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Effect of Silver Nanoparticles, Medium Composition and Growth Regulators on *in vitro* propagation of Picual Olive Cultivar

Hegazi E. S.[a], Aml R. M. Yousef[b]*, Abdallatif A. M.[a], Thanaa Sh. M. Mahmoud[b] and Mona K.M. Mostafa[b]

[a]*Pomology Department, Faculty of Agriculture, Cairo University, Giza, Egypt*

[b] *Horticultural Crops Technology Department, National Research Centre, 33 El-Bohouth St., Dokki, Giza, Egypt*

Abstract

Micro-propagation of olive provides the possibility of mass production true to type plants with appropriate attributes in short period of time, compared to conventional methods. Micropropagation of olive requires optimization of protocols adapted to specific cultivars requirements. This experiment was carried out during 2020 and 2021 seasons in tissue culture laboratory of Pomology Department, Faculty of Agriculture, Cairo University in order to evaluate the potential effects of Nano silver at 0, 5, 10 and 20 mg L^{-1} to improve sprouting percentage and shoot growth of Picual olive cv. The effect of nutrient media composition (MS modified, OM and OM modified) and cytokinins (BAP and Zeatin) on multiplication rate and shoot growth were examined. IBA and NAA (2 and 4 mg L^{-1}) were examined during rooting stage. The obtained results cleared that silver Nanoparticles (AgNPs) at 5 mg L^{-1} recorded the highest percentage of bud sprouting, shoot length, number of shoots/explant and number of leaves/ shoot. Olive medium (OM) recorded the highest shoot length, number of shoots/explant and number of leaves/ shoot compared with OM modified and MS modified media. Also, Zeatin at 4 mg L^{-1} recorded the highest shoot growth parameters during *in vitro* multiplication stage. During *in vitro* rooting stage, both the type and concentration of auxins have a significant influence on number of roots, root length and rooting % of Picual olive cultivar. In conclusion, it is recommended that very low concentrations of Nano silver particles to be used in plants tissue culture on olive media (OM) supplemented with Zeatin at 4mg L^{-1}.

Keywords: Nano-silver particles , disinfection, *in vitro* micropropagation, media composition, olive trees

1. Introduction

The olive tree (*Olea europaea* L.) is an ancient traditional crop tree found throughout the world but mainly farmed in Mediterranean countries, where is it considered one of the most suitable and best-adapted species to the Mediterranean-type climate [1,2]. Techniques of *in vitro* tissue culture have been widely employed in area of agriculture, horticulture, forestry and plant breeding. It is used for mass propagation, virus elimination, secondary metabolite production and *in-vitro* cloning of plants. Recently, plant tissue culture has been used for the conservation of endangered plant species through short and medium-term conservation [3]. Micropropagation technique allows propagating of fruit species which are hard to propagate conventionally [4]. These include axillary bud stimulation, organogenesis, and somatic embryogenesis [5]. Successful olive micropropagation is highly dependent on cultivar and shoot proliferation rate [6]. Various micropropagation factors like the source and age of explants, plant growth regulators (PGRs), media composition and carbon source affected multiple shooting [7].

Plant growth regulators (PGRs) play an essential role in determining the development pathway of plant cells and tissues in culture medium; Zeatin recorded the highest proliferation rate [7], similarly IBA (Indole-3-butyric acid) was the most efficient PGR in inducing root formation in the micro shoots, Naphthalene acetic acid and indole acetic acid help in promoting *in vitro* rooting. Media composition and carbon source also affected the regeneration efficiency [8, 9].

OM medium, MS medium and modified MS medium, are the most suitable media for olive micropropagation [10- 13]. Olive medium proved to be the most effective

*Corresponding author e-mail: amlgabr@yahoo.com

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one for resulting a better micro shoots compared to woody plant medium [14]. 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 role of NPs in callus induction, organogenesis, somatic embryogenesis, soma clonal variation, genetic transformation and secondary metabolite production [15]. 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 [16]. This capability of 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 [17].

The present study was undertaken to contribute the continuous search for efficient *in vitro* protocol for the micropropagation system of olive plants cv. Picual.

2. Experimental

2.1. Plant material

The current research was carried out at the laboratory of Pomology Department, Faculty of Agriculture, Cairo University. Olive shoots were collected from mature, 20-year-old, healthy olive trees (*Olea europaea* L.) cv. 'Picual' cultivated in the orchard of Pomology Department. Active growing shoots were collected from the tree in the morning during spring of 2020-2021 seasons.

2.2. Explant sterilization

Olive shoots were immediately brought to the laboratory; shoots were stripped of leaves, washed with tap water and divided into nodal cuttings. Surface sterilization was performed with 20% commercial bleach (5.25% sodium hypochlorite) for 10 min, followed by mercury chloride at 1000 mg L^{-1} for 5 min. Finally, explants were washed three times by sterile distilled water for 5 min.

2.3. Silver nanoparticles preparation

The silver nanoparticles were synthesised using the chemical reduction method of **[**18]; $AgNO_3$, $NaBH_4$ 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 $NaBH_4$ (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 colourless solution of $NaBH_4$-PVP was slowly changed from yellow to pale brown indicating the formation of silver nanoparticles

2.4. Silver nanoparticles characterization

The dimension and form of the produced nanoparticles, including the size and shape of the synthesised nanoparticles, was determined using transmission electron microscopy (JEOL JEM-1400, USA). A drop (2 ul) of Milli-Q water, which dissolved synthesised 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.

UV-Vis spectroscopy

The Ag nanoparticles were characterised using UV/VIS Spectrophotometer (T80, PG Instruments Ltd) in at the Cairo University Research Park. The scanning range for the samples was 200-700 nm. Millie-Q water was used as a blank reference.

2.5. Starting stage and silver nanoparticles treatments

During starting stage olive media (OM), due to **[**13**]**, was supplemented with the fresh prepared AgNPs **[**16**]**. Sliver nanoparticles were used at 0, 5, 10 and 20 mg L^{-1}. All media were supplemented with 30 g L^{-1} mannitol, 2mg L^{-1} Zeatin and 6g agar L^{-1} and autoclaved at 121°C for 15 min. Four explants were cultured on 50 ml of semi-solid medium and maintained in the growth chamber at 25±2°C and 16h photoperiod provided by cool-white fluorescent lamps. Four weeks later, the sprouted buds, number of shoot per explant, shoots length and number of leaves per shoot were recorded.

2.6. Multiplication stage

Multiplication stage was undertaken to explore and evaluate the effect of nutrient medium composition, Zeatin and Benzyl amino purine (BAP) concentration on proliferation rate of the explants and it included two parts.

Part1: Sprouted buds were transferred to one of the three different medium Table 1, namely olive media (OM), as described by [13], MS modified [19], and OM modified [20]. Each medium was supplemented with 2 mg L^{-1} Zeatin.

Part 2: Sprouted buds were transferred to olive medium was supplemented with three different concentrations of Zeatin or BAP (2, 4 or 6 mg L^{-1}). Sub-culture was performed every four weeks. All culture media combinations were supplemented with

30 g L^{-1} sucrose and 6.5 g agar L^{-1}, media pH was adjusted to 5.8 before adding agar and autoclaved at 121°C for 15 min. All cultures were maintained in growth chamber at 25°±2C and 16h photoperiod (provided with cool white fluorescent lamps).

Number of shoots per explant, shoot length, multiplication rate and number of leaves per shoot were determined; shoot multiplication was achieved by segmentation of elongated shoots at each subculture [21-22]. Thus multiplication rate was calculated as the total number of shoots per explant multiplies by the number of potentially nodal cuttings per shoot at subculture.

2.7. Rooting stage

The aim of this step was to explore and evaluate the response of olive shoot to auxin treatment and media composition during rooting stage. Shoots of the 3rd sub-culture (2-3 cm in length) were used for rooting treatments. The rooting steps were consisted of culturing olive shoots on three different medium namely olive media (OM) as reported by [13], MS modified [19], and OM modified [20] supplemented with 2mg L^{-1} of IBA; in a separated experiment to examine the effect of auxin type and concentration.

Olive shoots were cultured on olive medium (OM) supplemented with one of the following auxin treatments 2 and 4 mg L^{-1} of IBA and NAA. Darkening of rooting media was performed with 1g L^{-1} active charcoal. At the end of rooting stage root length (cm**),** root number per plant, and rooting percentage were calculated.

Statistical analysis

The design of this experiment was a complete randomized design (CRD) with three replications [23]. Data were subjected to analysis of variance (ANOVA) using procedure of MSTAT-C software statistical package [24]. Differences between treatments means were compared by using least significant difference (LSD) tests at 5% level of probability according to [25].

3. Results and discussion

3.1. Characterization of silver nanoparticles

TEM image of the synthesised silver nanoparticles (dark spherical objects) is shown in Figure 1, 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.

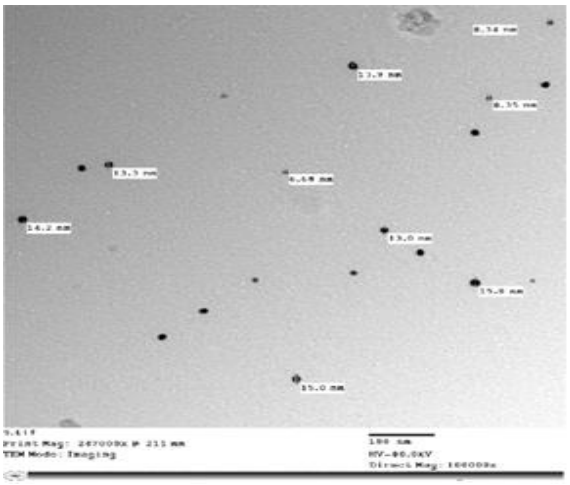


Figure1: The transmission electron microscope (TEM) micrographs of silver nanoparticles

3.2. UV-VIS spectroscopy

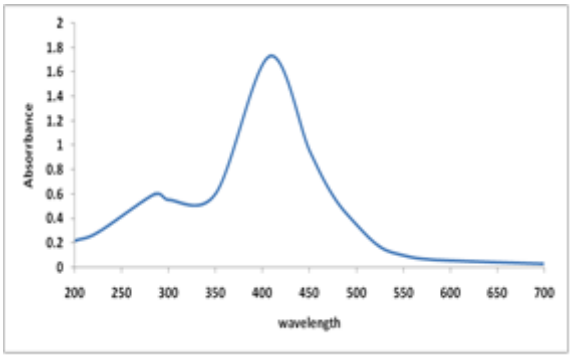
The UV-VIS spectrum of AgNPs is illilstrated in Figure 2. AgNPs have provided peaks of absorption between 200-700 nm. The UV-VIS spectrum of synthesised AgNPs gave absorbance peak at 407 nm.This absorption band is typical of AgNPs as reported perviousaly [26-27]. This single peak of the plasmon surface resonance revealed 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 [28].

Data presented in Table 2 showed the effect of adding different concentrations of silver nanoparticles (AgNPs) to OM on bud sprouting percentage and shoot growth of Picual olive during *in vitro* starting stage. It was obvious that the addition of silver nanoparticle to the culture medium had a significant effect on bud sprouting percentage and shoot growth of Picual olive compared with the control treatment. AgNPs at 5 mg L^{-1} recorded the highest bud sprouting percentage (82.33%), shoot length (4.45 cm), number of shoots/explant (1.54) and number of leaves/ shoot (11.13) followed by AgNPs at 10 mg L^{-1}. On the contrary, the higher concentration of AgNPs (20 mg L^{-1}) recorded the lowest value of bud sprouting %, number of shoots/explant and number of leaves/ shoot compared with the control.

These results are consistent with the previous observations Aghdaei *et al.* [29] and Hassan *et al.* [30] who found positive effects of nanoparticles (NPs) on improving growth vigour and development of the cultured olive explants *in vitro*. Moreover, silver nanoparticles treatment improved micropropagation efficiency [31-32], and increased survival and delayed

the explants senescence in many plant species [33], this may be due to the inhibition of ethylene action or its production; also, silver nanoparticles have shown stimulatory effects on *in vitro* plant regeneration [34]. The silver ion has a potent inhibitor on ethylene action [35] and stimulates the shoot regeneration in wheat or in maize tissue culture [36]. The obtained results confirm that, higher concentration of AgNPs (20 mg L^{-1}) has a negative effect on developing growth of olive shoots.

This result is in line with the previous studies by Vannini *et al.* [37] on wheat and Hassan *et al*. [30] on olive cultivars (Manzanillo, Picual and Koroneiki) since the high concentrations of AgNPs were frequently toxic to the plants and may otherwise delay or even inhibit the growth of plant tissues.



.Figure 2: UV/Vis spectrum of the synthesized silver nanoparticles

Table 1: Multiplication medium composition

Compound	Concentration (mg L^{-1})		
	MS modified	OM	OM modified
Ammonium nitrate[NH_4NO_3]	1031	412	1416.00
Potassium nitrate [KNO_3]	1500	1100.00	------
Boric acid [H_3BO_3]	12.40	12.40	12.40
Potassium dihydrogen phosphate [$KH_2 PO_4$]	255	340.00	265.00
Potassium iodide [KI]	0.83	0.83	--------
Potassium chloride [KCl]	250	500.00	---------
Potassium sulfate [K_2SO_4]	----------	----------	782.3
Sodium molybdate dehydrate [$Na_2 MoO_4 \bullet 2H_2O$]	0.25	0.25	0.2
Cobalt (II) chloride hexahydrate [$COCl_2 \bullet 6H_2O$]	0.025	0.025	-------
Calcium chloride [$CaCl_2$]	332.02	332.16	112.50
Calcium nitrate [$Ca(NO_3)2.2H_2O$]	300	416.92	1664.64
Magnesium sulfate [$MgSO_4$]	556.57	732.60	732.60
Manganese sulfate [MnSO4 • H2O]	16.90	16.90	33.80
Zinc sulfate heptahydrate [$ZnSO_4$ • 7H2O]	14.3	14.30	17.00
FeNaEDTA	36.70	36.70	44.63
Copper sulfate pentahydrate [$CuSO_4$ • 5H2O]	0.25	0.25	0.25
Nicotinic acid	5.00	5.00	1.0
Pyridoxine HCL	0.50	0.50	------
Thymine HCL	0.50	0.50	2.00
Glycine	2.0	2.0	2.00
Biotin	0.05	0.05	0.05
Folic acid	0.50	0.50	0.50
Glutamine	--------	--------	12
Myoinositol	100	100	100

Table 2: Effect of silver nanoparticles concentrations on bud sprouting percentage and shoot growth of Picual olive cultivar

Treatment	Bud sprouting (%)	Shoot length (cm)	No. of shoots/explant	No. of leaves/ shoot
Control	78.00 b	3.11 b	1.35 a	9.03b
AgNPs at 5 mg L^{-1}	82.33 a	4.45 a	1.54 a	11.13a
AgNPs at 10 mg L^{-1}	79.67 b	3.33 b	1.37 a	9.70 b
AgNPs at 20 mg L^{-1}	63.33 c	3.22 b	1.22 a	9.00 b

In this table and following ones, means followed by the same letter are not significantly different $p < 5\%$.

3.3. Multiplication stage

3.3.1. Effect of different nutrient media on shoot growth and multiplication rate of Picual olive cv.

Data in Table 3 showed the effect of different nutrient media compositions on shoot length, number of shoots/explant, number of leaves/ shoot and multiplication rate of Picual olive cultivar during the multiplication stage. According to the obtained results, olive shoot length was significantly affected by the type of nutrient media, while the number of shoots/explant and number of leaves/ shoot were slightly affected. OM medium recorded the highest shoot length, number of shoots/explant and number of leaves/ shoot compared with OM modified and MS modified media. The highest multiplication rate (%) was recorded for olive shoots on OM compared with MS and OM modified media.

3.3.2. Effect of types and concentrations of cytokinins on shoot growth of Picual olive cv.

The effect of different types and concentrations of cytokinins on in *vitro* growth of Picual olives growth on OM medium during the multiplication stage are shown in Table 4. It is clear that Zeatin was the most effective cytokinin for increasing the growth parameters compared with BAP. Zeatin at 4 mg L^{-1} recorded the highest shoot length (5.84 cm), while at 6 mg L^{-1}, it recorded the highest shoots/explant (1.72) and number of leaves/ shoot (10.78). As reported in earlier studies, *in vitro* multiplication efficiency in *Olea europea* species is widely dependent on culture medium, cytokinin type and its concentration [7]. Also, it seems that different olive tree cultivars require different basic medium formulations [38].

The results presented in the current study indicate that the best propagation performances attained for Picual olive cultivar were recorded for OM medium supplemented with Zeatin as a cytokinin. These results are in a line with those found by Rostami and Shahsavar [39], Hegazi *et al*. [40] and Hegazi *et al*. [41] they concluded that OM medium with 4 µM Zeatin appeared very satisfactory and gave the highest proliferation rate, number of shoots/explant, shoot height and number of leaves/shoot during multiplication stage. The improvement of growth achieved during the multiplication stage on the OM medium compared to OM modified and MS modified media is possibly due to the OM medium that enrich with potassium, and has been reported to affect the flux of other minerals such as nitrogen, phosphorous and carbon, and promotes the translocation of photosynthetic which in turn enhanced the quality of shoots [42, 14].

Table 3: Effect of nutrient media composition on shoot growth of Picual olive during *in vitro* multiplication stage

Media components	Shoot length (cm)	No. of shoots/ explant	No. of leaves/ shoot	Multiplication rate (%)
OM	5.47 a	1.69 a	10.50 a	7.18 a
OM modified	3.14 b	1.34 ab	8.87 ab	4.61b
MS modified	1.877 c	1.14 b	7.67 b	3.25c

Table 4: Effect of types and concentrations of cytokinins on the shoot growth of Picual olive cultivated on OM medium during *in vitro* multiplication stage

Cytokinin types and concentrations	Shoot length (cm)	No. of shoots/explant	No. of leaves/ shoot
Zeatin 2 mg L^{-1}	5.28 a	1.55 b	9.33 ab
Zeatin 4 mg L^{-1}	5.84a	1.58 ab	10.55 a
Zeatin 6 mg L^{-1}	5.44 a	1.72 a	10.78 a
BAP 2 mg L^{-1}	3.25 c	1.00 c	8.67 b
BAP 4 mg L^{-1}	4.33 b	1.09 c	9.44 ab
BAP 6 mg L^{-1}	4.33 b	1.14 c	10.00ab

3.4. Rooting stage

3.4.1. Effect of different nutrient media composition

As shown in Table 5 the nutrient media varied in its effect on the number of roots, root length and rooting percentage of Picual olive during *in vitro* rooting stage. Number of roots was not significantly affected by the different nutrient media**.** Concerning root length, MS modified medium produced the highest root length followed by OM without any significant difference among them. The highest rooting percentage (40.00%) was recorded with the OM medium, while OM modified medium recorded the lowest rooting percentage.

3.4.2. Effect of auxin types and concentrations

Both the type and concentration of auxins have significant influence on number of roots, root length and rooting percentage of Picual olive during *in vitro* rooting stage Table 6. The highest significant number of roots (3.25) was recorded in rooting medium supplemented with NAA at 4 mg L^{-1}, while the highest root length (8.72 cm) was recorded with rooting medium supplemented with NAA at 2mgL^{-1} without any significant difference than the previous concentration. The highest significant rooting percentage (58.67 %) was recorded for rooting medium supplemented with IBA at 4 mg L^{-1} followed by 4 mgL^{-1} NAA (46 %), while the lowest rooting percentage (14.67 %) was recorded in rooting medium supplemented with 2 mgL^{-1} IBA. Auxin is essential to induce rooting in the olive microcuttings as that exerts primary role in root formation by its involvement in successive and interdependent phases [43]. For rooting of olive, various auxins have been used, in particular IBA and NAA [44-45], the type and required concentration varies with different olive cultivars [7, 46]. The positive effect of NAA on the root number and root length may be due to that NAA is more persistent than IBA [47]. Hausman [48] has shown that in tissue culture media, IAA is photo-oxidized rapidly (50 % in 24 h), while IBA oxidized slowly (10 %) and NAA is very stable. Ansar *et al*. [49] reported that IBA produced rooting percentage higher than NAA and showed the tendency to increase with increasing concentration of IBA. This is probably because of IBA promoted the cell division of first root initials [50- 51], and may have triggered the early anticlinal cell division and root primordia formation than NAA. Sabatini *et al*. [52] reported that differentiation of phloem ray parenchyma cells into root primordia depends upon the type and concentration of the most appropriate auxin to become competent to respond to the organ genic signal.

Table 5: Effect of nutrient media on number of roots, root length and rooting% of Picual olive during *in vitro* rooting stage

Media	Number of roots	Root length (cm)	Rooting (%)
OM	1.50 a	8.08 a	40.00 a
OM modified	1.67 a	6.22 b	14.67 c
MS modified	1.55 a	8.67 a	23.33 b

Table 6: Effect of types and concentrations of auxins on number of roots, root length and rooting % of Picual olive during *in vitro* rooting stage

Auxins types and concentrations	Number of roots	Root length (cm)	Rooting (%)
IBA 2 mg L^{-1}	2.39 b	6.67 b	14.67 e
IBA 4 mg L^{-1}	2.65 b	7.90 ab	58.67 a
NAA 2 mg L^{-1}	2.75 b	8.72 a	39.0 c
NAA 4 mg L^{-1}	3.25 a	8.69 a	46.0 b

4. Conclusion

Based on the results obtained in the present study, it can be concluded that adding silver nanoparticles (AgNPs) to OM media at low concentrations of 5 mg L^{-1} improved the bud sprouting percentage and shoot growth. Zeatin at 4mg L^{-1} proved the better cytokinin for increasing the proliferation rate and growth parameters of olive cultivar during multiplication stage compared with BAP .Also, NAA proved to be better rooting hormone for Picual cultivar of olive in terms of rooting percentage, number of roots per rooted explant and root length as compared to IBA. Thus, these results will be helpful in the refinement of protocol for olive micropropagation in *in vitro*.

5. Abbreviations:

NPs: Nanoparticles; $HgCl_2$: Mercuric chloride; NaOCl: sodium hypochlorite; AgNPs: silver nanoparticles; NaBH4: Sodium borohydride; PVP: Polyvinylpyrrolidone; TEM: Transmission Electron Microscopy; CRD: completely randomized design; MS: Murashige and Skoog medium; OM: Olive media; BAP: 6-benzylaminopurine; NAA: Naphthalene acetic acid; IBA Indole-3-butyric acid; Ag: silver ion.

6. Conflicts of interest

The authors declare that they have no conflict of interest.

7. Formatting of funding sources

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