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Relevance of Nanoparticles on Micropropagtion, Antioxidant Activity and Molecular Characterization of *Sequoia sempervirens* L. Plant.

Iman M. El-Sayed[1], Walaa H. Salama[2*], Rasha G. Salim[3], Lobna S. Taha[1]

[1]*Department of Ornamental Plants and Woody Trees, Agricultural and Biological Research Division, National Research Centre (NRC), Egypt;*[2]*Department of Molecular Biology, Genetic Engineering and Biotechnology Research Division, National Research Centre (NRC), Egypt;*[3]*Department of Microbial Genetic, Genetic Engineering and Biotechnology Research Division, National Research Centre (NRC), Egypt.*

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Abstract

Micropropagation is essential in plant biology for the propagation of economic trees, plant improvement, genetic manipulation and bioactive compounds production. In recent years, the nanoparticles application has had successes in the improvement of plant characters, root induction, enhancing secondary metabolites. So, this study aims to examine the effect of three concentrations of Fe, Al, Zn and Ti nano oxides (2.5, 5 and 10 mg/L) on the improvement of growth behavior, pigment content, total phenolic and flavonoid contents, and specific activities of redox enzymes. In addition, to detect the genetic polymorphism between untreated and the best treated *Sequoia sempervirens* L. plant. Murashing and Skoog (MS) media at 75% of the original concentration containing 0.2 mg/L BA were individually fortified with nano Fe, Al, Zn, and Ti oxides, under standard control conditions *in vitro*. Selected morphological characters, total chlorophyll and total phenolic and flavonoid contents of treated explants were determined and compared with the untreated plant (control). The specific activities of peroxidase, catalase and polyphenol oxidase of plant groups were calculated using guaiacol, hydrogen peroxide and catechol as substrates under enzyme assay conditions, respectively. The results showed that the highest shoot number and length were 18.09 and 75.71 mm as well as the greatest root percentage and root number were 55.55% and 3.088 of shootlets treated with 10 mg/L Ti NPs. Whereas, the highest total chlorophyll, total phenolic and flavonoid contents were 220.35 mg/100g F.W, 3.41±0.28 mg GAE/g D.W and 1.67±0.12 mg CE/g D.W, respectively, with 5 mg/L Ti NPs treated shootlets. In addition, the treatment of shootlets with 10 mg/L Fe NPs has significantly increased the activities of the selected enzyme, whereas the maximum specific activity of polyphenol oxidase was 18.60 U/mg with shootlets fortified with 10 mg/L Ti NPs. Moreover, RAPD-PCR indicated that the superlative treatment with Ti NPs (10 mg/L) showed a low similarity comparable with control (74%). Briefly, Ti NPs shows a great enhancement in the morphological behavior of plant and, therefore, a high production *in vivo* in a short time.

Keywords: *Sequoia sempervirens* L., Nanoparticles, antioxidant enzyme, shooting, rooting, polymorphism, RAPD-PCR.

1. Introduction

Sequoia sempervirens L. known as redwood belongs to *Cupressaceae* Family (*Taxodiaceae*) and sets on the California Coast. Yet in addition, it is cultured in the whole world as ornamental plants in many gardens and parks. Many studies are interested in studying *S. sempervirens* L. because of its economic impact importance and on the other hand, for its beauty (Tosta *et al*., 2012). This species is being considered as an evergreen tree; also, it is the highest tree in the world and its diameter is very large (Farjon *et al*., 2006).

In vitro culture medium is an effective tool for faster growth and secondary metabolites induction (Khan, 2015 b). The methyl jasmonate (Me-J) and phenyl acetic acid (PAA) were used as elicitors for biosynthesis of many secondary metabolites *in vitro*. (Saeed *et al*., 2017; Kazmi *et al.,* 2019). Many compounds with medical importance were extracted from *Sequoia sempervirens* L. Arafat (2018) demonstrated the antitumor effect of the methanol extracts of various parts of Sequoia against colon, breast and lung tumors. Unfortunately, there are few *in vitro* studies on micropropagation of this coniferous tree by application varies sources of the explants (Sul *et al*., 1998). This may be according to previous studies mentioning that Sequoia is very difficult for formation roots, and its rooting percentage is very weak (Tosta *et al*., 2012; Sul and Korban, 2005).

Recently, various nanomaterials have been successfully used as new stimulators to improve the growth and characters of commercially important crops and plants as well as to enhance the accumulation and synthesis of the secondary metabolites. Therefore, the elucidation of the mechanism of interaction between plant and nanoparticles has been interesting to identify the activities of plants under control conditions in physiological, biochemical and

* Corresponding author e-mail: walaahsalama82@gmail.com.

molecular levels. (Khan *et al*., 2019; 2020 and Kumar *et al*., 2020).

In addition, application of modern agricultural biotechnology such as tissue culture technique can play an important role in improving the propagation of economic and commercial trees such as *S. sempervirens* L. Also, using the micropropagation tool for plants gave the highest production in a short time (Chadipiralla *et al*., 2020; Shatnawi *et al*., 2004; Chebet *et al*., 2003).

Furthermore, to meet the requirements of genetic variation in the horticulture industry, modern molecular techniques have been developed ranging from morphological characterization to different DNA-based markers include randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) (Ferdousi *et al*., 2013). For the conservation and utilization of plant genetic resources, the identification and characterization of germplasm is very crucial (Suvakanta *et al*., 2006). So, molecular marker is a perfect tool to characterize and preserve genetic assets of the plant. Besides, the Molecular characterization helps to determine the breeding behavior of species, individual reproductive success and consequently, the existence of gene flow, the movement of alleles within and between populations of the same or similar species, and its implications (Papa and Gepts, 2003).

In numerous plant species for population studies, genetic linkage mapping, and varieties analysis, RAPD procedure has been broadly used (Rout *et al*., 2003). The optimization of the RAPD analysis built upon the primers was selected. Though, this technique based on screening of several random sequences of primer to select primers gave positive amplification products. So, this work is implemented to detect the polymorphism between the control and three variable treatments of *Sequoia sempervirens* L. by using RAPD. Thus, this research will contribute basic knowledge in the aspect of their phylogenetic relationships and intra specific diversity.

In previous studies, different materials and methods were used as attempts to improve the induction of rooting formation in *Sequoia sempervirens* L. *in vitro* such as exposure of different laser radiation (red, blue, and green) (Taha *et al*., 2014), and various growth regulators as (NAA, BAP and Kinetin in the culture medium) (Menegnzzi *et al*., 2019). However, the results showed an improvement in shooting characters of *Sequoia sempervirens* L., but negative results in enhancing root formation (Taha *et al*., 2014; Menegnzzi *et al*., 2019). Therefore, the application of nanoparticles in MS culture media to understand the interaction between nanoparticles and *Sequoia sempervirens* L. plant gives attention not only to improve the morphological characters and stimulate the secondary metabolites of *Sequoia sempervirens* L. *in vitro* but also to enhance the root formation. So, this is the first attempt for studying the effect of the selected nano oxides (Fe, Al, Ti, Zn) on enhancing the root formation of the valuable *Sequoia sempervirens* L. tree *in vitro*.

This study aims to assess the effectiveness of different concentrations of the selected nano oxides (Fe, Zn, Al and Ti) on shooting and rooting ability, chemical composition, enzymes activities, and detection of the genetic variation of micropropagated *Sequoia sempervirens* L. Plantlets using *in vitro* culture technique for improving the formation of the roots of healthy quantitatively and qualitatively plants.

2. Materials and Methods

The experimental study was carried out during years 2018 and 2019 on *Sequoia sempervirens* L. at Central laboratories, Tissue Culture Technique Lab, Department of Ornamental Plants and Woody Trees, National Research Centre (NRC), Egypt.

2.1. Plant materials

The explants (stem node) of *Sequoia sempervirens* L. were taken from the adult tree at Orman Garden, Giza, Egypt, washed with liquid soap for 30 min, and then rinsed with running tap water for 1 h. The washed explants were immersed in 70% ethyl alcohol for 30 sec, then exposed to 15% sodium hypochlorite NaOH (Clorox +0.01% Tween 20) for 7 min, and then rinsed with sterile water three times. The explants were then sterilized in 0.1%$HgCl_2$ solution for 5 min, rinsed three times in sterile water under aseptic conditions.

2.2. Culture medium

The explants were cultured on MS culture medium at 3/4 strength of basal salts. The culture medium added with 0.2 mg/L of 6- benzyladenine (BA) and supplemented with sucrose 25g/L and solidified with 0.7% agar, adjusted to pH 5.7 with HCl and NaOH, then autoclaved at 121°C and 1.2Kg/ cm^2.

2.3. Culture condition parameters

The *in vitro* cultures were incubated in a growth chamber at 24 ± 1°C under fluorescent lamps with the light intensity of 3k lux at 16 h photoperiods.

2.4. Experiments treatments

The selected nanoparticles (NPs) used in this study were purchased from Sigma Co., USA, and the characterization of the selected NPs was done in Electron Microscope unit, NRC. The NPs were suspended in distilled water and dispersed uniformly according to Zafar *et al*. (2016) method. The MS culture media were grouped and treated separately by four nanoparticles Nano- Ferric Oxide (Fe NPs), Nano-γ-Alumina (Al NPs), Nano-Zinc Oxide (Zn NPs) and Nano-Titanium Oxide (Ti NPs) with varying concentrations (2.5, 5, and10mg/L) as follow:

S1: Control (3/4 strength of MS +0.2mg/L BA)
S2: Control + Fe NPs 2.5mg/L
S3: Control+ Fe NPs 5 mg/ L
S4: Control+ Fe NPs 10 mg/ L
S5: Control+ Al NPs 2.5 mg/ L
S6: Control+ Al NPs 5 mg/ L
S7: Control+ Al NPs 10 mg/ L
S8: Control+ Zn NPs 2.5 mg/ L
S9: Control+ Zn NPs 5 mg/ L
S10: Control+ Zn NPs 10 mg/ L
S11: Control+ Ti NPs 2.5 mg/ L
S12: Control+ Ti NPs 5mg/ L
S13: Control+ Ti NPs 10mg/ L

The specification of used nanoparticles is indicated in Table (1) and Figure (1; A, B, C and D).

Table 1. Specification of used nanoparticles.

Specification	Test method	
Nano- Iron Oxide		
Phase	Hematite	XRD
Particle size	<50 nm	TEM
Surface area	>50m^2/g	BET (P/Po: up to 0.35
Nano- γ- Alumina		
Phase	Gamma	XRD
Particle size	<100 nm	TEM
Surface area	>300m^2/g	BET (P/Po: up to 0.35
Nano- Zinc Oxide		
Phase	ZnO	XRD
Particle size	<30 nm	TEM
Surface area	~20m^2/g	BET (P/Po: up to 0.35
Nano- Titanium Oxide		
Phase	Anatase	XRD
Particle size	<50 nm	TEM
Surface area	>100m^2/g	BET (P/Po: up to 0.35

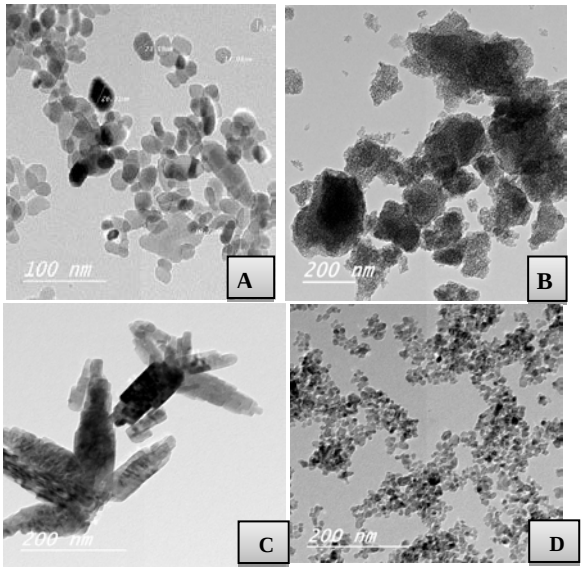

Figure 1. Scanning electron microscopy image of nanoparticles; A: Fe oxide NPs; B: Nano-γ-Alumina, C: Zn oxide NPs and D: Ti oxide NPs.

2.4.1. Shooting behavior

After incubation period of the experiment (three months), a number of formed shootlets per explant and Shootlet length (mm) were calculated for each group.

2.4.2. Rooting behavior

Percentage of roots formation (%), Number of roots /shootlet and Root length (mm) were determined for each group.

2.4.3. Hardening off

The number of roots produced, root length, and shoot height were registered after three months. Some of rooted plantlets were removed, washed, and then transferred from the rooting media to plastic pots containing a 1:1:1 ratio of peat, perlite, and clay. Newly potted plantlets were covered with polythene bags for three weeks before being moved to the green house.

2.5. Extraction and analysis

2.5.1. Photosynthetic pigments

The plants were grounded to powder using a mortar and extracting with 85% methanol. After that, the extracts were centrifuged for 10 min at 8000 rpm. The Photosynthetic pigments were assayed for the obtained supernatant according to Saric (1967) protocol using a UV–Visible spectrophotometer (UV-1280, Shimadzu, Japan). The equations and specific absorption in the wavelength are 660, 640, 440 nm to determine chlorophylls a, b and carotenoids, respectively.

2.5.2. Assessment of total phenolic and flavonoid content:

Total phenolic concentration was carried out according to Velioglu *et al.* (1998) using Folin-Ciocalteu reagent and gallic acid as a standard. Total phenols were monitored at 750 nm and the results are expressed as mg gallic acid equivalent (GAE)/ g dry weight tissue. Whereas, the total flavonoid content was calculated using a modified colorimetric method using $AlCl_3$ according to Zhishen *et al*. (1999) and used catechin as a standard. The absorbance was monitored at 510 nm and the results expressed as mg catechin equivalent (CE)/ g dry weight tissue.

2.5.3. Enzymatic assays

2.5.3.1. Peroxidase enzyme:

Peroxidase activities of the prepared plant extracts were carried out according to Miranda *et al.* (1995). The reaction mixture of 8 mM H_2O_2, 40 mM guaiacol and 0.1 ml crude extract to a final volume of 1 ml of 20 mM sodium acetate buffer, pH 5.5. The adjustment in absorbance at 470 nm was recorded for 1 min. One unit of peroxidase activity was defined as the enzyme concentration which increases the absorbance 1.0 per min under standard assay conditions and the specific activity is considered as units/mg protein.

2.5.3.2. Catalase enzyme:

The specific activity of catalase was determined according to Aebi (1974) using H_2O_2 as a substrate. The decrease in absorbance at 240 nm was recorded for 1 min and one unit of enzyme activity was defined as the enzyme concentration that hydrolyzes 1µm of H_2O_2per min under standard assay conditions. The specific activity is considered as units/mg protein.

2.5.3.3. Polyphenol oxidase enzyme:

The activity of polyphenol oxidase was assayed using catechol as a substrate according to Concellon *et al.* (2004). The increase in absorbance at 410 nm is recorded for 3 min. One unit of enzyme activity is defined as the enzyme concentration that causes a change of 0.1 in absorbance per min under standard assay conditions. The specific activity is considered as units/mg protein.

2.6. Extraction of genomic DNA.

Extraction of genomic DNA using DN easy plant mini kit (Qiagen Sciences, Maryland, USA) was carried out according to the manufacturer's instruction manual.

2.7. DNA fingerprinting

DNA fingerprinting was performed using ten RAPD-PCR primers to detect the polymorphism of three samples of *Sequoia sempervirens* L., these primers were synthesized by Metabion Corp., Germany. The sequences

of primers are showed in table (2). Reactions were performed in 25 µl assay mixture composed of 1x reaction buffer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, 0.2 µM of primer, 0.5 unit of *Taq* polymerase (Qiagen Ltd., Germany) and 50 ng of template DNA in sterile dist. water.

2.7.1. Amplification of RAPD-PCR

PCR amplification of the DNA was carried out using Perkin Elmer thermal cycler 9700. The temperature profile in the different cycles describes as follow: an initial strand separation cycle at 94°C for 5 min followed by 40 cycles comprised of a denaturation step at 94°C for 1min, an annealing step at 36°C for 1 min and an extension step at 72°C for 1.5 min, finally, the termination cycle for 10 min at 72°C. The PCR products were mixed with 5 µl of loading dye and resolved in 1.5 % agarose gel containing 0.5 mg/ml ethidium bromide in 1x TBE buffer at 100 volts using vertical gel electrophoresis apparatus. The resolved bands were visualized under ™XR+ Gel Documentation System (Bio-Rad).

2.7.2. Data analysis

For determination of genetic relationships between control and three selected treatment groups, the RAPD-PCR bands patterns were analyzed and compared with control group. The distinct and clear PCR products were scored as 1 for presence and 0 for the absence of bands. Bands of the same mobility have an identical score. The genetic similarity coefficient between control and the treatment groups were calculated according to Dice coefficient PAST program**.**

Table 2. RAPD primers name and sequence**.**

Primer name	Sequence (5'-3')
OPA-09	GGGTAACGCC
OPA-11	CAATCGCCGT
OPA-13	CAGCACCCAC
OPA-18	AGGTGACCGT
OPB-10	CTGCTGGGAC
OPB-11	GTAGACCCGT
OPB-12	CCTTGACGCA
OPB-15	GGAGGGTGTT
OPB-18	CCACAGCAGT
OPC-01	TTCGAGCCAG

2.8. Statistical analysis

The data were analyzed using a randomized complete design with three replicates per each treatment and were conducted using COSTATV-63 (Duncan, 1955); one way ANOVA (analysis of variance) was used to calculate the significance by new multiple range tests at $p<0.05$.

3. Results and Discussion

3.1. In vitro growth behavior

Micropropagation is one of the main advantages of use nanoparticles in agricultural biotechnology. Shooting and rooting characters that represent the *in vitro* growth behavior as a result of the application of four nanoparticles (Fe, Al, Zn and Ti oxides) at different concentrations (2.5, 5 and 10 mg/L) on *Sequoia sempervirens* L**.** were detected as shown in Table (3) and Figure 2. Shoot and root parameters include number and length of shootlets, rooting percentage, number and length of roots per shoot of untreated and treated groups were observed, calculated and compared with the control group (untreated explants in MS culture medium). Overall, number and length of shoots and roots of treated groups with different nanoparticles treatments were increased as compared to untreated group. Significantly, the highest values of shoot number and length were observed on shootlets that MS culture medium supplemented with Ti oxide NPs at concentration of 10 mg/L (18.09 and 75.71mm respectively) as compared to other treatments and control groups, whereas the minimum value of shoot number and length was observed in MS culture medium added with Zn oxide NPs at 5 mg/L (11.33 and 45.00 mm respectively). The same trend was observed for rooting behavior. Similarly, the highest rooting percentage and root number were recorded in the shootlets treated with Ti oxide NPs at 10 mg/L by (55.55% and 3.08) comparing with control and treated groups, while the longest root length was recorded on the shootlets treated with Al oxide NPs at 10 mg/L by 123.75 mm. In contrast, non-rooting was noticed in both untreated shootlets and medium treated with 2.5 and 5 mg/L of Zn oxide NPs.

These findings were confirmed by Zheng *et al*. (2005) who reported that Ti NPs increased germination and growth of spinach plant. Similarly, Albersheim *et al*. (2011) demonstrated that Ti NPs enhanced root and shoot length in the wheat seedling. These might be attributed to the small size of Ti NPs which help in increasing the ability to penetrate the seed and stimulate fast germination and growth of the plant. Furthermore, Tio_2 NPs was shown affectivity in the stimulation of gene expression of photosynthetic content, plant hormones metabolism and nitrogen metabolism (Yang *et al*., 2006; Albrecht *et al*., 2006) and these biosynthesis pathways might promote cell division, growth of plant and differentiation of plant cells (Song *et al*., 2013; Frazier *et al*.,2014). In other studies, Owje *et al*. (2019) improved the formation of roots of Fenugreek *in vitro* using Al NPs in the culture medium. Also, Zia *et al*. (2020) enhanced rooting reaction, number of roots/plant and root length in carnation cultivars by Ag NPs *in vitro*. Additionally, the application of Fe oxide NPs improved shoot and root growth parameters of *Moringa oleifera*, *Antigonon leptopus and stivia rebaudiana* plants (El-Sayed *et al*., 2019; El-Ziat *et al*., 2020; Khan *et al*., 2020). On the other hand, Desai *et al*. (2015) demonstrated that using Zn NPs reduced the morphological parameters of *Stevioside rebaudiana* plant *in vitro.*

Table 3. Effect of varied concentrations of four nanoparticles on *in vitro* shooting and rooting ability of *Sequoia sempervirens* L. plant.

Treatments	Shoot number	Shoot length mm	Root %	Root number	Root length mm
S1:Control ((3/4 strength of MS +0.2mg/L BA)	10.01 e	43.77 h	---	---	---
S2:Control+ Fe NPs 2.5 mg/L	12.00de	48.00 g	16.60 d	0.50 d	50.00 e
S3:Control+ Fe NPs 5 mg/L	12.90 cd	68.00 cd	33.33 c	1.50 c	122.00 a
S4::Control+ Fe NPs 10 mg/L	11.60 de	66.25 d	33.33 c	2.00 bc	70.50 d
S5:Control + Al NPs 2.5 mg/L	15.30 b	68.70 c	49.95 b	3.00 a	120.00 a
S6:Control + Al NPs 5 mg/L	10.19 e	60.36 f	16.65 d	0.60 d	50.00 e
S7:Control+ Al NPs 10 mg/L	13.50 b-d	63.66 e	49.95 b	2.06 bc	123.75 a
S8:Control+ Zn NPs 2.5 mg/L	12.00 de	47.33 g	---	---	---
S9:Control+ Zn NPs 5 mg/L	11.33 de	45.00 h	---	---	---
S10:Control + Zn NPs 10 mg/L	11.50 de	68.66 e	16.65 d	0.70 d	70.50 d
S11:Control+ Ti NPs 2.5 mg/L	11.88 de	61.66 ef	33.33 c	2.00 bc	80.33 c
S12:Control+ Ti NPs 5 mg/L	14.55 bc	71.25 b	33.33 c	1.50 c	118.33 a
S13:Control + Ti NPs 10 mg/L	18.09 a	75.71 a	55.55 a	3.08 a	110.66 b

Averages (means) having the same letter(s) within the same letter(s) within the same column are not significantly different according Duncan's multiple range tests at 5% level of probability.

3.2. Hardening off

Rooted plantlets of *Sequoia sempervirens* L. obtained from *in vitro* culture media supplemented with different concentration of Fe, Al, and Ti NPs were acclimatized and transferred to the greenhouse in the peat: perlite: clay (1:1:1 v/v/v) as shown in Figure 2.

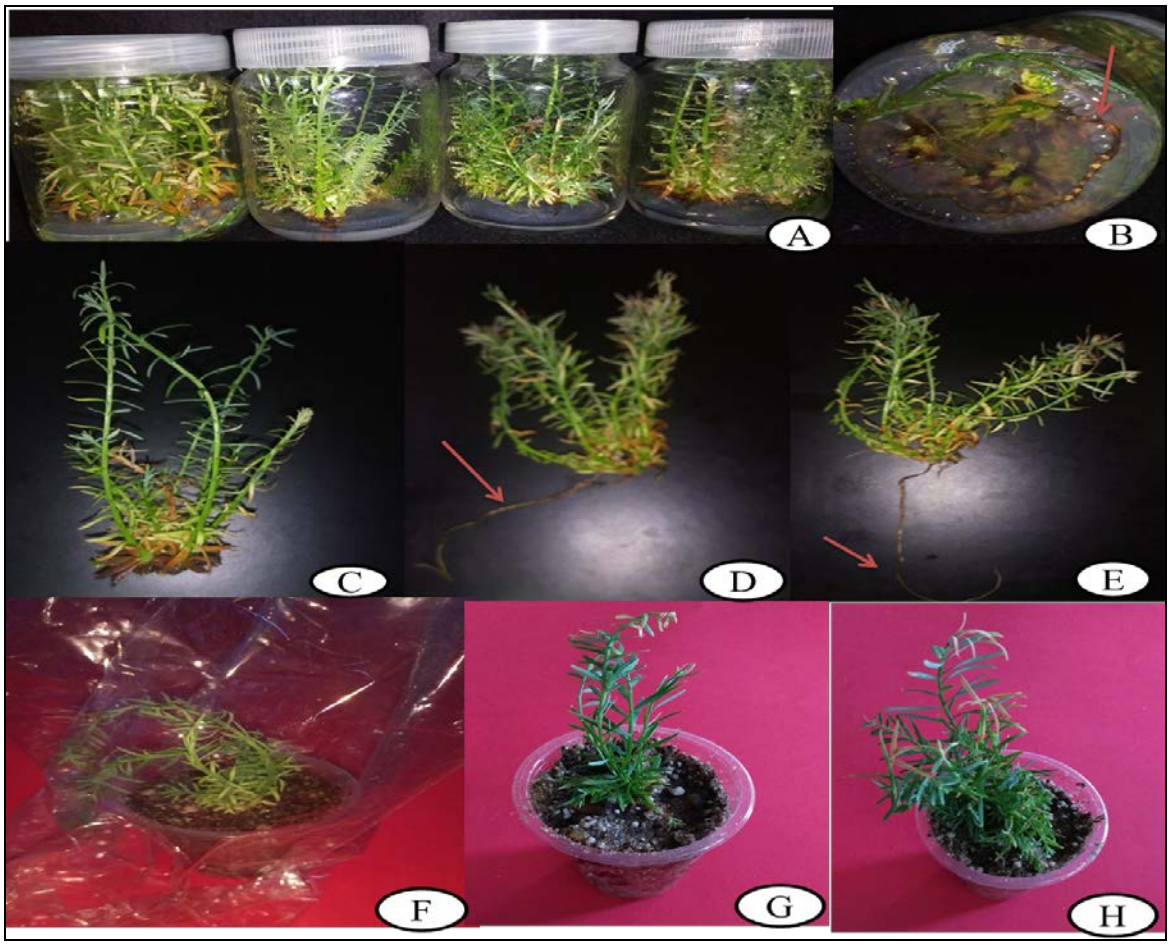

Figure 2. In vitro growth behavior; A: Shoot proliferation and rooting induction *in vitro* with application of 10mg/L Ti NPs, B: non-rooting plantlets (control), C: rooting on plantlets treated with Al NPs 10 mg/L and Ti NPs10 mg/L, D,E: Prepared plantlets to hardening off stage and F to H: Acclimatization plants to greenhouse.

3.3. Photosynthetic pigments

The influence of different concentrations of nanoparticles (2.5, 5 and 10 ppm) on both chlorophylls and carotenoids contents of treated shootlets were determined and compared with untreated (control). The results in Table (4) showed an increase in chlorophyll a, b, total chlorophylls and carotenoids contents in shootlets that were cultured on MS media supplemented with the various nanoparticles treatments. The highest contents of chlorophylls a, b, total chlorophyll and carotenoids were noticed with 5 mg/L Ti NPs (150.88, 69.37, 220.35 and

98.69mg/100g F.W., respectively). Whereas, the lowest contents of chlorophyll a, b, total chlorophyll and carotenoids were recorded with the addition of 2.5 mg/L Zn NPs (43.55, 19.38, 61.93 and 44.44 mg/100g F.W., respectively). It could be concluded that supplementation of Ti NPs in the culture medium was stimulated the accumulation of chlorophyll content in *Sequoia sempervirens* L. comparing with untreated (control) and other treatments.

These results are in agreement with Duhan *et al*. (2017) who reported that Fe and Zn NPs increased the leaf photosynthetic pigments and photosynthesis characters. In addition, El-Mahdy and Elazab (2020) demonstrated that using 1 and 2.5mg/L of Zno-NPs in culture medium increased chlorophyll a and b contents. This might be due to the presence of zinc and iron in both catalytic sites of many enzymes and structure of proteins and pigments that induced photosynthesis activity (Rout and Sahoo, 2015; Mohammadi *et al*., 2018).

Table 4. Effect of different concentrations of nanoparticles on photosynthetic pigments of Sequoia sempervirens L.

Pigment (mg/100g F.W.) / Treatments	Chlorophyll a mg /100 g F.W.	Chlorophyll b mg/100 g F.W.	Total chlorophyll mg/100 g F.W.	Carotenoids mg/100 g F.W.
S1:Contro l(3/4 strength of MS+0.2 mg/L)	40.78 e	19.22 h	59.0 k	42.33 l
S2:Control + Fe NPs 2.5 mg/L	45.99 de	20.63 gh	66.62 j	45.63 jk
S3:Control + Fe NPs 5 mg/ L	49.29 de	22.17 fg	71.46 i	49.55 i
S4:Control + Fe NPs 10 mg/ L	72.37 c	29.89 e	100.88 f	62.2 f
S5:Control + Al NPs 2.5 mg/ L	57.22 d	24.17 f	81.39 h	52.98 g
S6:Control + Al NPs 5 mg/ L	46.56 de	21.22 gh	67.78 ij	47.06 j
S7:Control + Al NPs 10 mg / L	88.57 b	38.79 d	127.03 d	66.1 d
S8:Control + Zn NPs 2.5 mg/ L	43.55 de	19.38 h	61.93 k	44.44 k
S9:Control + Zn NPs 5 mg / L	70.37 c	27.71 e	94.08 g	55.98 g
S10:Control + Zn NPs 10 mg / L	137.05 a	62.93 c	192.98 c	86.92 c
S11:Control + Ti NPs 2.5 mg/ L	83.44 bc	37.93 d	120.37 e	64.33 e
S12:Control + Ti NPs 5 mg / L	150.88 a	69.37 a	220.35 a	98.69 a
S13:Control + Ti NPs 10 mg / L	139.09 a	66.45 b	203.54 b	91.92 b

Average (means) having the same letter(s) within the same column are not significantly different according Duncan's multiple range tests at 5% level of probability.

3.4. Total Phenolic and flavonoid content

Data in Table (5) indicated the influence of individual supplementation of the selected nano oxides at different concentrations in culture media on the contents of total phenolics and flavonoid compounds of shootlets. The highest contents of phenolic and flavonoid compounds were 3.41 mg GAC/g tissue and 1.67 mg CE/g tissue of shootlets treated with Ti oxide NPs at 5 mg/ L, respectively. On the other hand, the minimal phenolic and flavonoid contents of shootlet were 1.05 mg GAC/g and 0.52 mg CE/g tissue estimated in shootlets treated with 2.5 mg/ L of Fe NPs treated group compared to other treatments and control groups, respectively. In addition, the increasing of concentrations of Zn NPs showed a gradual increase in the contents of total phenolic and flavonoid of treated shootlets compared with untreated explant. Whereas, the total phenolic and flavonoid contents were increased to 2.14 mg GAC/ g tissue and 0.95 mg CE/ g tissue with the treatment of Al NPs at 10 mg/ L compared with the other concentrations of Al NPs treatments and control. The total phenolic and flavonoid contents were significantly increased with zinc oxides nanoparticles treatments. This may be due to the ability of zinc to induce and accumulate secondary metabolites production (Javed *et al*., 2017)

These findings agreed with Raei *et al*. (2014) who reported that the maximum production of aloin was found when *Aloe vera* plant was treated with titanium oxide nanoparticles. Similarity, Al-oubaidi and Kasid (2015) showed that *Cicer arietinum* contents of gallic, chlorogenic, o-coumaric, tannic and cinnamic acids were increased when MS medium was augmented with TiO_2 NPs (4.5-6.0 mg/ L). Another study by Poborilova *et al.* (2013) reported that using Al_2O_3 NPs (0-100 gm/ L) in MS culture medium was increased the phenolic contents of tobacco plant. Similarly, Owje *et al*. (2019) indicated that supplementation of Al NPs in the culture medium increased lignin content of fenugreek plant. Also, addition of Ag NPs and Au NPs in culture medium resulted in high accumulation of phenolics and flavonoid contents of *prunella vulgars* L. plant (Fazal *et al*. 2016). Recently, Khan *et al*. (2020) noticed that fortification of high and low levels of Fe NPs increased total phenolics and flavonoids contents as well as increased accumulation of stevioside and rebaudioside of *Stavia rebaudiana* plant. Interestingly, we noticed a good correlation between these findings with shooting and rooting proliferation results and biochemical assays that were determined in this study. Thus, secondary metabolites accumulation and production are necessary to stimulate photosynthesis activity and increase the level of flavonoids, phenolics and tannins and to enhance the carrying of carbohydrate to different parts inside the plants (Ghasemzadeh *et al*., 2011; Khan *et al.*, 2020).

Table 5. Effect of different concentrations of selected nanoparticles on the contents of total phenolics (mg GAE/g tissue) and total flavonoids (mg CE /g tissue) of *Sequoia sempervirens* L.

Contents / Treatments	Total phenolics (mg GAE/ g tissue)	Total flavonoids (mg CE/ g tissue)
S1:Control ((3/4 strength of MS +0.2mg/ L BA)	1.43±0.12 g	0.79±0.05 f
S2:Control + Fe NPs 2.5 mg/ L	1.05±0.09 h	0.52±0.04 j
S3:Control + Fe NPs 5 mg/ L	1.41±0.12 g	0.68±0.05 hi
S4:Control + Fe NPs 10 mg/ L	1.78±0.15 e	0.85±0.07 e
S5:Control + Al NPs 2.5 mg/ L	1.65±0.14 f	0.79±0.06 f
S6:Control + Al NPs 5 mg/ L	1.05±0.095 h	0.65±0.04 i
S7:Control + Al NPs 10 mg / L	2.14±0.18 b	0.95±0.08 c
S8:Control + Zn NPs 2.5 mg / L	1.41±0.11g	0.72±0.06 gh
S9:Control + Zn NPs 5 mg / L	1.63±0.13 f	0.74±0.05 fg
S10:Control + Zn NPs 10 mg / L	2.03±0.16 c	0.91±0.08 cd
S11:Control + Ti NPs 2.5 mg / L	1.88±0.16 d	0.89±0.07 de
S12:Control + Ti NPs 5 mg / L	3.41±0.28 a	1.67±0.12 a
S13:Control + Ti NPs 10 mg / L	2.19±0.18 b	1.01±0.09 b

Data are mean of three replicate ± SD at P≥0.01. Means having the same letter(s) within the same column are not significantly different according Duncan's multiple range tests 5%level of probability.

3.5. Enzyme activity

The impact of different nanoparticles at 2.5, 5 and 10 mg/ L separately in MS culture media on the enzymatic activities of oxidoreductase enzymes such as peroxidase, catalase and polyphenol oxidase of *Sequoia sempervirens* L. shootlets was determined and described in Table (6). The application of different NPs was enhanced activities of tested enzymes compared with untreated explants. In addition, increasing the concentrations of these NPs was shown a dose-dependent increase in the activities of these enzymes. The treatment of explants with 10 mg L^{-1} of Fe NPs has significantly increased the activities of peroxidase, catalase and polyphenol oxidase to 51.60, 54.60 and 17.50 U/mg compared with other treatments and untreated explant. Whereas, the highest specific peroxidase activity was 70.50 U/mg for shootlets treated with 10 mg/L of Zn NPs comparable with other NPs and control explants. Similarly, the maximum activity of polyphenol oxidase was 18.60 U/mg for the group treated with 10 mg/L Ti NP comparable with control and other treated explants groups.

In agreement with our results, Lu *et al*. (2002) noticed the enhancement of antioxidant activity and nitrate reductase activity in soybean plant supplemented with TiO_2 NP during germination and growth. Similarly, Mathpal *et al*. (2015) observed the increase of activity of enzyme by using Zn NP, and attributed that to the role of Zn in the stimulation of enzymes incorporated in carbohydrate and protein metabolism of plant. Many researchers suggested that Zn and Fe NPs are playing the fundamental key of induction of different oxido-reductase enzymes which are responsible for phytohormone building, absorption of nutrients and metabolic biosynthesis (Dhir *et al*., 2011). Additionally, Owje *et al*. (2019) showed that using Al NPs increase the CAT activity of Fenugreek plant., and Khan *et al*. (2020) reported that applied Fe NPs at (90 µg/ L) increase the enzymatic activities of antioxidant enzymes (SOD, POD, and CAT) on *Stavia rebaudiana* plant.

Table 6. Effect of different concentration s of nanoparticles on activities of enzymes of *Sequoia sempervirens* L.

Determinations / Treatments	Peroxidase (U/mg)	Catalase (U/mg)	Polyphenol oxidase (U/mg)
S1:Control ((3/4 strength of MS +0.2mg/ L BA)	12.70	14.00	5.030
S2:Control + Fe NPs 2.5 mg/ L	13.10	17.30	5.30
S3:Control + Fe NPs 5 mg/ L	18.30	33.40	9.20
S4:Control + Fe NPs 10 mg/ L	51.60	54.60	17.50
S5:Control +Al NPs 2.5 mg/ L	13.00	17.30	6.40
S6:Control +Al NPs 5 mg/ L	13.70	24.40	7.50
S7:Control + Al NPs 10 mg / L	16.30	32.60	10.90
S8:Control + Zn NPs 2.5 mg / L	14.30	24.00	5.50
S9:Control + Zn NPs 5 mg / L	22.40	18.30	7.30
S10:Control + Zn NPs 10 mg / L	70.50	16.80	16.00
S11:Control + Ti NPs 2.5 mg / L	22.10	19.60	6.70
S12:Control + Ti NPs 5 mg/ L	39.50	21.80	17.60
S13:Control + Ti NPs 10 mg / L	29.70	41.70	18.60

3.6. DNA fingerprinting using RAPD-PCR

To study the genetic difference between the treatments lines (Fe NPs 10mg/L, Al NPs 10mg/ L, and Ti NPs 10mg/ L and control), ten selected RAPD primers were used. RAPD-PCR analysis offers the advantages of simplicity and rapidly conferred by the PCR product procedure to confirm the treatment lines 6 out of ten primers produced reproducible PCR products with a clear pattern for each line and showing easily scrabble RAPD profiles and informative as shown in Figure 3. The total bands were detected among the treatment lines and control (Table7). Only 23 of 52 total bands were polymorphic markers, and these primers produced multiple band profiles with a number of amplified DNA fragments varying from 4 to 13. The highest number of bands (13 bands) was generated by using the primer OPB-12, while the lowest was 4 bands and generated with primer OPC-01. Control and treatment lines gave distinct DNA fingerprint patterns. A dendrogram was constructed based on the RAPD-PCR data analysis as shown in Table (8) and Figure 4. The data also showed that the closest relationship between control and treatment Al NPs 10mg/ L was 89% and the low similarity with Ti NPs at 10mg/ L was 74%. This technique permits the characterization and detection of the diversity and identity between treated plantlets evaluated in this study. This application seems to be useful for organization and differentiation between the different treatments of Sequoia that may provide useful information on the level of polymorphism and diversity in Sequoia plantlets. These results would be useful for better management and differentiation of new clones. Toral *et al* (2009) also used RAPD-PCR to analyze the genotype identification and gene diversity for *Sequoia sempervirens* L. (D. Don) Endl. in Chile and concluded that the variation of RAPD marker is powerful in identifying genetic relationships between Sequoia clones, so in the future, the presence of more clones in the RAPD analysis and molecular data complementation with other techniques will improve the resolution of genetic relationships and the

potential use in *Sequoia* plantations in Chile. Correspondingly, Parsad (2014) established that RAPD technique is reliable and promising for the characterization of the *Hibiscus germplasm*. Thus, these RAPD markers show a capability for characterization and identification genetic diversity within the varieties in a species. This may also help in *Hibiscus* breeding system and conservation biology of these plants. In addition, Rafi *et al*. (2012) used RAPD-PCR to detect the association and the genetic variation between the geographical origin of 48 accessions of physic nut, *Jatropha curcas* L.

Table 7. The statistical analysis of RAPD-PCR primers used in this study

Similarity matrix between control and three treatment of *Sequoia sempervirens* L.				
Name	Control	Fe NPs 10 mg/l	Al NPs 10 mg/l	Ti NPs 10 mg/l
Control	100			
Fe NPs 10 mg/l	80	100		
Al NPs 10 mg/l	89	81	100	
Ti NPs 10 mg/l	74	75	70	100

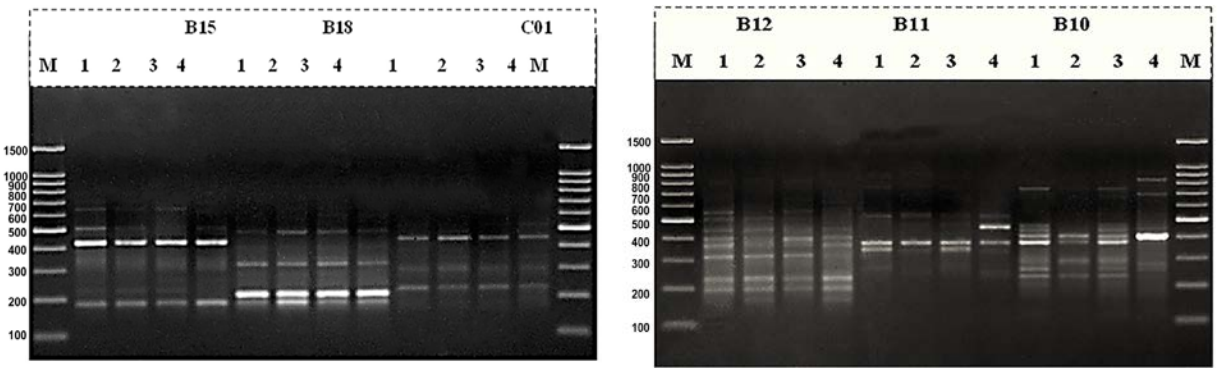

Figure 3. The PCR amplification profile of RAPD-PCR primers for the control and three treatment (Lane 1-4); **1:** Control, **2:** Fe NPs 10mg L^{-1}, **3:** Al NPs10 mg L^{-1}, **4:** Ti NPs 10 mg L^{-1} **and M:** 100 bp DNA ladder.

Table 8. The Similarity matrix based on analysis of RAPD-PCR of control and three treatments of Sequoia sempervirens L.

No	Name of primer	Monomorphic bands	Polymorphic bands	Number of Unique bands	Total bands	Polymorphism (%)	MW range (bp)	Mean of frequency
1	**OPB-10**	5	7	0	12	58	148-810	0.7
2	**OPB-11**	4	5	1	10	60	206-901	0.7
3	**OPB-12**	1	8	4	13	92	117-862	0.6
4	**OPB-15**	4	2	0	6	33	188-642	0.9
5	**OPB-18**	5	1	1	7	29	188-556	0.8
6	**OPC-01**	3	0	1	4	25	227-488	0.8
Total		22	23	7	52			4.5
Average		3.66	3.83	1.16	8.6	49.5		0.75

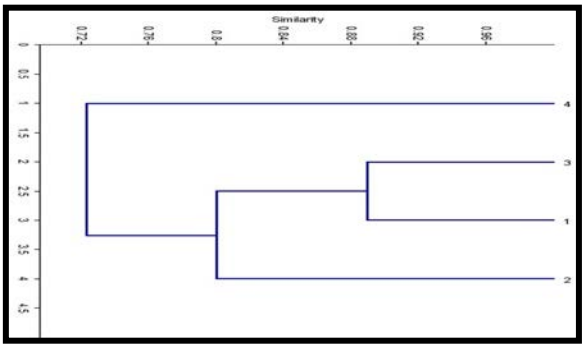

Figure 4. Dendrogram demonstrating the relationships between the control and treatments based on RAPD-PCR analysis. The numbers from 1 to 4 represent the following groups; 1: Control, 2: Fe NPs 10mg/l, 3: Al NPs 10 mg/l, 4: Ti NPs 10 mg/l.

4. Conclusion

Briefly, these findings demonstrated the benefits of using nanoparticles as an external elicitor to *Sequoia sempervirens* L. plant, and showed the high capability of Ti NPs compared with Fe, Zn and AL NPs for stimulation the shooting and rooting behavior includes shoot number, shoot length, root percentage, and root number, in addition, enhancement the content of photosynthetic pigments and secondary metabolites with increasing the activities of peroxidase, catalase and polyphenol oxidase enzymes. Additionally, RAPD-PCR is a good tool to study the genetic diversity between the control and the significant treatment Ti NPs 10 mg/L for shooting and rooting behavior. This will help in increasing the propagation of this economic tree and wide production of this valuable plant (growth), which will lead to an increase in the economic income.

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