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Nano-Silver Particles Eliminate the *in vitro* Contaminations of Olive 'Mission' Explants

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Abstract: Olive has been cultivated from ancient times. The traditional propagation methods of olive are laborious and with low efficiency. Now, *in vitro* techniques are an alternative for mass propagation of the species. Establishing a sterile culture is the most challenging step of plant tissue culture process. This study was conducted to evaluate the potential of nano-silver for elimination of contaminations in olive Mission single node explants on Murashige and Skoog half strength (MS/2) medium. Thirty days after culture, the percentages of infected and developed explants were calculated. The results demonstrated that 10% Clorox for 10 min after 70% ethanol for 1 min may be used for surface sterilization of olive explants. Nano-silver used as supplementary disinfectant treatments and microcuttings were submerged into various nano-silver solutions or nano-silver was added to the media. Submergence of microcuttings into various nano-silver solutions was effective to control the internal contaminations, but it also caused severe injuries to the explants. Adding nanosilver (4 mg L^{-1}) to media was fully effective to control explants internal contaminations and no harmful effects were observed on explants and their growth. In conclusion, using very low concentrations of nano-silver as a disinfecting agent in plants tissue culture media is recommended.

Key words: Decontamination, micropropagation, nano-silver, olive tree, tissue culture

INTRODUCTION

Native of the Mediterranean Sea basin, olive (*Olea europaea* L.) belongs to the Oleaceae family and its cultivation has been started from ancient times (Liphschitz *et al*., 1991). Now, olive is one of the most important commercial fruits tree grown in regions with Mediterranean climates.

Olive can be propagated by seed, cutting and sucker. The commonest method is the rooting of stem cuttings under mist system; however the efficiency of this method is very low. *In vitro* propagation techniques present a suitable high efficiency alternative for olive propagation (Rugini and Pesce, 2006). *In vitro* produced plants are true to type, clean (pest and disease free) and suitable for commercial production of a large number of plants. Successful micropropagation of olive cultivars has been reported by many researchers during the past three decades (Rugini, 1984; Bartolini *et al*., 1990; Rama and Pontikis, 1990; Dimassi-Theriou, 1999; Cozza *et al*., 1997; Troncoso *et al*., 1999).

Too many factors may limit micropropagation of higher plants, especially woody species (Houng *et al*., 2001). The most important limiting factor is *in vitro* contaminations (e.g., fungal and bacterial infections). There are some methods and chemicals available to control *in vitro* contaminations. However, the efficiency of some of these methods is low, and/or some of theme are too toxic. Antibiotics are used in controlling internal bacterial contaminations (Dabai *et al*., 2007). Using antibiotics also may adversely affect the growth and response of explants and may induce resistance in bacteria and generally they are not suggested for using in plants tissue culture laboratory (Dabai *et al*., 2007). Mercury chloride (AgCl) is widely used for controlling internal contaminations of woody plants. AgCl is very toxic and should be used with high cautions (Leifert and Woodward, 1997). Such chemicals not only are too toxic for the explants and the operator, but they may infect the environment. In addition, Martino *et al*. (1999) showed that olive explants are too sensitive to mercury chloride and it may kill the explants. Under such circumstance, finding an effective and safe substance for decontamination of the explants (especially for woody plants) is very important.

Nano-silver is a new and non-toxic material which shows high capabilities in eliminating microorganisms, e.g., fungus, bacteria and viruses. The detrimental effects of nano-silver have been shown on more than six-hundred microorganisms (Abdi *et al*., 2008). This capability of

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nano-silver is due to release of tiny particles of silver and so it is able to destroy not only the bacteria and fungus, but also the viruses (Sondi and Salopek-Sondi, 2004).

The aim of the current study was to evaluate the potential of nano-silver particles on decontamination of nodal segments of olive Mission for *in vitro* propagation.

MATERIALS AND METHODS

Plant materials: The experiment was conducted at tissue culture laboratory of the Department of Horticultural Science of Shiraz University (Iran) from spring to autumn 2008.

Branches of 9 years old olives cv. Mission brought from Kazerun Research Station, Kazerun, Iran. Explants included the single nodes and shoot apices. Leaves were removed and 2 cm uniform explants were washed thoroughly in running tap water for 20 min after pre-washing in solution contained 0.2-0.3% commercial detergent (Rika-Iran). Explants were submerged in a solution of 100 mg L^{-1} ascorbic acid plus citric acid 100 mg L^{-1} for 30 min to control the phenolic compounds (Brhadda *et al*., 2003).

Disinfestation by ethanol and clorox: Explants were submerged in 70% ethylic ethanol for 0, 0.5 and 1 min and after 3 times rinsing in sterile distilled water, they were submerged in 10% Clorox (Golrang-Iran) for 0, 5 and 10 min. The Clorox solutions contained 0.1% of a commercial detergent (Rika-Iran) as wetting agent. After Clorox treatments, explants were washed thoroughly for 3 times in sterile distilled water.

Disinfection treatments by nano-silver: Nano-silver (NanoCid 'L2000'-Iran) particles were used as disinfecting treatments after surface sterilization by 70% ethanol and 10% Clorox. Nano-silver treatments included: (1) submersion of explants in nano-silver solutions; (2) adding nano-silver to media.

- Explants were submerged in 0, 100, 200, 300 and 400 mg L^{-1} nano-silver solutions for 1 h (Abdi *et al*., 2008). The nano-silver solutions prepared in sterile distilled water
- Different amount of nano-silver (0, 4, 8 and 16 mg L^{-1}) were added to the media as disinfection agent and then media were autoclaved. Thirty days after culture the percentages of infected and developed explants were recorded

Medium and culture condition: The explants were placed on Murashige and Skoog half strength (MS/2) media supplemented with 3.0% sucrose (Brhadda *et al*., 2003). The medium was supplemented with 2.1 mg L^{-1} Benzyl Adenine (BA), 1.26 mg L^{-1} Gibberellic Acid (GA_3) and 0.6 mg L^{-1} Naphthalene Acetic Acid (NAA). The pH of medium adjusted to 5.7 by HCl or NaOH prior to adding 0.8% agar and then autoclaved at 121°C for 15 min (Bartolini *et al*., 1990). Culture room conditions were 25±3°C, 16 h photoperiod at 40 W m^{-2} irradiation.

Statistical analysis: The experimental design was a Complete Randomized Design (CRD) with four replications and 10 vessels per replication and 2 explants per vessel. Data were subjected to ANOVA. Means were compared with Tukey's HSD test at $p \leq 0.01$.

RESULTS

Results of disinfestations of olive single nodes by 70% ethanol and 10% Clorox are shown in Table 1. Results showed that ethanol may not be used singularly for controlling *in vitro* contaminations of olive explants. Clorox treatments reduced the number of infected cultures, significantly. The best results after using 10% Clorox obtained in 10 min treatment (up to 18%). However, ethanol treatments increased the efficiency of the Clorox treatments significantly. Increasing the period of treatments led to better control of *in vitro* contaminations and the best results were obtained in 70% ethanol for 1 min and 10% Clorox for 10 min (up to 51%). The explants were depressed for 24 to 48 h after long periods of ethanol and Clorox treatments, but no signs of chlorosis and/or necrosis were observed after these treatments.

Submerging the explants in nano-silver solutions after 70% ethanol and 10% Clorox treatment, wholly prevented the fungus and bacteria contaminations (100%) (Fig. 1). However, nano-silver submersion also affected the olive explants and very few explants developed these treatments (Fig. 1).

Adding nano-silver to the media following the elected 70% ethanol and 10% Clorox as surface sterilizing

Table 1: The effects of different time applications of 70% ethanol and 10% Clorox on controlling the *in vitro* contaminations

	70% ethanol (min)			
10% Clorox (min)	0	0.5	1.0	Average
0	6.1e†	9.4e	10.8e	8.7C
5.0	11.0c	19.7d	25.2e	18.6B
10.0	18.3a	32.5b	51.4d	34.6A
Average	11.8C	20.5B	30.5A	

†The means with the same capital or small case letters were not significantly different according to Tukey's test at $p \leq 0.01$

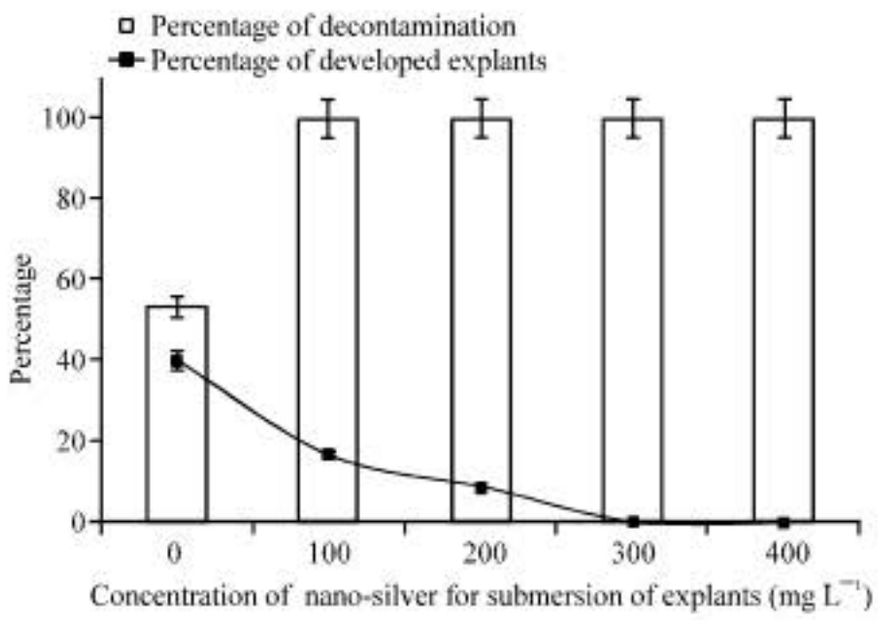

Fig. 1: The effects of submersion of explants in nano-silver solutions on decontamination and development of explants. Values are Mean±SE

Fig. 2: Explants grew normally after adding nano-silver particles to the media. The color of medium also changed from what… to dark yellow after adding silver particles, though it was still clear

treatment decreased the incident of fungus and internal contaminations significantly (Fig. 2). Adding nano-silver particles to the media resulted in reduction of *in vitro* contaminations incident less than 5%. The addition of different concentrations of nano-silver to the media were not significantly different on controlling the contaminations (Fig. 3). Presence of 6 mg L^{-1} nano-silver particles in the media seemed to reduce, compared with the other two concentrations, the growth of olive explants; however the differences were not significant.

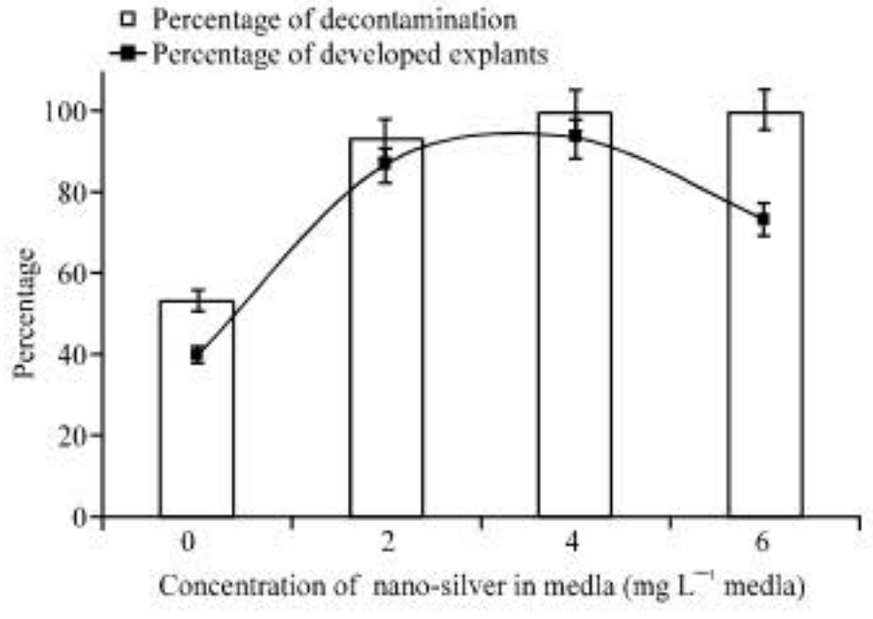

Fig. 3: The effects of using nano-silver in media on decontamination and development of olive explants. Values are Mean±SE

DISCUSSION

Establishing a sterile culture is the most challenging step in micropropagation of woody plants. In addition, this step is so laborious and costly. In the current study, potential of using nano-silver as a cheap and environmental friendly decontamination agent in micropropagation of olive Mission were investigated.

Results of this study showed that 70% ethanol and/or Clorox treatments are not highly effective in controlling *in vitro* contamination of olive explants. Ethanol pretreatments for 1 min before 10% Clorox treatments increase the efficiency of surface sterilization significantly. Mencuccini *et al*. (1997) and Briccoli *et al*. (2002) also suggested using of ethanol in decontamination procedure of olive explants. It has been shown that ethanol by eliminating the air bubbles on explants may increase the efficiency of Clorox and/or other decontamination treatments. Ethanol also may dissolve the waxes surrounding the young tissues and improves the penetration of decontamination factor into the tissues. On the other hand, ethanol also exerts antimicrobial properties by itself. However, the results of this study showed that ethanol may not decrease *in vitro* contaminations significantly. Based on the results of ethanol and Clorox experiment, the supplementary decontamination treatments are essential for disinfecting olive explants.

No study investigating the effects of nano-silver on decontamination explants of woody plants are reported in literature. Present results showed that nano-silver significantly prevent the incident of *in vitro* contaminations of olive explants. Abdi *et al*. (2008) also showed the effects of nano-silver particles on controlling

the internal contamination of valerian (*Valeriana officinalis* L.): these researchers obtained the best results of decontamination after submersing the surface sterilized valerian explants in 100 mg L^{-1} nano-silver solution for 60 min. Submerging the olive explants in nano-silver solutions after surface sterilization also showed that nano-silver is very effective in controlling fungus and bacterial contaminations. However, this method is not applicable for olive explants because of severe injuries and browning of the explants. On the other hand, adding nano-silver particles to the media controlled the *in vitro* contaminations significantly and did not affect the growth of explants.

The mechanism of action of nano-silver particles in terminating the microorganisms is not known clearly. However it has been shown that nano-silver particles release silver ions (Ag^+) slowly and the Ag^+ can destroy the cell structure of microorganisms (Lubick, 2008). Dibrov *et al*. (2002) stated that the effects of Ag^{++} on microorganisms may be due to their chemosmotic activity: these researchers showed that Ag^+ affects the phospholipids and destroy the cell membrane of microorganisms; Ag^+ also may substitute the sulphur in the -SH groups of cell membrane of microorganisms and destroy them. Tang *et al*. (2007) showed that the destructive effects of Ag^+ are related to production of active silver containing organic compounds, these compounds can attract the microorganisms and destroy their structure.

In the current study the harmful effects of nano-silver reported on higher plants for the first time. Such effects also may due to the adverse effects of high concentrations of Ag^+ on cell membrane of explants. These results are in contrast with those reported by Abdi *et al*. (2008) and show that differences in plants tissue and/or prolonged decontamination treatments may adversely affect the results of using nano-silver. Martino *et al*. (1999) also showed the sensitivity of olive explants to AgCl. However, the results of current study showed that low concentrations of Ag^+ in media are tolerable for olive Mission explants and may control the incident of contaminations.

CONCLUSION

The results of this study showed the effects of nano-silver particles on eliminating the fungal and bacterial contaminations of olive explants. Nano-silver eliminates the internal infections of *in vitro* explants and it is not toxic for the operator and for the environment. As the results showed, submerging of explants in high concentrations of nano-silver may kill them. At the end, it is suggested to add low concentrations of nano-silver particles in media of *in vitro* cultures of olive. The applicability of using nano-silver in plants tissue culture media as disinfecting agent to explants of other cultivars and/or species should be tested.

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