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Disinfecting Effects of Nano Silver Fluids in Gerbera (*Gerbera jamesonii*) Capitulum Tissue Culture

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ABSTRACT

Microbial contamination is one of the most important problems in plant tissue culture and various methods are employed to reduce it. Microbial contamination is an important barrier in the development of Gerbera tissue culture and micropropagation procedure even after disinfecting with normal methods. In this experiment the antifungal and antibacterial activity of nano-silver fluids was investigated in gerbera tissue culture and the effects of four different nano-silver concentration (25, 50, 100 and 200mg L^{-1}) in four soaking time of explants (15, 30, 60 and 180 min) were compared with two control treatments that included disinfecting explants with soaking them in Sodium Hypochlorite and Sodium Hypochlorite followed by Mercury Chloride aqueous solution. The explants were cultured on dedicated MS medium to evaluate the effects of NS on viability and other apparent properties. The analysis of variance resulted that there are significant differences in contamination rate, both among treatments and between control and treatments. The 200 mg L^{-1} nano-silver solution had successfully controlled bacterial and fungal contamination and had no undesired effects on regeneration of plantlets. According to reports on high levels of contamination in Gerbera tissue culture and also the hazardous environmental effects of mercury chloride, so nano-silver solution can be used as a low risk fungicide and bactericide in Gerbera capitulum tissue culture.

Key Words: Tissue Culture, Contamination, Gerbera, Nano-Silver particles

INTRODUCTION

To have a successful tissue culture process many controlled agents is needed, medium content, explant age, growth condition of parental plants and so on. However it has been reported that even with controlling all of these agents, microbial contamination from internal or external pathogenesis, cause defect to the process of tissue culture. Although many methods like sterilizing instruments, medium and plates under high temperature and pressure (autoclave) or disinfecting explants using Sodium hypochlorite, Mercury chloride or alcoholic solutions are applied to eliminate the problem of pathogens but because of the various methods of resistance in pathogens, after a period of time contamination outbreaks again. In the most of these cases antibiotics are used to diminish the problem (Smart et al. 1995, Kharrazi et al. 2011)). Despite of succession of this method in many cases, but because some of the biological processes that antibiotics affect on, are similar in explants and microorganisms, unexpected side effects like reduction of growth rate or epigenetic mutations has been reported (Pankhurst 1977).

Further studies proved that antibiotics can cause incidence of antibiotic-resistant pathogens through induced mutations or by preparing ground for natural selection, which finally lead to the necessity of new antibiotics (Taji et al. 1993). There are also reports on the appearance of the phytotoxic signs by the use of antibiotics in tissue culture medium (Pieric 1999). Leifert et al. (2000) reported some unexpected effects on *Clematis, Photinia, Hosta, Iris* and *Delphinium* tissue culture by using Rifampicin, Carbenicillin and Streptomycin. In addition, there are not any antibiotics that be able to control any pathogens in tissue culture (Taji et al. 1993). The application of activated charcoal, density gradient centrifugation, repeated subculture, low free-water medium, acidification or egg white lysozyme are also suggested to control the contamination of plant tissue culture (Herman 1996). Application of silver and its components like silver nitrate or silver sulfadazine to treat burns, wounds and infections had been common in the past centuries. However they lost their popularity by the production of antibiotics (Rai et al. 2009, Richard et al. 2002). So there are very rare reports about their use in the plant or animal tissue culture.

In the 16th century solid form of silver nitrate was called "*Lunar Caustic*" and used to remedy venereal disease, bone and perianal abscesses (Klasen 2000, Landsdown 2002). In the nineteenth century again infections of injuries were treated using silver nitrate (Rai et al. 2009). The introduction of pencillium in 1940s was the main reason that minimized the application of silver for bacterial infections. But using silver components restarted in 1960s with the introduction of a silver containing solution by *Mayer*. It possessed antibacterial

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properties against *Escherichia coli, Pseudomonas areoginosa* and *Staphylococcus areus* in addition of having no interfere with epidermal proliferation (Richard et al. 2002). In 1968 sulfadazine cream was made by the combination of silver nitrate and sulphonamide and was used as an antibacterial agent against *E. coli, S. areus, Klebsiela sp* and *Pseudomonas sp*. It also possessed anti fungal and anti viral activities (Fox and Modak 1974). Since rediscovering of silver biological activities, such a lot of studies have been done to clarify the new aspects of this multifunctional metal.

Nanotechnology is a growing field of science and technology and many aspects of its abilities to control environmental contamination has been proved (Castellano et al. 2007, Nomyia et al. 2004). In the last decades, the applications of metal nanoparticles, especially of silver, gold and platinum have been in the focus (Sondi and Salopek-sondi 2004). Based on many studies, metals at their nanoscale have unique chemical, physical and optical features, due to their high surface to volume ratio. The novel antimicrobial activity of nanosilver is a clear example of. However Shahverdi et al (2007) had studied the combination effects of several antibiotics and nanosilver in the microbial cultures, but the only existing report on the application of nanosilver in the plant tissue culture is limited to investigation of Abdi et al. (2008) that could decrease the contamination rate of *Valeriana officinalis* tissue culture. They also reported that their treatments had no side effects on leaf number, proliferation and number and length of explant roots. In our study, we also focused on the evaluation of antimicrobial activities and the best method of the treating nanosilver solutions on a highly contaminated tissue culture, *Gerbera jamesonii* (Shaban-pour 2009, Smart et al. 1995)

MATERIALS AND METHODS

To evaluate the potential of silver nanoparticles in controlling contamination of Gerbera (*G. jamesonii*) capitulum tissue culture, a completely randomized experiment with eighteen disinfecting treatment and each one with ten replicate was conducted through October 2009 to April 2010 in the tissue culture laboratory of ACECR[1] (Mashhad branch). Two of the treatments were candidate as controls (A and B). In control A, explants were disinfected only with Sodium hypochlorite solutions. Control B was defined as common method of disinfecting gerbera capitulum that is treating of explants in Sodium hypochlorite and then Mercury chloride respectively. The sixteen remain treatments were different concentrations of NS and soaking time of explants in which were applied after treating of explants with Sodium hypochlorite.

Preparing of explants and control treatments

In this investigation, we studied on immature capitulum of gerbera that were cut into slices with the diameter of 0.5 - 1 cm. After surface sterilization (30 min under tap water, 10 min soaking in 1.5% Sodium hypochlorite and 10 min rinsing with sterile water for three times) the control A explants were separated at this level. For preparing control B, ten of the surface sterilized explants were selected and soaked in 0.1 percent Mercury Chloride solution for 10 min and washed with sterilized water for three times under Sterile Fume hood.

Preparing sufficient concentration of nanosilver solutions

As mentioned above to evaluate the disinfecting potential and unexpected side effects of NS solutions four different concentrations (25, 50, 100 and 200 mg L^{-1}) and soaking time (15, 30, 60 and 180 min) of them was investigated. The desired concentrations were made by diluting *L2000® NS solution*[2] that contains 4000 mg L^{-1} nanosilver particles in pure water (Table 1).

Table 1. Method of preparing 400ml of various concentration of NS solution.

Desired Concentration (ml L)	Used Volume of L2000® stock (ml)	Volume of added distilled and deionised water (ml)
25	2.5	397.5
50	5	395
100	10	390
200	20	380

[1] Iranian Academic Center for Education, Culture and Research
[2] A product of *nanocid* company, Tehran, Iran

Treating explants using NS and culturing

This experiment was done to evaluate the abilities of NS solutions as a new disinfecting agent and comparing it with common sterilizing methods in gerbera tissue culture. the surface sterilization was done exactly like control treatments, in the next step, about 160 explants derived into 16 groups and each group (with ten replicates) were exposed to distinguished concentration of NS for a precise of time (Table 2). After sterilization all explants were cultured on MS medium supplemented with 4 mg L^{-1} BAA and 0.1 mg L^{-1} IAA. The contamination and some morphological traits (vitrification, proliferation and rooting) of each group (treatments) was investigated one and four weeks after culturing. The statistics for further analysis were gathered by counting vials containing bacterial or fungal colonies. In each replicate, infected cultured (bacterial or fungal) were recorded as "1" and not infected as "0". The data were bootstrapped using *Excel 2007*® with 100 sampling rate. Through this method qualitative data reformed to quantitative ones. The analysis of variance was done according to Duncan's multiple range tests at an alpha level of 0.01. To evaluate the compatibility of quantitative data and its qualitative equivalents, both of them were compared in table 3 and figure 1. Overlapping of both diagrams shows that statistical values have not changed through this method. After one month, the survived explants were subculture on a new MS medium with the same content and transferred to lighting condition to excite branching in, and also evaluating the effect of NS on them.

Table 2. Concentration and soaking time of used NS solution and the sign of each main treatments.

NS solution concentration (ml L^{-1})	Soaking Time (min)			
	15	**30**	**60**	**180**
25	C_1	C_2	C_3	C_4
50	D_1	D_2	D_3	D_4
100	E_1	E_2	E_3	E_4
200	F_1	F_2	F_3	F_4

Table 3. The average of qualitative data and their equivalent quantitative that were obtained through bootstrapping.

Treatments	Qualitative Data	Quantitative Data
A	0.7	0.6158
B	0.5	0.4075
C1	0.3	0.2909
C2	0.4	0.2956
C3	0.6	0.5960
C4	0.5	0.5130
D1	0.3	0.2799
D2	0.5	0.4275
D3	0.3	0.3070
D4	0.4	0.3804
E1	0.4	0.2076
E2	0.4	0.4014
E3	0.2	0.1826
E4	0.2	0.2146
F1	0.1	0.0914
F2	0.5	0.5135
F3	0.5	0.4045
F4	0.3	0.3028

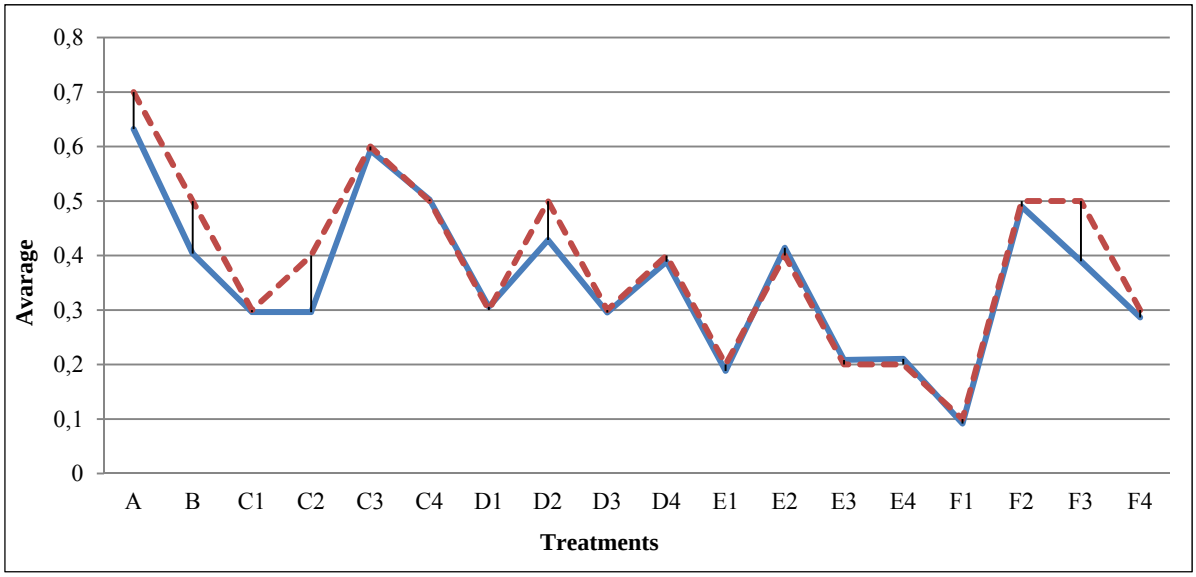

Figure 1. Comparing of the average of quantitative and their equivalent qualitative data that were obtained through bootstrapping.

RESULTS

According to average comparison and variance analysis (table 4) the eighteen treatments (table 2) were categorized into seven different groups (table 5). As it is shown, C_3 and treatment A (first control) showed the highest infection rate and placed in the same group (table 5) the second control (treatment B) and treatments E_2, D_4 and F_3 also showed no significant differences and placed in the same statistical group (table 5), however the appearance of pathogen colonies in the control treatments occurred earlier than in treatments exposed to NS solutions. The other treatments differ from controls significantly. The least infection was watched in the explants that were exposed to 200 mg L^{-1} of NS solutions for 15 min (treatment F_1- Table 5 and 6).

Table 4: Table of ANOVA on the disinfecting level of treatments

Source of Variation	Df	SS	MS	F
Treatment	17	3.48	0.20	100.32**
Error	162	0.33	0.002	--
Total	171	3.83		

Table5: Avarage comparison of NS treatments and controls according to Duncan's multiple range test (α=0.01)

Treatments	Concentration (mL L^{-1})	Soaking Time (Min)	Average*
C3	25	60	0.5960 a
F2	200	30	0.5135 b
C4	25	180	0.5130 b
D2	50	30	0.4275 c
F3	200	60	0.4045 cd
E2	100	15	0.4014 cd
D4	50	180	0.3804 d
D3	50	60	0.3070 e
F4	200	180	0.3028 e
C2	25	30	0.2956 e
C1	25	15	0.2909 e
D1	50	15	0.2799 e
E4	100	180	0.2146 f
E1	100	15	0.2076 f
E3	100	60	0.1826 f
F1	200	15	0.0914 g
Controls			
A	Only Sodium hypochlorite		0.6158 a
B	Sodium hypochlorite then Mercury Chloride		0.4075 cd

*Means with common letter have no difference statistically

Table 6: comparison of the percent of bacterial and fungal contamination of treatments.

Treatments	A	B	C_1	C_2	C_3	C_4	D_1	D_2	D_3	D_4	E_1	E_2	E_3	E_4	F_1	F_2	F_3	F_4	Average
Fungal contamination	40	30	10	40	30	30	30	20	30	30	10	30	10	20	10	10	20	20	22.3
Bacterial contamination	40	10	30	30	50	20	10	30	10	20	10	30	20	20	10	20	20	20	22.2
Sum	70	40	40	50	50	40	40	40	30	40	20	30	20	20	10	30	30	30	35

The reason of lower sum of bacterial fungal contamination percent in stared data, is that some of their replicate had both bacterial and fungal contamination together, so during counting the contamination proportion were counted two times, once in the bacterial contaminated group and once in fungal one.

Analysis also proved that using Sodium Hypochlorite and Nano-Silver can reduce both fungal and bacterial infection more effectively in compare to treatment A, as it was watched that the average percentage of infections decreased to 21.9% in NS containing treatments, and to 10% of fungal and bacterial infection in E_1, and to 10% of total infection in F_1 in contrast with control A that showed 40% bacterial and 40% fungal contamination.

These reductions in infection rates can prove the positive anti-pathogenic potential of NS solutions, in tissue culture. Results of comparing control treatments (A and B) also showed that the application of Sodium hypochlorite and Mercury chloride (control B) reduces the bacterial infection more sufficiently than fungal contaminations (Table 6).

DISCUSSION

Because of the importance of contamination controlling for achieving the goals of tissue culture, many different methods with its own advantages and disadvantages have been suggested till now. Applying Sodium hypochlorite, Mercury Chloride, alcoholic and antibiotic solutions are the most common examples. However the environmental side effects of Mercury chloride (Mutter et al. 2005, Counter and Buchanan 2004, Langford and Ferner 1999) and antibiotics are fading their use out, so the necessity to consider new antimicrobial agents is obvious. Karcher and Bock (1998) reported the increase of the chloroplast RNA editing sensitivity by use of Streptomycin and the lack of chloroplast differentiation in the higher concentrations. It is also reported that

Application of Antimycine A, even in low concentration such as 1mM in the tobacco tissue culture, inhibits electron cycling in mitochondria and photosystem I that leads to defect in ATP production and so results in formation of white and leached leaves (Horvath et al. 2000, Joet et al. 2001). However the application of antibiotics in animal tissue culture are more reliable rather than plants (Taji et al. 1993) but due to the incidence of antibiotic-resistant pathogens, the necessity of new antibiotics is rising sharply (Abdi et al. 2008) so studying other anti pathogenic agents having less side effects seems to be more necessary. Shahverdi et al (2007) investigated the antibacterial properties of various antibiotics in combination with NS particles. Their studies' results showed that the presence of NS significantly increased antimicrobial activity of Penicillin G, Amoxicillin, Erythromycin, Clindamycin and Vancomycin against *E. coli* and *S. aureus*. That can be applied as a method to minimize the use of antibiotics in tissue culture. The sterilization of Gerbera capitulum was studied by Shaban-pour (2009). He pretreated explants using 70% ethanol for 90 seconds and 15 min Sodium hypochlorite (1% aqueous solution) that resulted in only 34% disinfected explants.

In our investigation, using 1% Sodium hypochlorite for 15 and 20 min in combination with Mercury chloride was studied too; with this pretreatment 97 and 89 percent of explants remained disinfected respectively. The results also showed that pretreatment of explants in NS solutions not only decrease the bacterial and fungal contamination but also has no side effects on growth, branching and propagation rates. However the proportion of bacterial contamination in some treatments (D_4, E_2 and F_3) was equal to control B explants that were treated with Mercury chloride, but because of the high toxicity of Mercury chloride in environment and its stability (Counter and Buchanan 2004, Langford and Ferner 1999), NS solutions can be proposed to be substituted, the result of this study is in agreement with sondi and salopek-sondi (2004) that reported the antibacterial activity of NS against *E. coli* as a gram-negative model. They also observed that NS Particles damage the bacterial cell by interacting with the building elements of membrane. The studies of Cho et al. (2005) show the same results on *S. areus* that is a gram-positive bacteria. The tarriance of pathogen colonies occurring and the reduction of bacterial contamination rate of our studies are also in agreement with reports of Abdi et al. (2008).

Finally, according to the our succession in reducing microbial contamination in *G. Jamesonii* by utilization of nano-silver solutions and their preferability aspects like environmental compatibility, being low cost and also other scientific findings that shows NS is nontoxic to human and microorganisms are not likely to develop resistant against silver as it affects on a wide range of target in pathogens (Rai, 2009), the substitution of NS as a new generation of antimicrobial agent for tissue culture can be proposed, however in the other hand, according to the limited studies on the application methods, advantages and disadvantages of NS through the process of plant and animal tissue culture, it seems that much experimental trials are needed to understand the NS toxicity, its activity as a medium component or their effects on the other explant and pathogen species.

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REFERENCES

Abdi GR, Salehi H, and Khosh-Khui M (2008). Nano silver: a novel nanomaterial for removal of bacterial contaminants in valerian (*Valeriana officinalis* L.) tissue culture. Acta Physiology Plant 30: 709-714.

Castellano JJ, Shafii SM, Ko F, Donate G, Wright TE, and Mannari RJ (2007). Comparative evaluation of silver-containing antimicrobial dressings and drugs. Int Wound 4:114-122.

Cho KH, Park JE, Osaka T, and Park SG (2005). The study of antimicrobial activity and preservative effects of nanosilver ingredient. Electrochimica Acta. 51: 956-960.

Counter SA, and Buchanan LH (2004). Mercury exposure in children: a review. Toxicology and Applied Pharmacology 198: 209– 230

Fox CL, and Modak SM (1974). Mechanism of silver sulfadiazine action on burn wound infections. Antimicrobe Agents Chemother 5: 582-8.

Herman EB, (1996). Microbial contamination of plant tissue cultures. Agritech Consultants, Inc., Shrub Oak, USA.

Horvath EM, Peter SO, and Joët T (2000). Targeted inactivation of the plastid *ndhB* gene in tobacco results in an enhanced sensitivity of photosynthesis to moderate stomatal closure. Plant Physiol 123: 1337-1350.

Joët T, Couranc L, Horvath EM, Medgyesy P, and Peltier G (2001). Increased sensitivity of photosynthesis to antimycin A induced by inactivation of the chloroplast *ndhB* gene. Evidence for a participation of the NADH-dehydrogenase complex to cyclic electron flow around photosystem I. Plant Physiol 125: 1919-1929.

Karcher D, and Bock R (1998). Site-selective inhibition of plastid RNA editing by heat shock and antibiotics: a role for plastid translocation in RNA editing. Nucleic Acids Res 26: 1185-1190.

Kharrazi M, Nemati H, Tehranifar A, Bagheri A and Sharifi A (2011). *In Vitro* Culture of Carnation (*Dianthus caryophyllus* L.) Focusing on the Problem of Vitrification. J. Biol. Environ Sci 13:1-6

Klasen HJ (2000). A historical review of the use of silver in the treatment of burns (Part I, early uses). Burns 30: 1-9.

Landford NJ, and Ferner RE (1999). Toxicity of Mercury (Review). Human Hypertension 13: 651–656
Landsdown ABG (2002). Silver I: its antibacterial properties and mechanism of action. Wound Care 11:125-38.
Leifert C, Waites B, Keetley JW, Wright SM, Nicholas JR, and Waites WM (2000). Effect of medium acidification on filamentous fungi, yeasts and bacterial contaminants in Delphinium tissue cultures. Plant Cell Tissue Organ Cult 42: 149-155.
Mutter J, Naumann J, Schneider R, Walach H, and Haley B (2005). Mercury and autism: Accelerating Evidence. Neuroendocrinol Lett 26(5):439–446
Nomiya K, Yoshizawa A, Tsukagoshi K, Kasuga NC, Hirakava S, and Watanabe J (2004). Synthesis and structural characterization of silver (I), cobalt (II), aluminium (III) and complexes with 4-isopropyltropolone (hinokitiol) showing noteworthy biological activities. Action of silver (I)-oxygen bonding complexes on the antimicrobial activities. Inorg Biochem 98: 46-60.
Pankhurst CE (1977). Symbiotic effectiveness of antibiotic-resistant mutants of fast and slow-growing strains of Rhizobium nodulating Lotus species. Can. Microbiology 23:1026-1033.
Pierik RLM (1999). In Vitro Culture Of Higher Plants. Kluwer Academic Publishers., Dordreched, Netherlands.
Rai M, Yadav A, and Gade A (2009). Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances 27: 76-83.
Richard JW, Spencer BA, McCoy LF, Carina E, Washington J, and Edgar P (2002). Acticoat versus silver lon. The truth. Burns Surg. Wound Care 1: 11-20.
Shaaban-pour K (2009). Effects of hormones and genotype on gerbera micro propagation. M.Sc. Thesis. Islamic Azad University, Tehran, Iran, 120p
Shahverdi AR, Fakhimi-phard A, Shahverdi HR, and Minaian S. (2007). Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. Nanomedicine, Nanotechnology, Biology and Medicine 3: 168-171.
Smart DR, Ferro A, Ritchie K, and Bugbee BG (1995). Use of antibiotics to reduce rhizoplane microbial populations in root physiology and ecology investigations. Physiol Plant 95: 533-540.
Sondi I, and Salopek-Sondi B (2004). Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. Colloid and Interface Science 275: 177-182.
Taber RA, Theilen AM, Falkinham JO, and Smith RH (1991). *Mycobacterium scrofulaceum*: a bacterial contamination in plant tissue culture. Plant Science 78: 231-236.
Taji AM, Dodd WA, Williams RR (1993). Plant Tissue Culture Practice. University Of New England. London.