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Improve *In vitro* Multiplication of Olive Shoots Using Environmental-Safe Chitosan, Selenium, and Silver Nanostructures

Osama M. Darwesh [1,*], Sayed A.M. Hassan [2], Abdou M. Abdallatif [3]

[1] Microbial Nanotechnology group, Agricultural Microbiology Dept., National Research Centre, Dokki, Cairo, 12622, Egypt; darweshosama@yahoo.com (O.M.D.);
[2] Tissue Culture Technique Lab., Central Laboratories Network and Pomology Dept., National Research Centre, Dokki, Cairo, 12622, Egypt, sayed_ebead@yahoo.com (S.A.M.H.);
[3] Pomology Dept., Faculty of Agriculture, Cairo University, Giza, 12613, Egypt; (A.M.A); abdo.abdullatif@agr.cu.edu.eg
* Correspondence: darweshosama@yahoo.com (O.M.D.);

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Abstract: Olive micropropagation has lately become a potent method for bulk multiplication. Olive trees are traditionally propagated commercially through stem cuttings or grafting. The current study aimed to examine the potential for some olive cultivars' *in vitro* growth and multiplication and the effectiveness of silver, chitosan, and selenium nanoparticles as microbial decontamination agents. Transmission electron microscopy carried out validation and characterization of the biosynthesized nanoparticles. The tested nanoparticles showed varying antimicrobial activity; when it came to preventing microbiological contamination, AgNPs were quite successful. The genotype substantially impacted shoot growth and multiplication rate; Koroneiki and Picual cultivars showed superior growth than Manzanillo cv. The development and multiplication rate of *in vitro* olive shoots was considerably altered by adding nanoparticles to the culture media. Silver nanoparticles reported greater values for shoot number, shoot length, leaf number, and multiplication rate than the chitosan and selenium nanoparticle treatments. The findings concluded that nanoparticles were effective *in vitro* disinfection agents and had a good impact on olive shoots' development and multiplication rate (especially AgNPs).

Keywords: *Olea europaea*; micropropagation; disinfectant; nanotechnology; environmental-safe; AgNPs; nanochitosan.

1. Introduction

One of Egypt's most significant fruit crops is the olive (*Olea europaea*), which has historically been grown throughout the Mediterranean region [1]. Olive trees are typically grown in arid desert environments because they are well adapted to semi-arid environmental conditions [2,3]. Olive fruits, oil, and leaves are rich sources of valuable nutrients and bioactive pharmaceutical materials [4]. There is widespread agreement that olives and olive oil are good for human health [5]. The hard-rooting cultivars of olive trees are grafted onto seedlings or clonal rootstocks, and olive trees are typically propagated by leafy stem cuttings [6]. According to reports, olive micropropagation is a potent method for the bulk generation of pathogen-free and true-to-type cultivars. It provides an important tool for genetic advancement and germplasm preservation [7,8]. The success of olive *in vitro* propagation is affected by several factors, including plant genotype, growth medium, cytokinin type, and concentration.

The main issues with olive micropropagation are typically contamination with various types of microorganisms, oxidation of phenolic chemicals, and delayed shoot growth [9–12]. Eliminating microbial contamination is one of the fundamental prerequisites for the efficient start, growth, and development of cultured plant tissues. Microbial contamination is a severe issue with *in vitro* propagation [13,14]. Although ethyl alcohol, mercury chloride, and sodium hypochlorite are frequently used to sterilize plant materials to remove microbial contamination, these chemicals are harmful to plant tissues. They have an inhibiting effect on explant survival, growth, and development [15,16]. More research is therefore required to increase the effectiveness of olive micropropagation while inhibiting the growth of diseases. Recently, nanotechnology has received much attention; it is anticipated to find use in various agricultural domains, such as boosting plant growth, yield, nutritional value, and secondary metabolite synthesis. Additionally, nanotechnology attempts to create pesticide and nutrient delivery systems, lowering environmental contamination [17-19]. Excellent chemical, mechanical, and electrical properties of nanoparticles depend on the type of material used, the size of the particles, and how well they are absorbed and transported inside plant tissues [20,21]. Numerous nanoparticles, such as silver, chitosan, titanium dioxide, and zinc oxide, have antibacterial properties that are effective against several pathogen genera [22,23]. Previous studies in plant tissue culture have demonstrated the beneficial effects of NPs on the formation of calluses, shoot growth, and multiplication [24,25]. However, the genotype of the plant and the type, size, and concentration of the nanoparticles significantly impact how well plants perform when exposed to nanoparticles [26]. AgNPs treatments stimulate growth and delay explants senescence of *in vitro* growth of Tecomella undulate [27] and Brassica juncea [28]. The antimicrobial activity of chitosan nanoparticles against different plant pathogens was reported previously [29,30]. Chitosan application improves plant growth and photosynthesis and increases plant resistance to abiotic stress [31,32]. Chitosan NPs have the advantages of biocompatibility, biodegradability, and safety [33]. Selenium NPs have wide applications in medical and agricultural fields [34]. It was reported to stimulate callus induction, improve tobacco morphogenesis, and increase shoot length and shoot number of *in vitro* growing artichoke [35]. More research is needed to fully understand the potential impact of nanoparticles on in vitro plant tissue culture. The current study, therefore, sought to ascertain how silver, chitosan, and selenium nanoparticles affected microbial contamination and how they affected the micropropagation of three important olive cultivars in an Egyptian environment (Koroneiki, Picual, and Manzanillo).

2. Materials and Methods

2.1. Chemicals.

Absolute ethanol, ascorbic acid, chitosan, silver nitrate, sodium hydroxide, and acetic acid were all of the analytical grade and came from the Chem Lab in Zedelgem, Belgium; sodium selenite, SD Fine-Chem Limited in Mumbai, India; and tri-sodium polyphosphate from Lab-Scan Analytical Sciences in Sowinskiego, Poland (Dop Organik Kimya- Ankara, Torkiya). All solutions were made using conventional techniques and deionized water.

2.2. Preparation of silver, selenium, and chitosan nanoparticles.

Silver nanoparticles (AgNPs) were prepared using the supernatant-free cells of *Fusarium oxysporum* [36]. The cell-free supernatant was collected by filtering after a 250 ml

flask containing 100 ml of potato dextrose broth (PDB) medium was inoculated with *Fusarium oxysporum* and incubated for 3 days at 28 C on a rotating shaker (Whatman No.1). The 1:1 mixture of the cell-free supernatant and 1M silver nitrate solution was incubated under static conditions for 24 hours at room temperature. AgNPs were produced by centrifugation at 10,000 rpm for 10 min, three deionized water wash cycles, absolute ethanol wash cycles, and oven drying at 50 °C [37]. In the case of selenium nanoparticle preparation, stock solutions of 100 mM sodium selenite and 50 mM ascorbic acid were prepared in deionized water. The sodium selenite solution and ascorbic acid were combined dropwise while being stirred magnetically (at 600 rpm) at room temperature until the final ratio of ascorbic acid to sodium selenite was 1:4. Until the colorless solution turned light orange, the mixtures were left to react [38]. Tri-sodium polyphosphate (TPP) was used in the ionic gelation process to create chitosan nanoparticles [39]. The pH of the chitosan solutions was adjusted to 5.9 using 1M sodium hydroxide solution, and TPP solution was prepared by dissolving 0.1g in 100ml deionized water. The medium molecular weight chitosan was dissolved in 100 ml of 1% (v/v) acetic acid solution by continuous stirring overnight at room temperature [40]. Chitosan nanoparticles were made by continuously swirling (550 rpm) a 35 ml chitosan solution while adding 14 ml of TPP solution dropwise. Centrifugation at 10,000 rpm for 10 min was used to extract chitosan nanoparticles. The pellet was cleaned with distilled water, completely ethanol, and air dried.

2.3. Characterization of the synthesized nanoparticles.

Utilizing transmission electron microscopy, the size and morphology of the produced nanoparticles have been examined (JEOL, JEM 1400, USA). Samples were created by drop-coating a synthetic nanoparticle solution onto copper grids coated with carbon. The additional solution was removed after the films on the TEM grids had been let to stand for two minutes, and the grid was then left to dry before being measured. TEM images were captured using an 80 kv accelerating voltage.

2.4. Effect of synthesized nanoparticles on in vitro microbial contamination.

Silver at 5 and 10 mg l^{-1}, selenium at 2.5 and 5 mg l^{-1}, and chitosan at 40 and 60 mg l^{-1} were added to the non-sterilized Rugini olive medium [41] supplemented with 30 g l^{-1} mannitol and 6 g l^{-1} agar in order to validate the effectiveness of the synthesized nanoparticles on microbial contamination. These additions were compared to non-sterilized free nano (autoclave sterilized free nanoparticles medium). The prepared media were placed in glass Petri dishes, which were then incubated at 25°C for 48 h with a 16-hour photoperiod. Fungus or bacteria colony formation was observed by visually inspecting the incubated Petri dishes. The visible colonies were counted, and the contamination percentage was calculated in relation to the negative control treatment.

2.5. Plant materials and explants preparation.

Active growing shoots of 30 to 40 cm were collected during June and July from mature own-rooted olive trees of 'Koroneiki', 'Picual', and 'Manzanillo' cultivars, grown at an experimental olive orchard (031°12'65"E longitude, 30°00'48"N latitude, Giza, Egypt). Olive shoots were gathered from each cultivar and transported right away to the tissue culture facility, where they were defoliated, cut into nodal cuttings, and repeatedly washed under running water. The nodal segments were treated with 0.1% mercuric chloride ($HgCl_2$) for 5 minutes

after being surface sterilized with 20% commercial bleach containing 5.5% sodium hypochlorite for 10 min. The nodal segments were then rinsed with distilled and sterilized water.

2.6. Effect of the synthesized nanoparticles on in vitro performance of olive explants.

Nodal cuttings that had undergone sterilization were grown in Rugini olive medium [41] with the addition of 30 g mannitol, 2.5 mg zeatin, and 6 g agar. The media were autoclaved at 121°C for 15 minutes after the pH was adjusted to 5.8. The cultures were cultured in a growth room at 25°C with a cool white fluorescent lamp providing 4000 lux light intensity for 16h/8h light/dark photoperiods. Four weeks later, the sprouted axillary shoots were moved to fresh Rugini olive medium supplemented with one type of the aforementioned nanoparticles, including chitosan NPs at 40 and 60 mg l^{-1}, selenium NPs at 2.5 and 5 mg l^{-1}, and silver NPs at 5 and 10 mg l^{-1}, in addition to the control free nanoparticles medium. Before autoclaving, the nanoparticles were introduced to the proliferation medium. Each treatment consisted of 20 jars, and four explants were grown in a 200 ml jar with 50 ml of semi-solid media and incubated as described previously. The sub-culture was performed every four weeks. In the 3rd subculture, the number of shoot per explant shoots length, multiplication rate, and the number of leaves per shoot was determined. The multiplication rate is calculated as the total number of shoots per explant multiplied by the number of potentially nodal cuttings per shoot [12].

2.7. Experimental design and statistical analysis.

The experiment was carried out in a completely randomized design, and the assumptions of normality were tested using Shapiro–Wilk's test [42]. The effect of olive genotype, nanoparticle treatments, and their interaction was examined using a two-way analysis of variance using the SAS software (version 9.0; SAS Institute, Cary, NC, USA) on normally distributed data [43]. Three replicates per treatment were used to calculate the mean and standard error (SE), and repeated Duncan range tests with a significance level of 0.01 were used to determine whether there were any significant changes within and between treatments [44].

3. Results and Discussion

3.1. Characterization of the synthesized nanoparticles.

The TEM instrument characterized the produced silver, chitosan, and selenium nanoparticles. The diameter and shape of the tested nanoparticles are illustrated in Figure 1. TEM micrograph demonstrated the formation of a spherical shape with a narrow range of particle size distribution; the spherical nanoparticles were produced with sizes of 5-15 nm (Silver NPs), 15-35 nm (Selenium NPs) and 20-50 nm (Chitosan NPs). Analysis of the TEM micrograph demonstrated that the mean size of the obtained nanoparticles is comparable to the particle size that has been reported in previous studies for silver [45], selenium [46], and chitosan nanoparticles [26]. Therefore, the prepared nanoparticle in our study represented a typical nanoparticle in terms of shape and size.

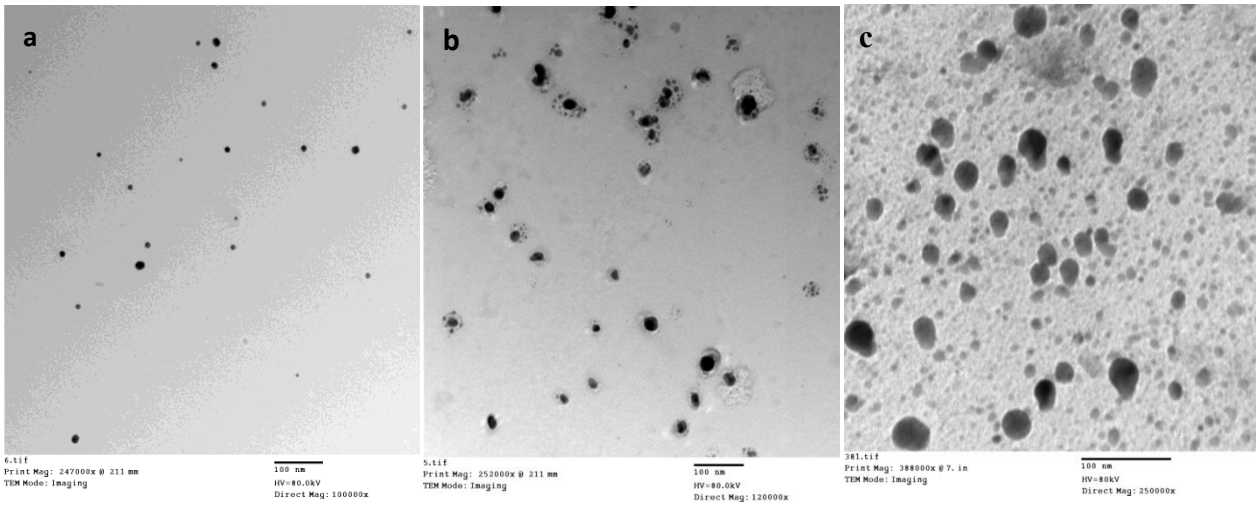

Figure 1. the transmission electron microscope (TEM) micrographs of (**A**) silver; (**B**) selenium; (**C**) chitosan nanoparticles.

3.2. Effect of synthesized nanoparticles on in vitro microbial contamination.

The obtained results suggested a substantial difference between the various nanoparticle types and concentrations on microbial contamination with regard to the variation in inhibitory potential of the produced nanoparticles on *in vitro* microbial contamination (Figure 2). When compared to the negative control plates, silver nanoparticles at a concentration of 10 mg l^{-1} recorded the lowest value of microbial contamination in the tissue culture media. Selenium nanoparticles achieved the greatest value, while chitosan nanoparticles came in second. The results indicated the feasibility of applying the nanoparticles in the tissue culture medium as antimicrobial agents without sterilization. The inhibition potential of nanoparticle agents *in vitro* growth of microorganisms confirms our previous studies about the antimicrobial activity of the tested nanoparticles [19,39].

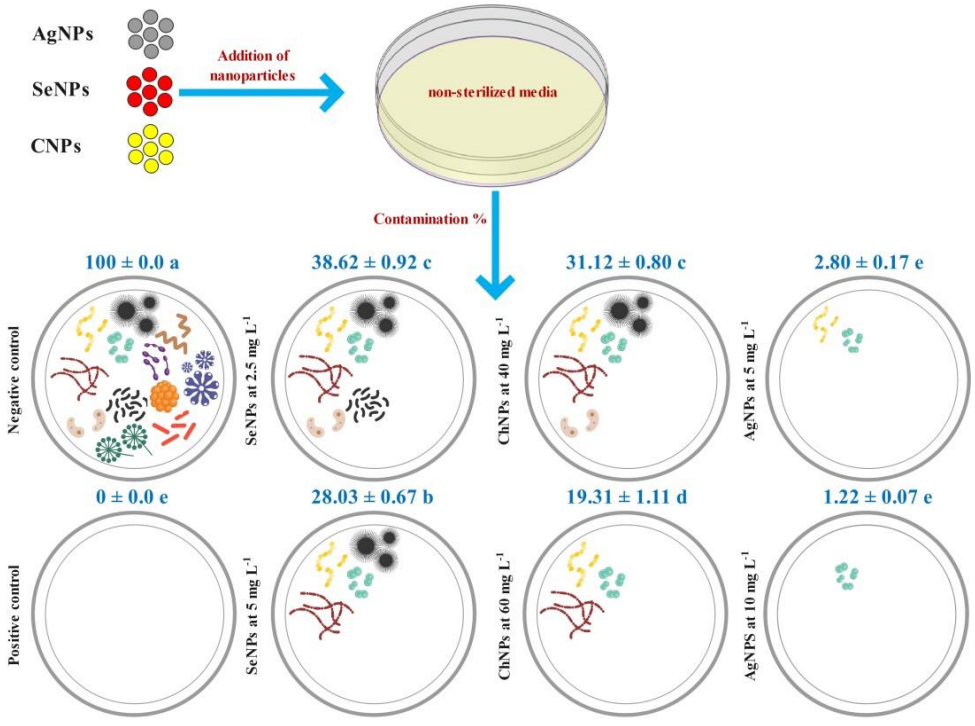

Figure 2. Flowchart about the effect of silver, selenium, and chitosan nanoparticles on *in vitro* microbial contamination; AgNPs, silver nanoparticles; SeNPs, selenium; ChNPs, chitosan nanoparticles; negative control (non-sterilized medium); positive control (autoclaved medium).

Silver nanoparticles (AgNPs) recorded the lowest contamination percentage, statistically comparable to media sterilized by autoclaving, indicating that silver NPs have a great potential for removing microbial contamination in the culture medium (Figure 2). Silver's significant toxicity to various microbes may be the cause of the high antimicrobial activity of AgNPs [13,17]. Moreover, the small particle size (5-15 nm) of the obtained silver nanoparticles is important for interactions and binding silver with cell membrane proteins, resulting in cell death [47].

Chitosan nanoparticles showed relatively high antimicrobial activity compared with selenium and control treatment; the antimicrobial activity of ChNPs against different plant pathogens was reported previously [29,30]. Chitosan NP affects cell membrane permeability and inhibits DNA replication [48]. Our results showed that SeNPs have low antimicrobial activity compared to AgNPs and ChNPs; the low toxicity of SeNPs may be due to the negative charge of SeNPs, resulting in a relative aversion of the bacterial membrane [49]. Also, higher concentrations of SeNPs may be required to affect microbial contamination significantly. A previous study showed that 50 mg l^{-1} is the minimum dose for inhibiting *E. coli* and *S. aureus* [50]. According to Nguyen *et al*. [49], SeNPs exhibited dose-dependent antimicrobial properties; 10 mg l^{-1} inhibited *in vitro* growth of *S. aureus* but did not affect *E. coli*, *Salmonella*, and *L. monocytogenes*.

3.3. Effect of the synthesized nanoparticles on in vitro performance of olive explants.

According to the data in Table 1, both plant genotype and nanoparticle significantly impacted the in vitro growth of olive shoots. Picual recorded a larger number of shoots/explant than Koroneiki and Manzanillo. Selenium NPs treatments recorded the lowest value for the number of shoots/explant, while silver NPs treatments recorded the maximum number of shoots/explant (1.770.43 and 2.280.56, respectively). There was no statistically significant difference between the chitosan NPs and the control treatment.

When compared to Picual and Manzanillo cv., Koroneiki cv. had the longest shoots, as indicated in Table 2. It is clear that, compared to the control treatment, adding nanoparticles to the culture media impacted the growth of in vitro cultivated olive shoots. Treatments with nanoparticles substantially impacted shoot length; SeNPs at 5 mg l^{-1} recorded the lowest value, and AgNPs at 10 mg l^{-1} the highest value.

Table 1. The effect of nanoparticles type and concentration on shoot number of different olive cultivars.

Treatments	Concentration (mg l^{-1})	Manzanillo	Picual	Koroneiki	Mean
Selenium NP	2.5	1.00±0.00 g	1.50±0.05 e	1.30±0.05 f	1.27±0.07D
	5	1.00±0.00 g	1.00±0.00 g	1.00±0.00 g	1.00±0.00E
Silver NP	5	1.20±0.05f	2.00±0.00bc	2.12±0.02 b	1.77±0.14B
	10	1.83±0.05 cd	2.00±0.11bc	3.00±0.05 a	2.28±0.18A
Chitosan NP	40	1.00±0.00 g	1.80±0.20 d	1.30±0.00 f	1.36±0.11C
	60	1.00±0.00 g	1.00±0.00 g	1.00±0.00g	1.00±0.00CD
Control	--------	1.00±0.00 g	2.00±0.00bc	1.00±0.00 g	1.33±0.16CD
Mean		1.14±0.06 B	1.61± 0.09 A	1.53±0.15 A	

Values of interaction (treatments x cultivars) followed by different lowercase letters are significantly different ($p < 0.01$). Mean values of treatment or cultivar followed by different uppercase letters are significantly different ($p < 0.01$); each value represents a mean of three replicate± standard error (SE).

As axillary shoot production is severely constrained by the high apical dominance of olive shoots, olive shoot multiplication is accomplished by segmenting elongated shoots at each subculture [6,12]. Comparing Picual cv. to both Manzanillo and Koroneiki olive cultivars,

the rate of multiplication of the farmed olive branches was higher (Table 3). Regarding multiplication rate, there was a significant difference between the nanoparticle treatments; silver NPs at both concentrations increased it compared to the other treatments for the studied olive cultivars, whereas selenium NPs at 5 mg l^{-1} and chitosan NPs at 60 mg l^{-1} produced the lowest value.

Table 2. The effect of nanoparticles type and concentration on shoot length (cm) of different olive cultivars.

Treatments	Concentration (mg l^{-1})	Manzanillo	Picual	Koroneiki	Mean
Selenium NP	2.5	6.0±0.57c	6.0±0.57c	7.0±0.57bc	6.33±0.33B
	5	3.0±0.00d	4.0±0.00d	3.0±0.00d	3.33±0.16D
Silver NP	5	6.0±0.57c	6.0±0.00c	8.0±0.57b	6.67±0.41B
	10	7.0±0.00bc	8.0±0.57 b	10.0±0.00 a	8.33±0.47A
Chitosan NP	40	7.0±0.57bc	6.0±0.00 c	6.5±0.28bc	6.50±0.24 B
	60	4.0±0.00d	5.5±0.00c	6.0±0.57c	5.17±0.34C
Control	-------	7.0±0.00bc	6.0±0.00 c	7.0±0.57bc	6.67±0.24B
Mean		5.71± 0.35 B	5.92± 0.26 B	6.78± 0.46 A	

Values of interaction (treatments x cultivars) followed by different lowercase letters are significantly different ($p < 0.01$). Mean values of treatment or cultivar followed by different uppercase letters are significantly different ($p < 0.01$); each value represents a mean of three replicate± standard error (SE).

Table 3. The effect of nanoparticles type and concentration on multiplication rate of different olive cultivars.

Treatments	Concentration (mg l^{-1})	Manzanillo	Picual	Koroneiki	Mean
Selenium NP	2.5	3.0±0.12d	3.2±0.05d	2.2±0.12e	2.80±0.16C
	5	1.2±0.00fg	1.3±0.00f	1.2±0.00fg	1.23±0.07E
Silver NP	5	3.2±0.00d	4.3±0.05b	4.8±0.12 a	4.10±0.24B
	10	4.3±0.17b	4.0±0.00c	5.0±0.00 a	4.43±0.16A
Chitosan NP	40	2.0±0.00 e	2.0±0.00e	1.3±0.12f	1.77±0.12D
	60	1.0±0.00 g	1.2±0.00fg	1.1±0.00fg	1.10±0.03E
Control	--------	2.0±0.00e	2.0±0.00e	1.1±0.00fg	1.70±0.15D
Mean		2.38± 0.24 B	2.57±0.26 B	2.38±0.36 A	

Values of interaction (treatments x cultivars) followed by different lowercase letters are significantly different ($p < 0.01$). Mean values of treatment or cultivar followed by different uppercase letters are significantly different ($p < 0.01$); each value represents a mean of three replicate± standard error (SE).

The data in Table 4 demonstrated that Picual cv. had the most documented leaves. There was a statistically significant difference in the number of leaves between the various nanoparticle treatments compared to both Manzanillo and Koroneiki olive. The highest leaf number was obtained with silver NPs at 10 mg l^{-1}, followed by silver NPs at 5 mg l^{-1}, while the lowest leaf number was obtained with selenium NPs at 5 mg l^{-1} and chitosan NP at 60 mg l^{-1}.

Table 4. The effect of nanoparticles type and concentration on leaf number of different olive cultivars.

Treatments	Concentration (mg l^{-1})	Manzanillo	Picual	Koroneiki	Mean
Selenium NP	2.5	9.0±0.57cd	10.0±0.57c	6.0±0.57fg	8.33±0.66C
	5	4.0±0.00hi	5.0±0.00gh	4.0±0.00 hi	4.33±0.17E
Silver NP	5	8.0±0.00de	10.0±0.57c	12.0±0.57b	10.00±0.62B
	10	12.0±0.57b	10.0±0.57c	14.0±0.57a	12.00±0.65A
Chitosan NP	40	6.0±0.00fg	7.0±0.00ef	6.7±0.33efg	6.55±0.18D
	60	3.0±0.00 i	6.0±0.00fg	6.7±0.66efg	5.22±0.60E
Control	--------	7.0±0.57ef	7.0±0.00ef	6.0±0.00fg	6.67±0.24D
Mean		7.00± 0.64 B	7.85± 0.45 A	7.90± 0.76 A	

Values of interaction (treatments x cultivars) followed by different lowercase letters are significantly different ($p < 0.01$). Mean values of treatment or cultivar followed by different uppercase letters are significantly different ($p < 0.01$); each value represents a mean of three replicate± standard error (SE).

The nanoparticle application may stimulate or inhibit effects on plant growth and development; the impact of nanoparticles on plant growth depends on particle size, shape, concentrations, plant genotype, and age [51].

The outcomes demonstrated that nanoparticles had a beneficial impact on olive cultivars' *in vitro* multiplication and shoot growth. As previously observed, silver ion (Ag^+) prolongs senescence and increases the survival of plant shoots growing *in vitro* [27], improves somatic embryogenesis [52], organogenesis [53], and increases shoot multiplication rate and plant growth [54]. Due to the impact of Ag+ as an ethylene blocking agent, AgNPs increased the number of shoots per explant in *Brassica juncea*, *Tecomella undulate* Roxb., and *Vanilla planifolia* [25]. Selenium is not a necessary nutrient for higher plants, and their effects vary depending on concentration. Selenium at low concentrations can stimulate plant growth, whereas higher doses have an inhibitory effect. It was reported that selenium nanoparticles stimulate callus induction and improve the morphogenesis of tobacco and artichoke [35]. Recent studies showed that selenium stimulates shoot growth and increases the fresh and dry weight of *in vitro* growing olive shoots [56]. The greater absorption and mobility of SeNPs in the *in vitro* growing olive shoots may account for the observed results, which showed that SeNPs had a detrimental influence on the growth of shoots of several olive cultivars. Additionally, little information is available regarding chitosan NPs' effects on plant development when used *in vitro*. Chitosan NPs added to the culture medium at the proper concentration encourage the growth of the shoots. The toxicity of chitosan NP was higher than that of the chitosan bulk type, which may be attributable to the differences in physicochemical properties of chitosan NP compared with the chitosan bulk type[26]. However, the higher dosage drastically reduced plant growth and development. The application of nanoparticles in culture media should be optimized to avoid the toxicity of high concentrations; according to our findings, the higher concentration of selenium and chitosan NPs had a negative effect on olive shoot growth. This is true despite the fantastic performances and the encouraging results of nanoparticle application under *in vitro* conditions. Numerous studies have demonstrated that greater NP concentrations negatively impacted plant regeneration, organogenesis, shoot growth, and cell viability [20]. NPs affect mitotic activity and change different plant species' DNA structure and gene expression [57]. According to Nakasato *et al*. [58], a high concentration of chitosan NP severely inhibited germination and negatively affected the growth of *Zea mays*, *Brassica rapa*, and *Pisum sativum*. Se toxicity may be due to the replacement of sulfur atoms by selenium in sulfur-containing amino acids, which results in changes in protein structure and function; simultaneously, selenium can cause oxidative stress and cellular damage and disrupts plant metabolism, and reduce plant growth. Therefore, the effects of different types and concentrations of NPs on plant tissue should be optimized to determine the optimum dose with minimal phytotoxicity [54].

4. Conclusions

According to the obtained results, the tested nanoparticles showed varied antimicrobial activity; AgNPs were highly effective in inhibiting in vitro microbial contamination, and ChNPs and SeNPs showed low antimicrobial activity. The addition of nanoparticles to the culture medium significantly affected the growth and multiplication rate of *in vitro* growing olive shoots. Higher concentrations of chitosan and selenium NPs had a negative impact on the growth behavior and multiplication of olive shoots grown in vitro; however, AgNPs had a good effect on both.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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