect to means and percentages, respectively. The experiments were repeated 3 times.

Results and Discussion

The NS solution was successful after surface sterilization. The most effective disinfection treatment is the treatment by 100 mg l^{-1} NS for 60 minutes after the explants have been rinsed in sterilized distilled water. This treatment differed significantly from other treatments. Moreover, this treatment had no negative effect on the micro-propagation of the different subcultures (Fig. 2).

Usually, after surface sterilization, the NS solution had an acceptable effect on the control of bacterial contaminants without adversely affecting the growth of *Catherenthus* micropropagation. However, it was not effective to control the fungi in this experiment. The differences between the effects of NS treatment before and after sterilization on the surface may be due to the fact that the NS solution in NS is directed towards the medium after sterilization towards the end of the explants.

After cutting, the sterilized explants were immersed into the NS solution before being introduced into the culture vessel. Before the surface is sterilized, the explants are washed with distilled water and transferred to the medium prior to surface sterilization.

Our results showed that low concentrations used and NS low exposure times were effective in decontamination of Catherenthus explants. The results of this study (Table 1) showed that the addition of nanosilvers to the tissue culture medium significantly reduces bacterial contamination compared with the control. The use of the 100 mg/l concentration of SNps in MS medium eliminated bacterial contamination (0.0%). The increase in the amount of nanoparticles in the medium from 5mg/l to 25mg/l significantly reduced the rate of bacterial contamination from 66.70% to 33.30% and then to 16.30% when 75 ml was added. However, the overall elimination of fungal infection was not achieved even at the highest level of AgNP (100 mg/l) used, but the use of AgNP showed a decrease in fungal growth compared with the control whereas 100 mg/l significantly reduced fungal infection rate from (83.3% to 33.3%) (Table 1).

It is apparent from this study that higher concentrations of AgNP are required to effectively control fungal growth. This is also in line with what has been reported in previous studies, such as a concentration of 200 mg/ l.

Silver and its compounds have long been used as antimicrobial agents (**Brown and Anderson 1968, Russell and Hugo 1994, Herman 1996 and Abdi, et al., 2008).** The most important silver compound currently used is silver sulfadiazine (AgSD), although silver metal, silver acetate, silver nitrate and silver protein also have an antimicrobial effect.

The use of AgNO3 as a silver compound against infection in tissue culture is common (**Herman 1996).** Ag NP has successfully controlled bacterial and fungal infection without any adverse effect on regeneration of lemongrass plantations (**Fakhrfeshani et al., 2012**). It has also been reported that NS can be an effective tool for eliminating contaminants from tissues only if the correct dose and exposure time are

used (**Mahna et al., 2013**). However, NS has not yet become a universal decontaminant and our result will contribute to future research to find an effective way to control microbial contamination in the plant tissue culture.

However, in the NS solution treatment, they were washed prior to the surface sterilization explants with distilled water and then transferred to the medium. One method has been proposed by **Salehi and Khosh-Khui (1997)** to control bacterial contamination in C. *rotheus*. They used the gentamicin solution after surface sterilization. The use of NS can be more comfortable and less toxic than using antibiotics in the medium. Furthermore, the use of other methods of controlling infection as the first medium acidification and subsequent pH adjustment to normal conditions (**Leifert et al., 2000**) and filtering the microbial culture (**Hussain et al, 1994)** may be methods slow in tissue culture techniques. To show acceptable antibacterial activity in this study is consistent with the findings of other researchers (**Nomiya et al., 2004, Sondi and Salopek-Sondi 2004).**

Fig 1. Effect of a nano-silver at increasing concentrations in media on contamination and survival (A) *C. roseus.* As explants, apical shoot cut from field-grown were used and contaminated. (B) and on regeneration (=explants with shoots). Regeneration percentages were calculated from explants without contamination.

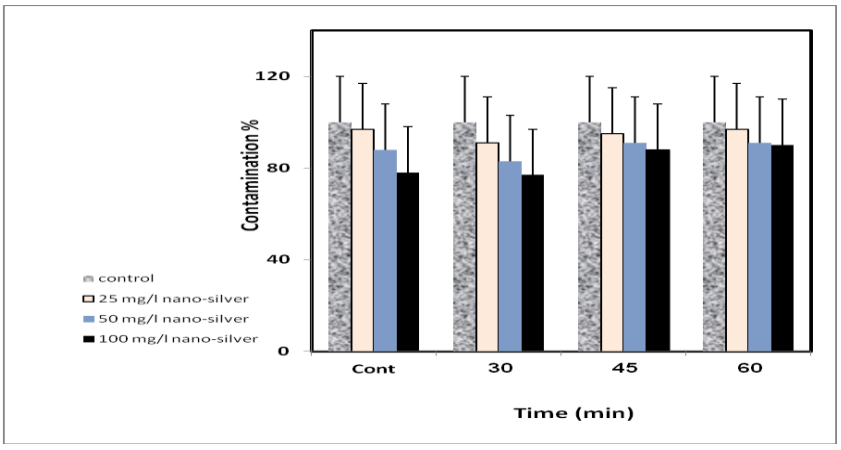

Fig. 2 Effect of different concentrations of NS solution at different time treatments before surface sterilization on contamination %. The data are the mean ± SD of 5replicates.

Table 1: Effect of AgNPs added to the culture media on contamination % with bacterial and fungal contamination. The data are the mean ± SD of 5replicates.

	Nano silver concentration mg/l					
Contamination %	**control**	**5**	**25**	**50**	**75**	**100**
Fungal contamination	**83.3±4.5**	**83.3±11.2**	**66.05±9.34**	**34.5±3.4**	**26.4±1.7**	**0.0**
Bacterial contamination	**83.3±6.43**	**66.7±12.5**	**66.7±7.22**	**33.3±2.66**	**0.0**	**0.0**

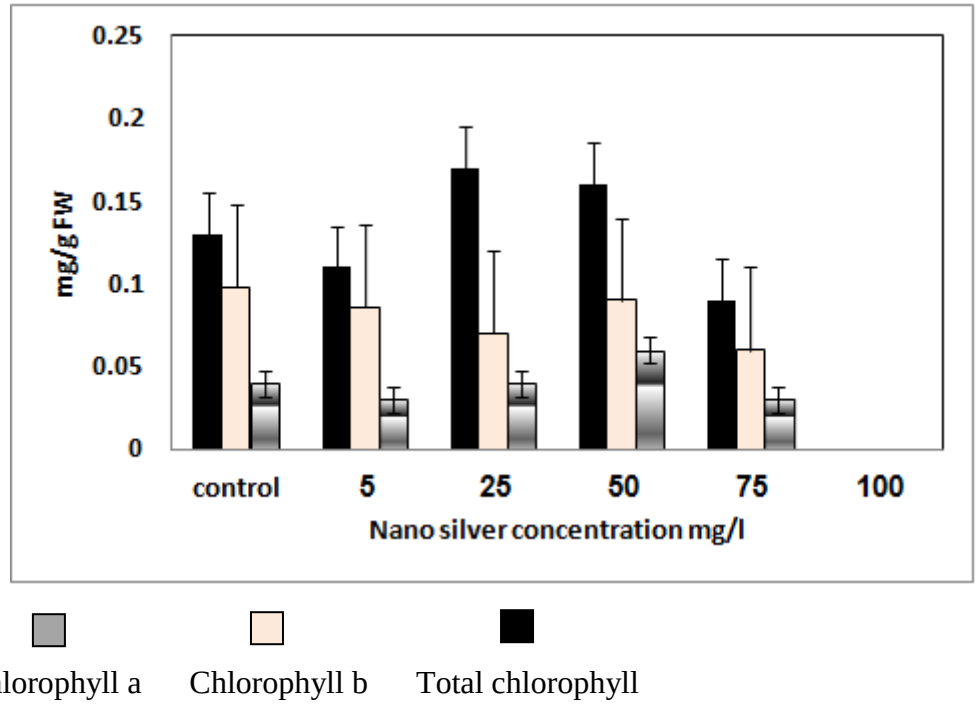

Figure (3): The effect of nano silver concentration on the chlorophyll (mg g-1 FW) content of Catharanthus roseus (L.). The data are the mean ± SD of 5replicates.

Chlorophyll a content in *in vitro* shoots decreased in 5 and 75 mgl^{-1} treatments comparing to control, while significantly increased by application with nano-Silver at 50 mgl^{-1} (Fig.3). All nano-Silver concentrations had no significant effects on chlorophyll b content. Total chlorophyll significantly increased (<0.001) comparing to control. (Fig. 3) applications of AgNPs improve total chlorophyll and increased chlorophyll content, thus providing a substantial improvement in photosynthesis.

Vegetable green chlorophyll in plant, algae and cyanobacteria, this biomolecule is considered to be the most important molecule of photosynthesis, enabling the plants to absorb energy from light. **(Abdolsamad et al., 2015)**.

AgNPs were used for changes in the content of biomolecules such as chlorophyll and *β*-carotene in plants and algae. Silver NPs are capable of producing a number of reactive oxygen species, **Rippka, 1972**, which causes oxidative damage to cells by lipid peroxidation and oxidation of thiol groups into the protein and DNA. (**Park et al., 2014).**

Alkaloid content

Total alkaloid content significantly increased in plants challenged with nano silver at 25 mg/l and 5mg/l (0.26 – 0.18 mg/l respectively) . whereas total alkaloid content of explants grown under higher treatment 75 and 100 mg/l with no detection for any alkaloids. Treatment with 50mg/l, however, had no significant effect on total alkaloid content (Table 2).

The contents of secondary metabolic products cultured cells and organs significantly increased and optimized the culture medium composition, incorporation of precursors and elicitor, and supplying apropriadas.83 culture conditions, 88-90 NP from the in vitro culture of the plant can be used as a source of food and as a helper. **Poborilova et al**. (2013) reported that the added culture of TNF Al_2O_3 (10 and 100 mg m/l) of tobacco cell suspension cultures significantly increased the phenolic content. The accumulation of phenols in cells was dose-dependent and the duration of exposure. **Zhang et al**. **(2013)** investigated the possible substitution of Ag NP to increase the artemisinine content of hairy root cultures at *Artemisia annua.* Artemisinin production increased 3.9 times when cultures were treated with 900 mg/l Ag NP for 3 days. Likewise, the highest content of artemisinine (2.2-fold increase versus control) was obtained from cell suspension cultures at 24-hour A 24 hours post-treatment 5 mg/ L Co NPs (**Ghasemi et al., 2015**). Conclusion Co-NP treatment increased the artemisinin content due to down regulation of SQS and DBR2 genes.

All the above studies confirm that NPs in plant cells and organ cultures are used as successful and promising bioactive compounds. Further studies are needed to assess the potential for the production of secondary metabolites by different NPs content in plant tissue culture and appropriate mechanisms.

Table 2: Effect of different concentrations of nanosilver on alkaloid content % of *Catherensus* explants after four weeks and eight weeks.

Alkaloid %	**Nano silver concentration mg/l**					
	control	**5**	**25**	**50**	**75**	**100**
after 4 weeks	0.18±0.02	0.22±0.03	0.26±0.05	0.21±0.02	0.0	0.0
after 8 weeks	0.21±0.01	0.22±0.06	0.31±0.02	0.18±0.01	0.0	0.0

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تأثير الجسيمات النانوية نترات الفضة على عمليات التعقيم وانتاج القلويات في زراعة الانسجة لنبات الوينكا

للسيدة الدكتورة

هبه شاهين

مـــــن

مدرس بقسم بايوتكنولوجيا النباتية معهد الهندسة الوراثية والتكنولوجيا الحيوية بجامعة مدينة السادات

الملخص العربى

يمثل التلوث الميكروبي مشكلة خطيرة في زراعة الأنسجة النباتية ويتم استخدام تقنيات مختلفة للحد منها.

وقد أجريت تجربة لتقييم إمكانات nanosilver (NS) لإزالة الملوثات البكتيرية من زراعة الانسجة للاجزاء النباتية المختلفة لنبات الوينكا

غالباً ما يتم استخدام المواد النانوية وخصوصاً الفضة النانوية (NS) كعامل مضاد للميكروبات في حقول علمية مختلفة ، بما في ذلك نشر النباتات في المختبر. التجارب المتضمنة NS تتم في خطوتين ، أولا (قبل وبعد التعقيم السطحي جنبا إلى جنب مع السيطرة) في ثلاث تركيزات (٢٥ و ٥٠ و ١٠٠ ملغ /لتر) في ثلاث مرات (٣٠ و ٤٥ و ٦٠ دقيقة). ثانياً ، تمت زراعة النباتات على وسط متوسّط مكمل بتركيزات NS مختلفة في تركيبة مع ٥ ملغ /لتر من كينتين و ٠.١ ملغ / لتر من NAA وأظهرت النتائج أن استخدام محلول ١٠٠ ملغ /لتر من نانو الفضة بعد تعقيم السطح أدى إلى أعلى نسبة (٨٩٪) من الاجزاء المنزرعة النظيفة وكان فعالا تماما للسيطرة على العدوى البكتيرية عند تقييمها بعد أربعة أسابيع من الاستزراع. وفقا للبيانات التي تم الحصول عليها في التجارب ، تم استنتاج أن NS لديها قدرة جيدة للقضاء على الأمراض البكتيرية في تقنية زراعة الأنسجة النباتية NS .يعزز بقوة إنتاج قلويدات عند إضافتها إلى وسط النمو في تركيز ١٠٠ملغ /لتر