Evaluation the potential of Nano silver for removal of bacterial contaminants in valerian (*Valeriana officinalis* L.) tissue culture

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ABSTRACT

An experiment was conducted to evaluate the potential of nano silver to remove bacterial contaminants of valerian tissue culture. Treatments were using nano silver at two stages (before and after surface sterilization along with control) with three rates (30, 60 and 120 mg l^{-1}) at three times of soaking (30, 60 and 180 min). Nodal explants were cultured on MS medium supplemented with different concentration of Kinetin (0, 2, 4 and 5 mg l^{-1}) and 0.1 and 0.2 mg l^{-1} of NAA. Optimum result for plant regeneration was obtained in media containing 4 mg l^{-1} Kin and 0.2 mg l^{-1} NAA. Results showed that using 120 mg l^{-1} of nano silver solution after surface sterilization resulted in the highest percentage (89%) of disinfected explants. Nano silver solution did not affect the characters measured. On the basis of the data obtained in this experiment, it was concluded that nano silver had a good potential for removing of bacterial contaminants in plant tissue culture procedures. As this is the first report on application of nano silver in *in vitro* culture techniques, further investigations on other plant species are needed to clarify the effectiveness of nano silver for removal of bacterial contaminants in tissue culture of other crops.

Key words: Contamination, Explant, Medium, Micropropagation, Nanobiotechnology

INTRODUCTION

The genus *Valeriana* (*Valerianaceae*) encompasses nearly 250 species found mainly in the northern temperate regions. *Valeriana officinalis* L. has been widely use in traditional medicine in Iran as well as in the pharmaceutical industry. Valeriana have sedative, antispasmodic and ansiolitic properties due mainly to their production of iriod esters known as valepotriates, in the plant rhizomes (Nishiya *et al*., 1992). The great interest in valepotriates for therapeutical use and their uncertain and limited supply from field harvesting have led to the investigation of alternatives for the production of the biologically active plant constituents. Plant regeneration has been described from shoot tip and axillary bud explants, from callus and from embryo-like structures derived from suspension cultures of *Valeriana wallichii* (Mathur *et al*., 1988; Mathur and Ahuia, 1991; Mathur, 1992), from adventitious shoots, from seedlings, callus and suspension cultures of *Valeriana edulis* ssp. procera (Enciso-Rodríguez, 1997; Castillo *et al*., 2000) and from shoot buds of *Valeriana jatamansi* (Kaur *et al*., 1999). Successful tissue culture of all plants depends on the removal of exogenous and endogenous contaminating microorganisms (Buckley et al. 1994; Constantine 1986). Fungi and bacteria are the most common microorganisms to be found on or in plant tissues. To eliminate bacterial contamination during *in vitro* propagation different methods have been developed in the last few years (Barrett and Cassells 1994; Hussain et al. 1994). Antibiotics are commonly used in the medium to eliminate unwanted contaminants from plant systems (Smart et al. 1995). Theoretically, it might seem that all contamination problems could be overcome by the incorporation of one or more antibiotics into the culture medium. However, antibiotics are frequently phytotoxic otherwise may retard or inhibit plant tissue growth. Also, prolonged exposure of cells or tissues to antibiotics can result in the development of resistance through genetic change (mutation) in the genes within the organelles (the cytoplasmic genes or cytoplasmic DNA). Most antibiotics have been shown inhibitory effects in the plants. Streptomycin and chloramphenicol are inhibitors of protein synthesis; rifampicin inhibits nucleic acid synthesis, and penicillin inhibits cell-wall membrane synthesis (Pankhurst 1977). Streptomycin alters the sensitivity of chloroplast RNA editing, and at phytotoxic levels, white tissue results, owing to the lack of chloroplast differentiation (Karcher and Bock 1998). Inhibition of the cyclic electron flow in chloroplast photosystem I and mitochondrial ATP production inhibition in tobacco by antibiotics such as antimycin A at as low concentration as 1 mM resulted in malformed and bleached leaves (Horvath et al. 200; Joe¨t et al. 2001). Phytotoxicity of rifampicin, carbenicillin and streptomycin in *Clematis*, *Delphinium*, *Hosta*, *Iris* and *Photinia* cultures is also reported (Leifert et al. 1992). Using antibiotics in the media inhibit both multiplication and rooting of *Delphinium* shoot cultures (Leifert et al. 2000). Teixeira da Silva et al (2003) reported a decrease in explant survival and biomass reduction, malformation of roots and inhibition of shoot formation in chrysanthemum, and also in tobacco endoreduplication by application of antibiotics in the media. Nano silver has shown to have

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antibacterial, antifungal and antivirus effects (Nomiya et al. 2004; Sondi and Salopek-Sondi 2004). Studies have demonstrated that silver ions interact with sulfydryl (-SH) groups of proteins as well as the bases of DNA leading either to the inhibition of respiratory processes (Bragg and Rannie 1974) or DNA unwinding (Batarseh 2004). Inhibition of cell division and damage to bacterial cell envelopes is also recorded (Richards et al. 1984) and interaction with hydrogen bonding processes has been demonstrated to occur (Russell and Hugo 1994). Interruption of cell wall synthesis resulting in loss of essential nutrients has been shown to occur in yeasts (Wells et al. 1995) and may well occur in other fungi. Antiviral activity of silver ions has been recorded and interaction with –SH groups has been implicated in the mode of action (Thurmann and Gerba 1989). Silver ions clearly do not possess a single mode of action. They interact with a wide range of molecular processes within microorganisms resulting in a range of effects from inhibition of growth, loss of infectivity through cell death. The mechanism depends on both the concentration of silver ions present and the sensitivity of the microbial species to silver. Contact time and temperature can impact on both the rate and extent of antimicrobial activity (Dibrov et al. 2002). Using antibiotics in medium as mentioned above have mutation risks or may show *in vitro* inhibitory effects. Therefore, using antibiotics without application in the medium may reduce mutation risks and inhibitory effects of them. Nano silver have antimicrobial effects at low concentrations. However, so far there is little report on using nano silver to eliminate microorganisms in tissue culture procedures. The present study was conducted to obtain an optimum micropropagation procedure and evaluate the potential of nano silver to eliminate fungal and bacterial contaminants in *Valeriana officinali*s L. explants without application in the medium. The effects of nano silver solution (NSS) on growth, proliferation rate and rooting were studied and compared with non nano silver (NS) treated materials.

MATERIALS AND METHODS

Nano silver preparation

The nano-particles used in this experiment were silver particles 35 nm (average) in size. Figure 1 show the TEM micrograph of Ag nano-particles. The base working fluid was pure water. Silver (Ag) nano-fluids were prepared using a two step method. Ag nano-particles were prepared first. They were produced using a catalytic chemical vapor deposition method (Nanocid Company Method). The silver nano-particles were then added to pure water. No surfactant was used in the Ag-nano-fluid suspensions. The mixture was prepared using an ultrasonic homogenizer. Nano-fluid concentrations of 30, 60 and 120 mg l^{-1} were used in this study. Some atomic and physical properties of nano silver used in this study are shown in Table 1.
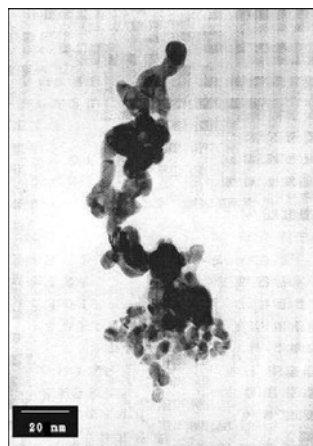
Figure 1. Transmission electron microscopy (TEM) micrograph of Ag nano-particles (35 nm)

Table 1. Properties of nano silver used in this study

Physical properties		Atomic properties	
Density (near r.t.)	10.49 g cm^{-3}	Oxidation states	1 (amphoteric oxide)
Liquid density at m.p.	9.320 g cm^{-3}	Electronegativity	1.93 (Pauling scale)
			1st: 731.0 kJ mol^{-1}
Melting point	1234.93 K (961.78 °C, 1763.2 °F)	Ionization energies	2nd: 2070 kJ mol^{-1}
			3rd: 3361 kJ mol^{-1}
Boiling point	2435 K (2162 °C, 3924 °F)	Atomic radius	160 pm
Heat of fusion	11.28 kJ mol^{-1}	Atomic radius (calc.)	165 pm
Heat of vaporization	258 kJ mol^{-1}	Covalent radius	153 pm
Heat capacity	(25 °C) 25.350 J (mol·K)$^{-1}$	Van der Waals radius	172 pm
Atomic mass	107.8682(2) g mol^{-1}	Crystal structure	Cubic face centred
Electron configuration	[Kr] 4d^{10} 5s^{1}		
Electrons per shell	2, 8, 18, 18, 1		

Plant materials and culture conditions

Greenhouse grown valerian (*Valeriana officinalis* L.) plants were used in this study. These plants were tested by culturing the stem explants in potato dextrose agar (PDA) medium for internal contamination assay. After testing they were divided in two groups:

1- with internal contamination
2- without internal contamination

Group 1: With internal contamination

For group 1, a number of 20-25 cm stems were cut and transferred to the laboratory immediately. They were cut to length of about 0.5-1 cm and prewashed in water supplemented with 10 drops of a weak household detergent solution for 10 min and then placed under running tap water at least for 30 min. Nano silver solution in different concentrations (30, 60 and 120 mg l^{-1}) and exposure times (30, 60, 180, 300, 600 and 1200 min) were used at two stages of before and after surface sterilization along with control. Initial experiment showed that in high exposure times (300, 600 and 1200 min) explants turned to bleach. Therefore, high exposure time's results were omitted.

For the treatment of without surface sterilization, after prewashing in water and running with tap water, nodal segments were dipped at appropriate times and concentrations of NSS. After this treatment, the explants were rinsed 4 times with sterilized distilled water. For the treatment of before surface sterilization, after dipping explants in NSS, the explants were surface sterilized with 70% ethanol for 1 min and 10% Clorox (containing 5.25% sodium hypochlorite) for 1 min and then rinsed 4 times with sterilized distilled water. For the treatment of after surface sterilization, after rinsing with sterilized distilled water, as mentioned before, the explants were dipped in NSS with appropriate concentrations at different times. After recut, the sterilized explants were dipped in NSS before being transferred to the culture vessels. After sterilization, about 1 cm single node explants were cultured on a modified MS (Murashige and Skoog 1962) medium containing salts, organic constituents, 30 g l^{-1} sucrose, 8 g l^{-1} agar and 4 mg l^{-1} Kin and 0.2 mg l^{-1} NAA (Between different treatments Kinetin (0, 2, 4 and 5 mg l^{-1}) and 0.1 and 0.2 mg l^{-1} of NAA, 4 mg l^{-1} Kin and 0.2 mg l^{-1} NAA showed best response and selected for evaluation the effect of different methods of NS application for disinfection on shoot regeneration and rooting of Valerian) (Data not shown) . The pH of media was adjusted to 5.8 by 0.1 N HCl before autoclaving for 15 min at 121 °C and 1.5 kg cm^{-2} pressure. Cultures were kept under a 16h photoperiod of 1.5 klux light intensity emitted by 2 cool white fluorescent lamps at 25±3 °C. For rooting, shoots were cultured in media containg different concentration of NAA (0, 0.5, 1.5 and 2). Between different treatments for rooting 0.5 mg/l NAA showed best response (Data not shown).

Group 2: Without internal contamination

Explants in this group were cut to length of about 0.5-1 cm and prewashed in water supplemented with 10 drops of a weak household detergent solution for 10 min and then placed under running tap water at least for 30 min. Then, the explants were surface sterilized with 70% ethanol for 1 min and 10% Clorox (containing 5.25%

sodium hypochlorite) for 1 min and then rinsed 4 times with sterilized distilled water. Thereafter, they were transferred to MS medium containing salts, organic constituents, 30 g l^{-1} sucrose, 8 g l^{-1} agar and 4 mg l^{-1} Kin and 0.2 mg l^{-1} NAA (Betwen different treatments 4 mg l^{-1} Kin and 0.2 mg l^{-1} NAA showed best response). Other culture condition in this group was similar to group 1.

Data collecting

The percentages of infected explants were recorded 3 days after culture for without surface sterilization treatment. For estimation of size and growth of bacterial and fungal colonies the grades of 1 (the lowest) to 5 (the highest) contamination were given. For other treatments, the percentages of infected explants were recorded 3 weeks after cultures. The experiment was conducted as a completely randomized design in a factorial arrangement with 4 replications and each replicate with 10 explants. Means were compared using Duncan's new multiple range test (DNMRT) at 5% probability level. Impact of the NS on subsequent shoot formation and rooting were assessed in four subcultures with 4 weeks intervals.

RESULTS

Group 1

In general, using NSS without surface sterilization (SS) did not affect contamination. In control, visible fungal contaminations were observed only 3-5 days after culture. While, the bacterial contaminations were observed 7-10 days after culture, in the other treatments, colony appearance was delayed at least 6-8 days and 12-18 days for fungal and bacterial contamination, respectively. The size and growth of colonies varied significantly among treatments. In the control treatment, growth of the colonies was quick. Whereas, in the other treatments depend on exposure time and concentration of NSS the growth was negligible (Table 2).

Table 2. Effects of NSS on appearance, colony size, growth and fungal and bacterial contamination percentages of *Valeriana officinalis* L. single node explants.

Treatments (Time+Concentration)	Contamination (%)		Appearance (day)		Size[¶]		Growth[§]	
	Fungal	Bacterial	Fungal	Bacterial	Fungal	Bacterial	Fungal	Bacterial
Control	100 a[†]	100a	3-5	8-10	5.00a	5.00a	5.00a	5.00a
30 (min)								
30 mg l^{-1}	97 a	98 a	12	6	4.50b	4.75ab	4.50b	4.75ab
60 mg l^{-1}	97 a	97 a	12	6	4.00c	4.00c	4.00bc	4.00c
120 mg l^{-1}	90 b	97 a	14	6	3.50d	4.10c	3.50c	4.00c
60 (min)								
30 mg l^{-1}	91 b	95 a	13	5	3.50d	4.25bc	3.50c	4.25bc
60 mg l^{-1}	91 b	94 a	13	7	3.00e	3.75cd	3.10d	3.75cd
120 mg l^{-1}	89 b	88 ab	15	7	2.40e	3.10d	2.50e	3.00d
180 (min)								
30 mg l^{-1}	90 b	78 c	14	7	2.75e	2.20e	2.75de	2.00e
60 mg l^{-1}	90 b	73 c	17	7	2.00 f	1.10f	2.10f	1.20f
120 mg l^{-1}	88 b	68 d	18	8	1.00g	1.10f	1.00g	1.00f

†In each column, means followed by the same letters are not significantly different using DNMRT at 5% probability level
Ranking from 1, the smallest colony to 5, the largest colony
Ranking from 1, the lowest growth to 5, the highest growth

In general, cultures subjected to NSS treatment before SS showed low percentage of disinfected valerian explants (Table 3). In all the treatments, the percentage of fungal contamination was zero. Among the treatments, highest percentages of disinfection (32%) were observed when the explants were dipped in 120 mg l^{-1} NSS for 180 min.

Table 3. Comparisons among different treatments used for disinfection of *Valeriana officinalis* L. single node explants 3 weeks after culturing on the medium.

Treatments (Time+Concentration)	Before SS Contamination (%)	After SS Contamination (%)
Control	98a[†]	99a
30 (min)		
30 mg l^{-1}	94b	90b
60 mg l^{-1}	94b	91b
120 mg l^{-1}	92b	83c
60 (min)		
30 mg l^{-1}	93b	78c
60 mg l^{-1}	92b	71d
120 mg l^{-1}	85bc	63e
180 (min)		
30 mg l^{-1}	76c	32f
60 mg l^{-1}	76c	29f
120 mg l^{-1}	66cd	11g

†In each column, means followed by the same letters are not significantly different using DNMRT at 5% probability level.

Using NSS after SS was successful. Treatment with 120 mg l^{-1} of NS for 180 min after rinsing the explants in sterilized distilled water was the most successful disinfection treatment. This treatment had significant differences with the other treatments. Also, this treatment did not have any negative impact on measured characters in micropropagation of valerian in four subsequent subcultures.

Group 2
As mentioned above explants in this group did not show any contamination during culture period. Comparing this group with explants obtained from NSS treatment after SS did not show any significant differences in measured characters (proliferation rate, leaf number, percentage of fresh weight, number of rooted explants, number of roots, and root length) in micropropagation of valerian in four subsequent subcultures (Table 4) (Fig 2).

Table 4. comparing the different group on Shoot Regeneration (%),No. of shoots/explant, Average shoot length (cm), No. of Leaves, Root Number/Shoot, Rooted shoot % and Root Length (mm) of Valeriana officinalis at 4th subculture in media containing 4 mg l^{-1} Kin and 0.2 mg l^{-1} NAA.

Group	Shoot Regeneration (%)	No. of shoots/explant	Average shoot length (cm)	No. of Leaves	Root Number/Shoot	Rooted shoot %	Root Length (mm)
Group 1	89.3 ± 2.2 a	9.3 ± 1.2 a	4.1 ± 0.2 a	6.8±0.2 a	10.58±1.2a	100 a	34.7±0.9 a
Group 2	91.3 ± 2.2 a	8.9 ± 1 a	4.4 ± 0.1 a	6.5±0.5 a	10.1±2.2a	100 a	34.7±0.5 a

†In each column, means followed by the same letters are not significantly different using DNMRT at 5% probability level.
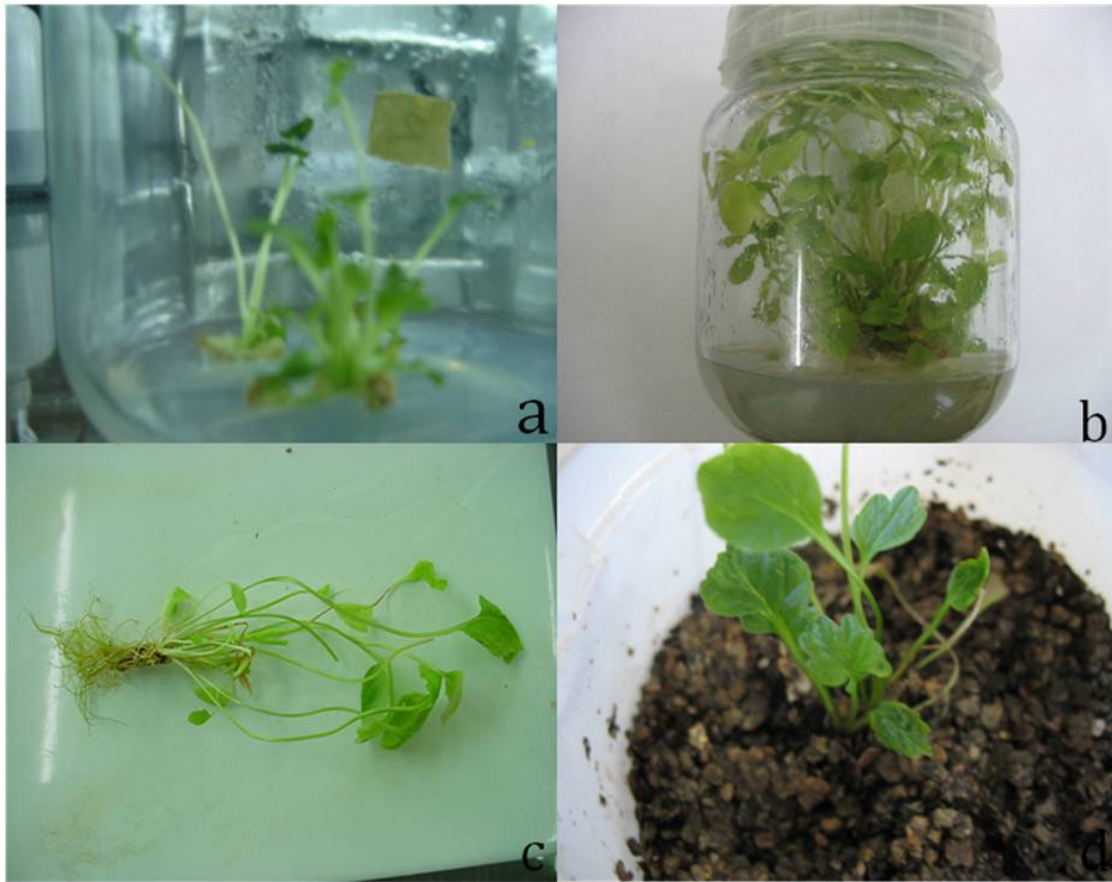
Figure 2. Valerian nodal segments Response to different treatment in selected media. (A and B) The emergence of large number of shoots from the nodal segment on MS medium supplemented with 4 mg l^{-1} Kin and 0.2 mg l^{-1} NAA 25 days after culture. (C) Rooting of a regenerated shoot in B5 medium supplemented with 1.5 mg l-1 NAA 21 days after culture. (D) two-week-old acclimatized plants growing in greenhouse.

DISCUSSION

Silver and its compounds have long been used as antimicrobial agents (Brown and Anderson 1968; Russell 1994). The most important silver compound currently in use is silver sulfadiazine (AgSD), although silver metal, silver acetate, silver nitrate, and silver protein have antimicrobial effect, too. Using AgNO$_3$ as silver compound against infection in tissue culture is common. Our results showed that silver in nano size can similarly control the bacterial infection in tissue culture conditions. Also, subcultures indicated that bacterial contaminations were removed because the late appearance of contamination was not observed in subsequent subcultures. In general, using NSS after SS had acceptable influence on the bacterial contaminants control without any adverse effects on growth characters in micropropagation of valerian. However, it was not effective in controlling the fungi in this experiment. The differences in the effects of NS treatment before and after surface sterilization may be due to presence of nano silver in NSS treatment after SS at the cut end of the explants inside the medium. After recut, the sterilized explants were dipped in NSS before being transferred to the culture vessels. But, in NSS treatment, before SS explants were washed with distilled water and then transferred to the medium. A method has been suggested by Salehi and Khosh-Khui (1997) for controlling bacterial contamination in miniature roses. They used gentamicin solution after surface sterilization. Using nano silver may be more convenient and less toxic than using antibiotics in the medium. Furthermore, using other methods of controlling the infection like first acidification of the medium and later regulation of pH to normal condition (Leifert et al. 2000) and microbial culture filtrate (Hussain et al. 1994) may be time consuming methods in tissue culture techniques. Showing acceptable antibacterial activity in this investigation is in agreement with results obtained by other investigators (Nomiya et al. 2004; Sondi and Salopek-Sondi 2004).

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