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Effect of Silver Nanoparticles on Microbial Contaminants and Growth of *in vitro* Olive Shoot cv. Sourani

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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. Silver nanoparticles (AgNPs) show high capabilities in eliminating microbial contamination. Therefore, this study was conducted to evaluate the potential of silver nanoparticles for eliminating microbial contaminations during the establishment stage of olive micropropagation. Validation and characteristion of the synthesized nanoparticles was carried out by UV/VIS Spectrophotometer and Transmission Electron Microscopy (TEM).The effect of AgNPs was investigated either by surface immersing olive explants in AgNPs solution at (0, 100, 200 and 400 mg L^{-1}) or adding to the olive media at (0, 5, 10 and 20 mg L^{-1}). The obtained results cleared that a submergence of micro-cuttings into various AgNPs solutions was effective to control microbial contaminations; AgNPs at concentration of 400 mg L^{-1} recorded the lowest contamination percentage (6.6%). Also, surface sterilization by AgNPs at different concentrations had a significant effect on bud sprouting percentage and shoot growth. Moreover, the addition of silver nanoparticle at 10 mg L^{-1} to the culture medium recorded the highest bud sprouting percentage (91.88%), while AgNPs at 20mg L^{-1} recorded the highest value of shoot length and leaves number. Meanwhile, the highest number of shoots /explants was recorded for AgNPs at 5 mg L^{-1} without any significant differences with the other concentrations. In conclusion, it is recommended to use silver nanoparticles as a disinfectant agent to sterilize olive explants, also the additions of nanoparticles to the culture medium were efficient in control contamination of explants and improve their growth.

Key words: *Olea europea* · Micropropagation · Sterilization · AgNPs · Contaminations

INTRODUCTION

Olive (*Olea europaea*) is one of the traditionally cultivated fruit crops in the Mediterranean region [1]. Olive fruits and leaves are rich sources of valuable bio-actives pharmaceutical materials [2]. The benefits of olive products to human health have been widely recognized [3]. Olive trees are traditionally propagation by cuttings and grafting on clonal or seedling rootstocks [4]. Micropropagation of olive has been reported as a powerful technique for mass production of pathogen-free and true to type cultivars [5, 6]. Generally, microbial contamination and slow shoot growth are the major problems of olive micropropagation [7]. Microbial contamination is a serious problem, of *in vitro* propagation; eliminate the microbial contamination is one of the basic requirements for successful initiation of cultured plant tissues [8, 9]. To eliminate microbial contamination, plant materials must be surface sterilized; disinfection procedures usually involve using of sodium hypochlorite, ethyl alcohol, mercury chloride and antibiotics; nevertheless, these substances are frequently toxic to plant tissues and have shown inhibitory effects on explant growth and development [10, 8]. Recently, nanotechnology has received much attention in diverse fields of science and technology. Nanoparticles (NPs) application has successfully led to the elimination of microbial contaminants from explants and had a positive

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role in callus induction, organogenesis, somatic embryogenesis, genetic transformation and secondary metabolite production [11-13]. Nano-silver shows high capabilities in eliminating microorganisms, the detrimental effects of nano-silver have been shown on more than six-hundred types of microorganisms; silver ions interact with many processes at the molecular level in bacterial cells, resulting in the inhibition of their growth and even death [8]. This capability of nano-silver is due to release of tiny particles of silver so it is able to destroy not only the bacteria and fungus, but also the viruses [14]. Presence of AgNPs in the media reduces the microbial contamination in tissue cultures of olive cv. 'Mission' [15]. The aim of this study was to evaluate the possibility of using different concentrations and application methods of silver nanoparticles to eliminate microbial contaminants and improve the *in vitro* growing olive Sourani shoots.

MATERIALS AND METHODS

Plant Materials: Active growing shoots of Sourani olive cv. were collected from mature, healthy olive trees growing in the orchard of Pomology Department, Faculty of Agriculture, Cairo University, Giza, Egypt. Olive shoots were collected from the tree in the morning during spring of 2020-2021. Olive shoots were immediately brought to the laboratory; shoots were stripped from leaves, washed with tap water and divided into nodal cuttings.

Silver Nanoparticles Preparation: The silver nanoparticles were synthesized using the chemical reduction method of [16]. $AgNO_3$, $NaBH_4$ and PVP were dissolved in deionized water to form aqueous solution of $AgNO_3$ (0.1 M), $NaBH_4$ (0.01 M) and PVP (0.01 M), respectively. The aqueous solutions of PVP (0.01 M) and NaBH4 (0.01 M) were mixed at a volume ratio of 1:1. About 500 ml of this solution was transferred to a beaker and agitated with a magnetic stirrer before adding the $AgNO_3$ (0.1 M) solution. Upon addition of silver nitrate drop by drop, the colorless solution of $NaBH_4$-PVP was slowly changed from yellow to pale brown indicating the formation of silver nanoparticles.

Silver Nanoparticles Characterization by UV/VIS Spectroscopy and TEM: The AgNPs were characterized using UV/VIS Spectrophotometer (T80, PG Instruments Ltd). The scanning range for the samples was 200-700 nm. Millie-Q water was used as a blank reference. The dimension and form of the AgNPs nanoparticles, was determined by transmission electron microscopy (JEOL JEM-1400, USA). A drop (2 ml) of Milli-Q water, which dissolved synthesized nanoparticles, was placed on a carbon grid (C-grid). The size was obtained by measuring the diameter of particles presented in the TEM image. The images were obtained at a bias voltage of 40-120 kV.

Antimicrobial Properties of Synthesized Silver Nanoparticles: In order to validate the efficiency of the silver nanoparticles as disinfectant agents, filtration sterilized (22µ; Joanlab Equipment CO., LTD, China) solution of AgNPs at 5, 10 and 20 mg L^{-1}, were added to the non-sterilized Rugini olive medium [17] supplemented with 30g L mannitol and 6 g L agar; the experiment including comparative negative control treatment (non-sterilized free nanoparticles medium) and positive control treatment (autoclave sterilized free nanoparticles medium). The prepared media were dispensed in glass petri dishes and incubated at 25°C with 16 h photoperiod for 48h; fungus or bacteria colony formation was monitored by periodical visual examining of culture plates and the visible colonies were counted and the contamination percentage was calculated in relation to the negative control treatment.

Effect of AgNPs Treatments on Microbial Contamination and Growth of Olive Shoots

Effect of Immersion of Olive Explant in AgNPs Solution: Olive explants (single node) were submerged in filtration sterilized solutions of 0, 100, 200 and 400 mg L^{-1} of AgNPs for 30 min after surface sterilization with 20% commercial bleach (5.25% sodium hypochlorite) for 5 min. Olive explants were cultured on 50 ml of semi-solid medium Rugini olive medium supplemented with 30 g L^{-1} mannitol, 2.5 mg L^{-1} zeatin and 6 g L^{-1} agar. The pH was adjusted to 5.8 and the media were autoclaved at 121°C for 15 min. The cultures maintained in the growth chamber at 25±2°C and 16h photoperiod provided by cool-white fluorescent lamps. Four weeks later contamination percentage, sprouting percentage, the number of shoots per explant, shoot length, numbers of leaves per shoot were recorded.

Effect of Adding AgNPs to Culture Medium: Sterilized olive explants [18] were cultured on 50 ml of semi-solid Rugini olive medium supplemented with filtration sterilized AgNPs (0, 5, 10 and 20 mg L^{-1}). All media were supplemented with 30g L^{-1} mannitol, 2mg L^{-1} zeatin and 6g agar L^{-1}. Four weeks later contamination percentage, sprouting percentage, the number of shoots per explant, shoot length, number of leaves per shoot were recorded.

Statistical Analysis: The design of this experiment was a complete randomized design (CRD) with three replications [19]. Data were subjected to analysis of variance (ANOVA) using MSTAT-C software statistical package [20]. Differences between treatments means were compared by using least significant difference (LSD) tests at 5% level of probability according to [21].

RESULTS AND DISCUSSION

Characterization of Silver Nanoparticles: The UV-VIS spectrum of AgNPs is illustrated in Figure (1A). The UV-VIS spectrum of synthesized AgNPs gave absorbance peak at 407 nm. This absorption band is typical of AgNPs as reported previously [22]. The single peak of the plasmon surface resonance indicated that the AgNPs were spheres with a broad size distribution; other researchers have confirmed the PSR peak at 400-450 nm as a signal of AgNPs synthesis [23]. TEM image of the synthesized silver nanoparticles (dark spherical objects) is shown in Figure (1B), the size ranged from 6.68 nm to 15 nm. The TEM images indicated that the AgNPs were spherical in shape and well scattered in the solution and the nanoparticles were separated from each other without any aggregation.

Antimicrobial Properties of AgNPs: Regarding the variation in inhibition potential of the tested silver nanoparticles concentration on *in vitro* microbial contamination (Table 1 and Figure 2), the obtained results showed that, there was a significant difference between the different nanoparticles concentrations on microbial contamination; silver nanoparticles treatments recorded the lowest value of microbial contamination compared with the negative control plates.

Table 1: Effect of silver nanoparticles on *in vitro* microbial contamination

Treatment	Microbial contamination (%)
Negative control (Non-sterilized)	100 a
Positive control (Autoclaved)	0.00 c
AgNPs at 5mg L^{-1}	4.40 b
AgNPs at 10mg L^{-1}	3.33 b
AgNPs at 20mg L^{-1}	3.67 b

Means in each column followed by the same letter are not significantly different $p< 1\%$.

The obtained results indicated the feasibility for applying the silver nanoparticles in the tissue culture medium as antimicrobial agents. The inhabitation potential of nanoparticles agent on *in vitro* growth of microorganisms confirms the previous studies about the antimicrobial activity of nanoparticles [24, 25, 13]. The obtained results showed that, silver NPs has a high potential for eliminates microbial contamination in culture medium; AgNPs recorded the lowest contamination percentage, which is statistically similar to media sterilization by autoclaving. The high antimicrobial activity of AgNPs may be attributed to the strong toxicity of silver ions to a wide range of microorganism [8, 26, 27]. Moreover, the small particle size of the obtained silver nanoparticles is important for interactions and binding of silver ions with cell membrane proteins, which resulting cell death [14].

Effect of Immersion of Olive Explant in AgNPs Solution Contamination Percentage: Data in Figure (3) shows the effect of explant immersion in different silver nanoparticles concentrations on contamination percentage of olive explants during starting stage. Surface disinfection by immersing Sourani olive explant in silver nanoparticles (AgNPs) significantly reduced the contamination

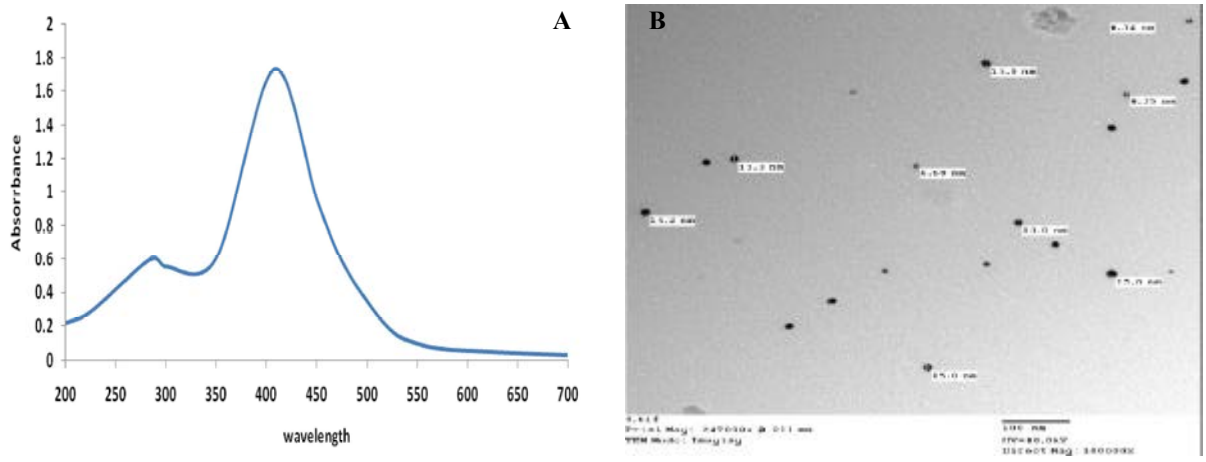

Fig. 1: UV/V is spectrum (A) and Transmission electron microscope (TEM) micrographs (B) of silver nanoparticles

Fig. 2: Microbial contamination percentage affected by different concentrations of AgNPs

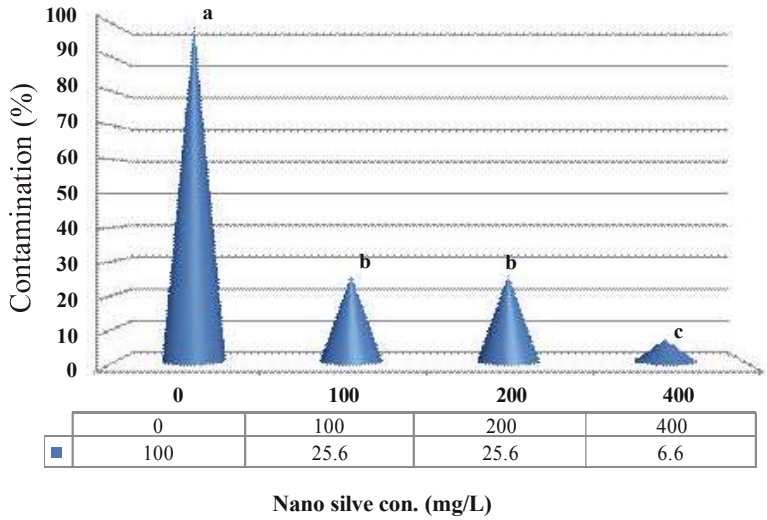

Fig. 3: Effect of AgNPs on contamination percentage of Sourani olive cultivar

percentage compared with control treatment which recorded the highest value of contamination (100%). The lowest contamination percentage was noticed with silver nanoparticles at 400 mg L^{-1} (6.6 %), followed by 200 and 100 mg L^{-1} with no significant difference between them.

It's clear from the above results that contamination percentage of Sourani olive cultivar had positively affected with submersion in silver nanoparticles at different concentration. According to [28] AgNPs successfully controlled bacterial and fungal contamination without any harmful effects on regeneration of the lemon grass explants. It has been reported that AgNPs can be an efficient tool for removing contaminants from plant tissues, but the right dose and exposure time are to be used [29]. Abdi *et al*., [8] used AgNPs solution at three concentrations (25, 50 and 100 mg) for 30, 60 and 180 minutes for disinfection of *Valeriana officinalis* L. explants; the higher concentration of AgNPs solution for a longer period of time caused higher mold fungal reducibility. Pretreatment of explants with AgNPs solution not only decrease the bacterial and fungal contamination but also has no side effects on growth, multiplication rates and regeneration of plantlets [28].

Bud Sprouting Percentage: Bud sprouting percentage of Sourani olive cultivar was affected by submersion in silver nanoparticles (AgNPs) as a surface sterilization with different concentrations (100, 200 and 400 mg L^{-1}) during *in vitro* starting stage (Figure 4). AgNPs at 200 mg L^{-1} recorded the highest percent of bud sprouting (75%), while AgNPs at 100 mg L^{-1} recorded the lowest percent (64%).

Shoot Length and Shoot Number: It's clear from the data in Figure (5) that immersion of olive explants in silver nanoparticles at different concentrations increased the shoot length; the highest value (5 cm) was recorded at 400 mg L^{-1} followed by 200 (4.89 cm) and 100 mg L^{-1} (4.83 cm) respectively. Immersion of olive explants in silver nanoparticles has significantly increased shoot number

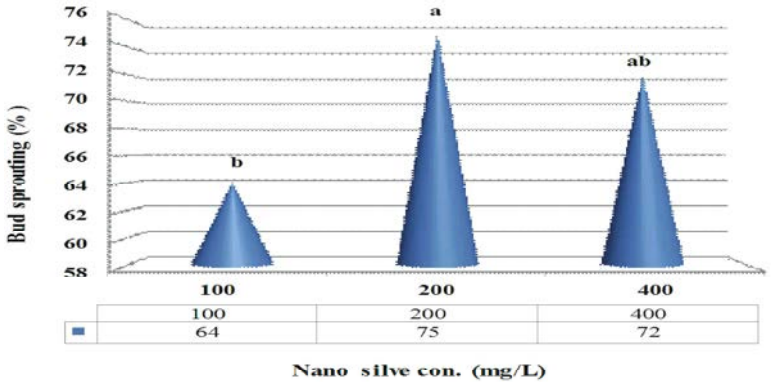

Fig. 4: Effect of AgNPs on bud sprouting percentage of Sourani olive cultivar

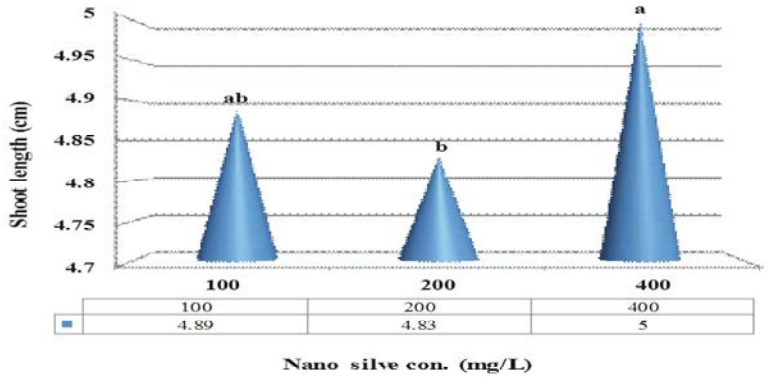

Fig. 5: Effect of AgNPs on shoot length (cm) of Sourani olive cultivar

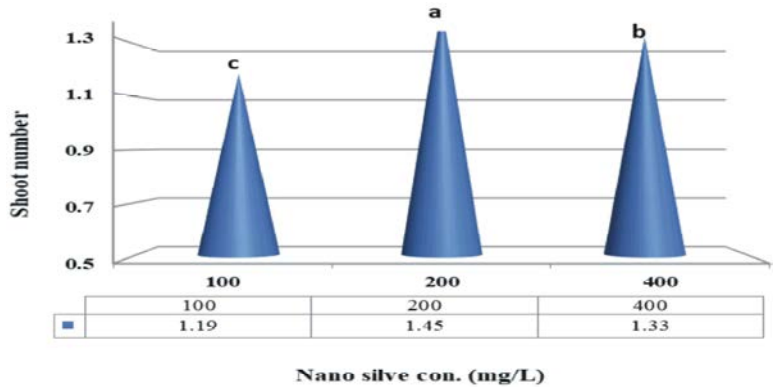

Fig. 6: Effect of AgNPs on shoot number of Sourani olive cultivar

(Figure 6). Analysis of results showed that 200 mg L^{-1} of AgNPs recorded the maximum number of shoots (1.45), while the minimum number of shoots (1.19) was recorded in 100 mg L^{-1} of silver nanoparticles.

Leaves Number / Shoot: Leaves number of Sourani olive cultivar was significantly affected with silver nanoparticles treatments as a surface sterilization with different concentrations (Figure 7). Silver NPs at 100 and 200 mg L^{-1} recorded the highest leaf number (11.33), while 400 mg L^{-1} recorded lowest leaf number (11.11).

Effect of Adding AgNPs to Culture Medium

Effect on Bud Sprouting: Data presented in Figure (8) showed that Ag NPs concentration in culture media had a slight effect on sprouting percentage of Sourani olive cultivar; Ag NPs at 10 mg L^{-1} recorded the highest bud sprouting percentage followed with Ag NPs at 5 mg L^{-1}

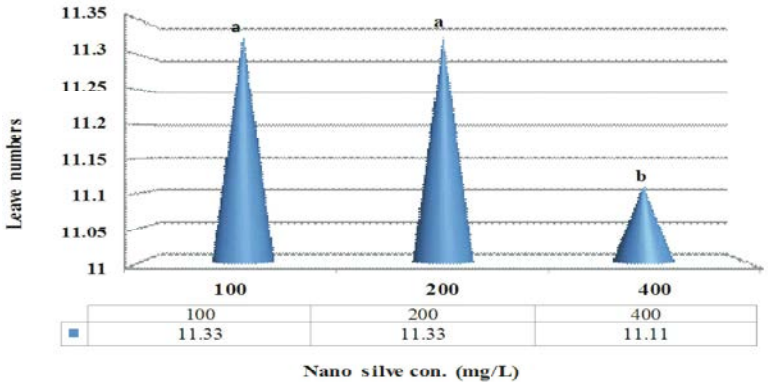

Fig. 7: Effect of AgNPs on leaves number of olive cultivars

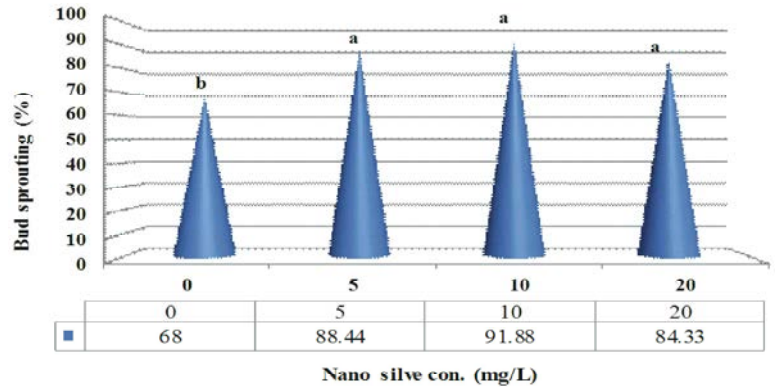

Fig. 8: Effect of adding AgNPs to culture medium on bud sprouting of Sourani olive cultivar

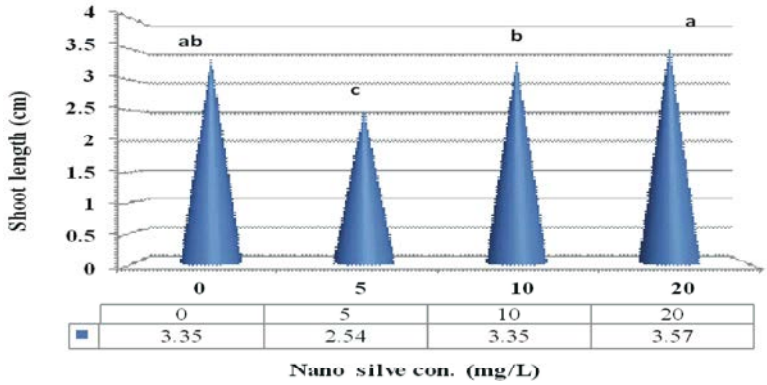

Fig. 9: Effect of adding AgNPs to culture medium on shoot length of Sourani olive cultivar

while increasing Ag NPs concentration to 20 mg L^{-1} in the growth medium had a negative effect on bud sprouting percentage and recorded lower value.

Effect on Shoot Length and Shoots Number: As shown in Figure (9) shoot length was significantly affected with nanoparticles treatments. It is evident that the addition of nanoparticles to culture medium had significantly affected growth of *in vitro* cultured Sourani olive shoots compared with the control treatments; AgNPs at 20 mg L^{-1} recorded the highest shoot length (3.57 cm), while AgNPs at 5 mg L^{-1} recorded the lowest value (2.54 cm).

According to the data illustrated in Figure (10 and 11) there was a slight difference between different Ag NPs concentrations; the highest number of shoots /explants was recorded for AgNPs at 5 followed by 10 mg

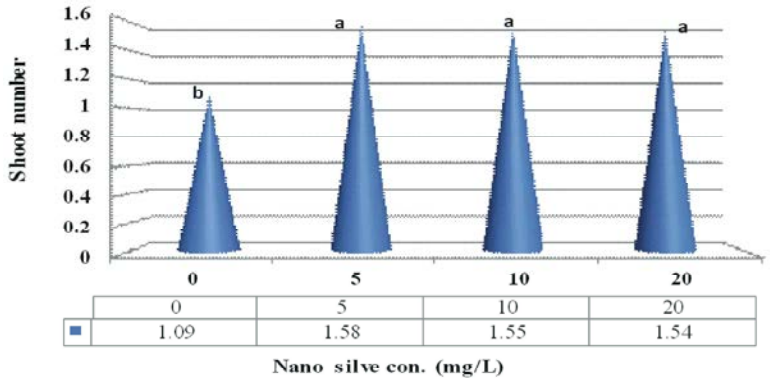

Fig. 10: Effect of adding AgNPs to culture medium on shoots number of Sourani olive cultivar

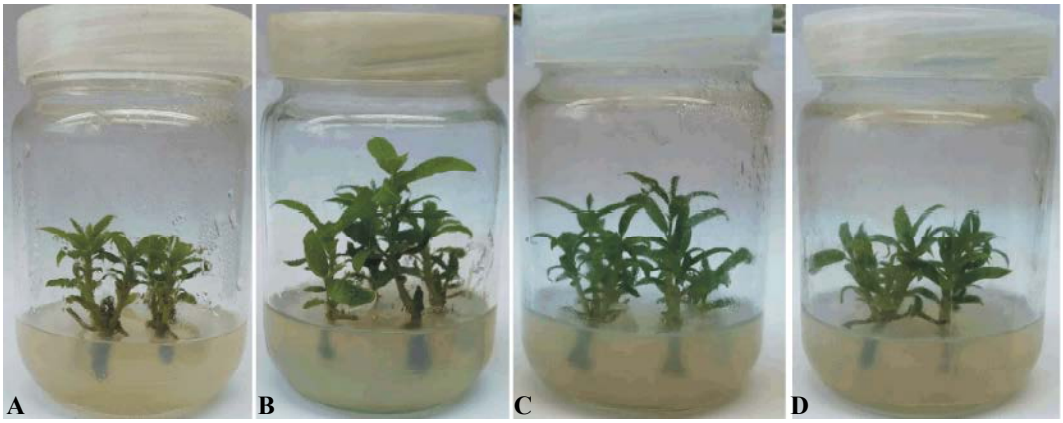

Fig. 11: Effect of AgNPs on *in vitro* growth of olive shoots of Sourani cultivar; control (A) AgNPs at 5mgL^{-1} (B), AgNPs at 10mgL^{-1} (C) and AgNPs at 20mgL^{-1} (B).

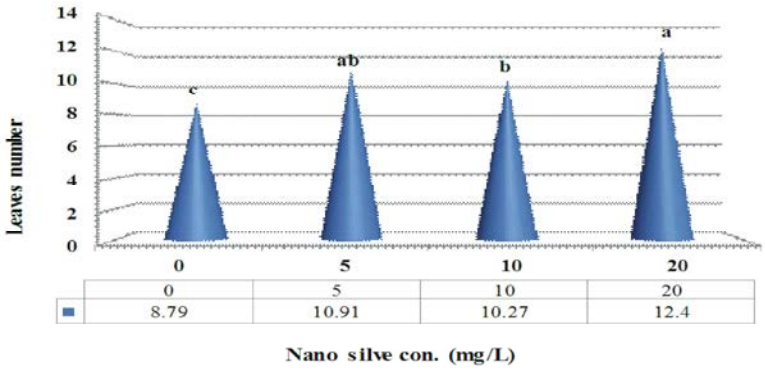

Fig. 12: Effect of adding AgNPs to culture medium on leaves number of Sourani olive cultivar

L^{-1} (1.58 and 1.55 respectively), while the high Ag NPs treatments was recorded lower shoot number (1.54) compared with the other concentration, while the control treatment recorded the lowest value of number of shoots /explants.

Effect on Leaves Number: Data presented in Figure (12) showed that number of leaves was significantly affected by nanoparticles treatments. Silver NPs at 20 mg L^{-1} produced the highest leaf number (12.40) followed by silver NPs at 5 and 10 mg L^{-1} (10.91 and 10.27 respectively) while control recorded the lowest leaf number (8.79).

The addition of silver nanoparticle to the culture medium had a positive effect on *in vitro* growth of Sourani cultivar compared with the control. This agrees with the previous studies showed that the nanoparticles application may lead to stimulatory effects on plant

growth and development; the impact of nanoparticles on plant growth depending on particle size, concentrations and plant genotype [30-32, 18]. The obtained results indicated the positive effect of AgNPs, in improving olive micropropagation. As reported previously silver ion (Ag^+) has a positive effect on plant tissue culture *e.g.,* increased survival and delayed explants senescence [33], improve somatic embryogenesis [34, 35], organogenesis [36, 12], increase shoot multiplication rate and plant growth [37]. Shoot growth and number of shoots per explant were increased in *Brassica juncea, Tecomella undulate* Roxb. and *Vanilla planifolia* cultured on medium supplemented with AgNPs [38- 41], which was attributed to the effect of Ag^+ as an ethylene blockage agent [42]. Several studies have shown that, higher concentrations of NPs had adverse effects on shoot growth and plant regeneration [43- 45]. Therefore, the effects of different types and concentration of NPs on plant tissue should be optimized in order to determine the optimum dose with minimal phytotoxicity [11].

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