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Research Article
Antioxidant Activity, Active Compounds and Oil Characterization of *in vitro Sequoia sempervirens* Cultures

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Abstract

Background and Objective: *Sequoia sempervirens* is a very valuable tree for ornamental and industrial purposes. It could be used in the paper, timber and pulp industries. A comparative study was conducted to optimize shootlets and callus micropropagation under the effect of some factors, their contents of some active compounds and essential oil ingredients comparing with the mother plant as the source of shoots. **Materials and Methods:** The effects of some factors influencing the various stages of *Sequoia sempervirens* micropropagation from the culture of nodal explants to achieve an *in vitro* culture of shootlets, callus tissues were investigated and chemically analyzed for their active compounds contents and the essential oil ingredients. **Results:** The highest survival percent (100%) was obtained with Clorox 10% (for 10 min) then immersing in MC 0.2% (for 5 min) or Clorox 20% (for 10 min) followed by MC 0.1% (for 5 min). More proliferated shootlets could be achieved on half or 3/4 strengths of MS (Murashige and Skoog) media supplemented with BA (Benzyl Adenine) at 0.1 and 0.2 mg L^{-1}. *In vitro* rooting ability was best on half strength of MS medium supplemented with 0.2 mg L^{-1} of BA. Callus growth was promoted on MS medium supplemented with 2, 4-D (2,4-dichlorophenoxyacetic acid). The highest contents of chlorophylls were recorded with 1/2 MS strength added with 0.1 mg L^{-1} of 2ip (N6-(2-Isopentenyl) adenine). Different concentrations of 2iP and BA produced significant effects on antioxidant activity, total phenols and flavonoids contents. Terpinene-4-ol, Eugenol and ɣ-Terpinene contents were increased in the essential oil extracted from shootlet compared to those extracted from the mother plant. **Conclusion:** The optimal conditions were successfully established for *in vitro* growth of *Sequoia sempervirens* with potential for secondary metabolite production.

Key words: *Sequoia sempervirens*, micropropagation, secondary metabolites and oil composition

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sequoia sempervirens is an evergreen gymnosperm tree. It is a member of the family Cupressaceae (formerly Taxodiaceae). The reddish-brown heartwood is fire-resistant. Its Leaves are dimorphic, including linear and scale-like leaves[1]. The tree is very valuable not only for ornamental purposes as trees can reach its length up to 110 m but also for industrial purposes as it grows so strongly, rarely suffers from insect attack or disease and it is resistant to hardly winds and other poor climatic conditions[2]. It could be used in the paper industry, timber industry (plywood) and pulp industry. Propagation by seeds is common for *S. sempervirens*, although seed germination is very variable where many seeds are often empty, the embryos are either deformed or infected with diverse parasites and viable seeds are difficult to store[3].

Tissue culture needs a particular medium and culture conditions[4]. Minerals are essential constituents of the culture medium. There are big choices of combinations of macro and micronutrients. MS medium[5] is the most vastly used culture medium because the majority of plant cell cultures respond to it positively. It is categorized as a rising salt medium compared with many other formulations, where it contains high levels of potassium, nitrogen and some of the microelements, particularly manganese and boron[6]. The full power of salts in media confirmed it good for different species, however, in some types, the reduction of salts concentration to ½ or ¼ of full level produced the best results for *in vitro* growth[7].

Cytokinin is one of the plant growth regulators pivotal for plant growth and development and is known to stimulate cell division and differentiation[8]. Different types of cytokinins can also encourage lateral bud growth and thus lead to multiple shoot formation through breaking shoot apical dominance[9]. The most used cytokinins in tissue culture are BA, 2iP, KIN and TDZ. The efficiency of BA to encourage axillary shoots growth *in vitro* is well described by Nobre *et al.*[10]

The establishment of protocols for culture the medical plants using different growth regulators to enhance the production of bioactive compounds is required for commercial and research application. Bioactive compounds were found to be accumulating in culture cells at a higher level than those in natural plants through optimization of culture conditions[11].

In Egypt, through the valuation of the status of shrubs and trees, it was detected that *Sequoia sempervirens* is threatened extinction in natural Egyptian Suburbs where there is one tree of it in El Orman Garden[12]. *Sequoia sempervirens* tree in Egypt could be proliferated by tissue culture techniques because it is threatened, where, it never produces seeds and neither propagated vegetatively[13].

The *S. sempervirens* oil chemical constituents were rarely reported before in Egypt. Although it has a lot of medical and commercial uses, it has not attention been paid to estimate the volatile oil[14].

This study aimed to attain a suitable micropropagation protocol for *Sequoia sempervirens* tree and callus induction under the effect of some factors besides a comparative study of the tree (mother plant) as the source of shoots explants and the obtained proliferated shootlets as well as callus tissues contents of some active compounds and analyze the essential oil to find out its ingredients for all those sources.

MATERIALS AND METHODS

Study area: This study was conducted in Tissue Culture Technique Laboratory-Central Labs-Ornamental Plants and Woody Trees Department, National Research Centre (NRC), Egypt, during the years of 2019-2020 on *Sequoia sempervirens* tree.

Culture establishment: Nodal explants (4-5 cm) of *Sequoia sempervirens* were collected from an adult tree in Orman Garden, Giza, Egypt and washed using Septol soap for 30 min then rinsed with running tap water for 1 hr. The washed explants were immersed in 70% ethanol for 30 sec, then exposed to different disinfectants under aseptic conditions as follow:

- Clorox 10% for 10 min
- Clorox 20% for 10 min
- MC 0.1% for 5 min
- MC 0.2% for 5 min
- Clorox 10% for 10 min+MC 0.1% for 5 min
- Clorox 10% for 10 min+MC 0.2% for 5 min
- Clorox 20% for 10 min+MC 0.1% for 5 min
- Clorox 20% for 10 min+MC 0.2% for 5 min

After 2 weeks incubation on ½ MS at 24±1°C under fluorescent lamps with the light intensity of 2000 lux at 16 hrs photoperiods. The following has been estimated:

$$\text{Aseptic cultures (\%)} = \frac{\text{Contamination jars}}{\text{Total cultured jar}} \times 100$$

$$\text{Explants survival (\%)} = \frac{\text{Number of explants survival}}{\text{Total explants cultured in jar}} \times 100$$

Shoot proliferation and rooting ability: Initiated explants obtained from the previous experiment were cultured on MS

medium at different powers (full, ¾ and ½ MS), full MS alone was free of plant growth regulators (control), while three-quarter or half MS were supplemented with different cytokinin types [Benzyl Adenine (BA) and N6-(2-Isopentenyl) adenine (2iP)] and concentrations [0.1 and 0.2 mg L^{-1}]. The explants were re-cultured into this media two times, at 60 days intervals. Data were recorded proliferation rate (Explant survival %, shootlet length (mm) and shootlet number/explant) and rooting characters as rooting (%), roots number/shootlet and roots length (mm).

Callus culture development: For callus induction, symmetrical size (about 1 cm long) of nodal explant was cultured on MS medium that containing 2.5% sucrose, 8% agar and was supplemented with different auxin types [2, 4-dichlorophenoxyacetic acid (2, 4-D) and 4-Chlorophenoxy-acetic acid (p-CPA)] and concentrations [1, 2 and 3 mg L^{-1}] plus 0.2 of Benzyl Adenine (BA). Six types of callus media were used and each medium type consisted of 5 replicates (jars) and each replicate contained 3 explants. Cultures of all various types of callus media were incubated in a growth chamber in dark at 24±1°C for 3 months.

Incubation conditions: For all stages, the cultures were incubated under 24±1°C under fluorescent lamps with a light intensity of 2000 lux at 16 hrs light/8 hrs darkness daily.

Photosynthetic pigments: Chlorophyll a, b and carotenoids were extracted from shootlets (mg/100 g) according to Yang *et al.*[15].

Active compounds

Sample preparation: The dried samples were ground to a fine powder. The powder sample (0.25 g) was extracted with 10 mL of methanol for 24 hrs. The supernatant was recovered and used for the determination of antioxidant activity, total phenols and flavonoids.

Antioxidant activity: The free radical scavenging activity using the 1.1-diphenyl-2-picrylhydrazyl (DPPH) reagent was determined according to Brand-Williams *et al.*[16]. About 0.75 mL of the extracted sample was added to 1.5 mL of prepared methanolic DPPH solution was added and stirred the decolorizing process was recorded after 30 min of reaction at 517 nm by spectrophotometer and compared with a blank.

$$\text{Antioxidant activity} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Total phenols: The total phenolics content of the methanolic extract was determined according to Singleton *et al.*[17] by Folin-Ciocalteu's reagent. The absorbance was determined using at 765 nm by spectrophotometer. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed.

Total flavonoids: Total flavonoids were estimated using the method of Ordonez *et al.*[18] using Aluminum chloride, the absorbance was measured at 420 nm by spectrophotometer. Total flavonoids content was calculated as quercetin equivalent to a calibration curve.

Essential oil extraction and analysis: The content in essential oils of micropropagated was compared to that of the mother plant. For this, leaf tissues (5 g) were ground in a mortar with pestle chilled and extracted in 20 mL Hexane for 4 hrs at 40±2°C under reflux. The solvent was evaporated in a rotary evaporator and the residue resuspended in 1 mL diethyl ether[19]. Volatile compounds were analyzed and identified by GC using DsChrom 6200 Gas Chromatograph equipped with a capillary column DB-WAX 122-7032 Polysillphenylene-siloxane 30 m×0.25 mm ID×0.25 µm film.

Experimental design and data analysis: The layout of the experiment was designed in a completely randomized design and a test of Least Significant Difference (LSD) at 0.05 was used for comparison among means according to Steel and Torrie[20].

RESULTS

Culture establishment: To establish an *in vitro* culture of *Sequoia sempervirens*, different disinfectants treatments such as Clorox (10 and 20%) and/or Mercuric Chloride (MC) at 0.1 and 0.2% each alone or together for various durations (5 or 10 min) were applied in Fig. 1. The results showed that using Clorox at 10 or 20% for 10 min followed by MC at 0.1 or 0.2% for 5 min caused the highest percent of aseptic cultures (100%). It could be also noticed that using those two types and concentrations of disinfectants (Clorox and MC) together was favoured for obtaining decontaminated cultures with the highest survival of explants relatively comparing with those obtained when each disinfectant was used alone. While, the cultured explants could survive with highest percent (100%) with the treatments of Clorox 10% (for 10 min) followed by MC 0.2% (for 5 min) or Clorox 20% (for 10 min) followed by MC 0.1% (for 5 min).

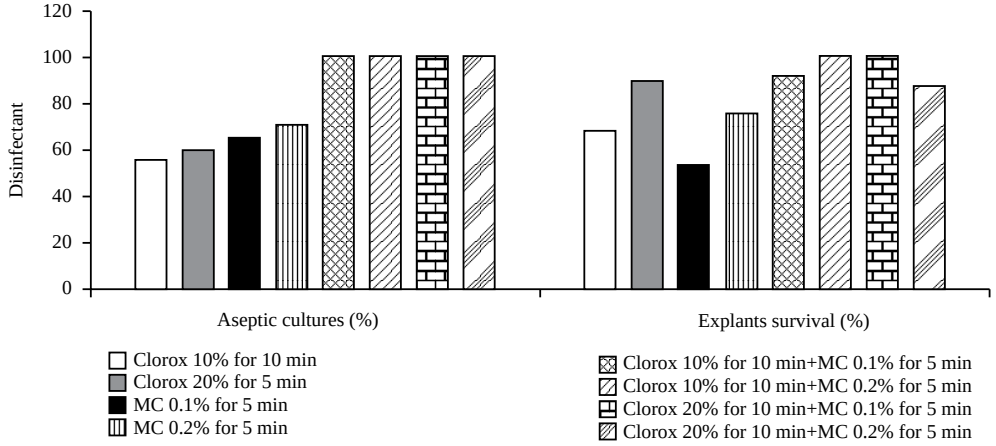

Fig. 1: Effect of different disinfectants on the aseptic and survival percent of *Sequoia sempervirens* explants
MC: Mercuric chloride

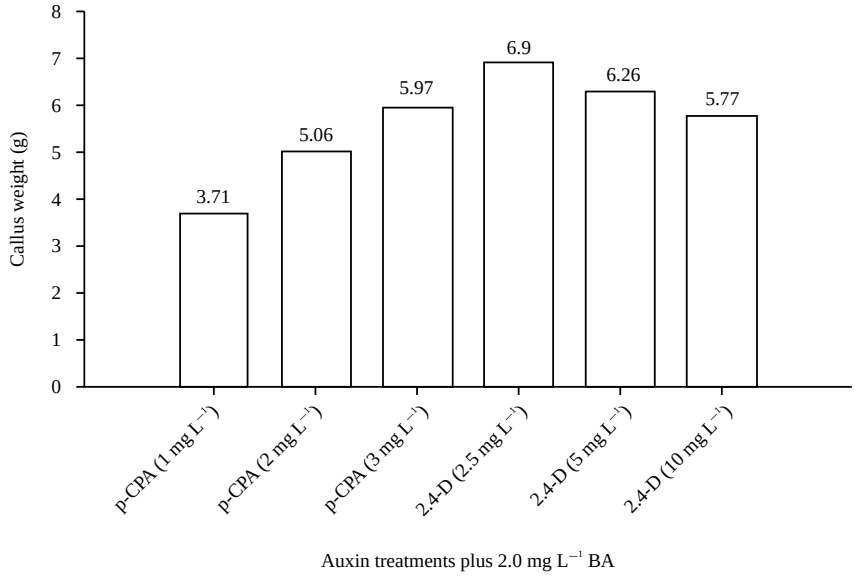

Fig. 2: Callogenesis potentiality response of *Sequoia sempervirens* explants to various auxin types and concentrations in MS culture media
p-CPA: 4-Chlorophenoxy-acetic acid, 2, 4-D: 2, 4-dichlorophenoxy acetic acid

Shoot proliferation and rooting ability: As shown in Table 1 and Fig. 3a and b, the explants could survive with the highest percent (100%) when MS power was decreased to ½ or ¾ strengths irrespective of the type or concentration of the cytokinin which was used compared with control (full strength of MS free of hormone). However, more proliferated shootlets per explant (5.37, 6.10, 5.37 and 5.50, respectively) could be achieved on half and 3/4 strengths of MS media supplemented with BA at 0.1 and 0.2 mg L^{-1}. This illustrated that lowering the power of MS media had a promotion effect on shootlet proliferation. Meanwhile, the achieved shootlets grown to the longest ones (71.49 mm) on ¾ strength of MS supplemented with 0.2 mg L^{-1} of 2ip as compared to control and other treatments. *In vitro* rooting ability (rooting %, number and length of roots) was in highest values (42.40%, 1.4 and 20.0 mm, respectively) on half strength of MS medium supplemented with 0.2 mg L^{-1} of BA.

Callogenesis potentiality: The potentiality of nodal explant to form callus tissues was affected by various auxin types and concentrations in MS media that contained BA (2.0 mg L^{-1}) as shown in Fig. 2. The response of callus growth to the treatment of 2, 4-D at 2.5 mg L^{-1} in MS media shown in Fig. 3c

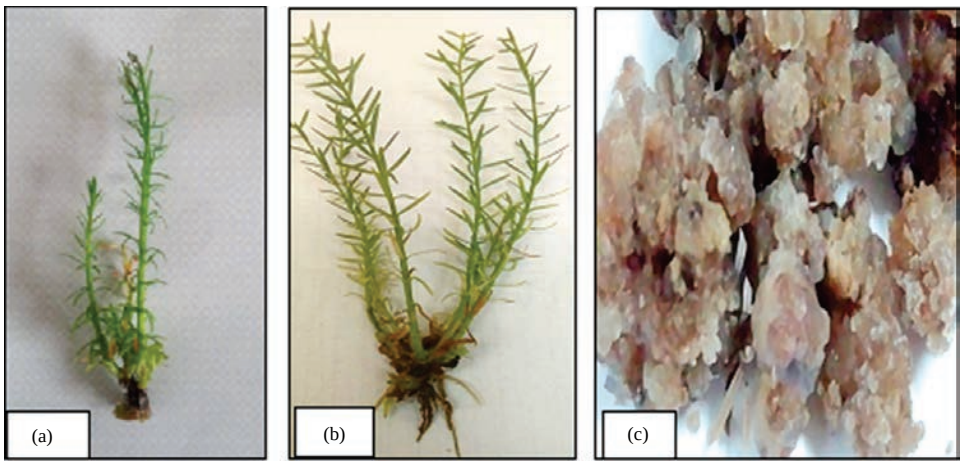

Fig. 3(a-c): Shoot proliferation, rooting and callogenesis of *Sequoia sempervirens* explants responses to various culture media
(a) Control, (b) 1/2 MS+0.2 mg L^{-1} BA and (c) 2, 4-D at 2.5 mg L^{-1}+BA at 2 mg L^{-1}

Table 1: Shoot proliferation and rooting ability of *Sequoia sempervirens* effecting by the power of MS media and various cytokinin types and concentrations

Treatments (mg L^{-1})	Explant survival (%)	Shootlet length (mm)	Shootlet number/ explant	Root in g (%)	Roots number/ shootlet	Roots length (mm)
Control (full MS free of hormones)	90.2	51.70	2.67	25.33	0.36	5.00
1/2 MS+0.1 BA	100.0	56.77	5.37	33.43	1.33	11.47
1/2 MS+0.2 BA	100.0	51.23	6.10	42.40	1.4	20.00
1/2 MS+0.1 2iP	100.0	63.07	2.50	0.00	0.00	0.00
1/2 MS+0.2 2iP	100.0	60.73	3.17	0.00	0.00	0.00
3/4 MS+0.1 BA	100.0	50.6	5.37	16.20	1.23	8.37
3/4 MS+0.2 BA	100.0	52.56	5.50	16.40	0.73	16.37
3/4 MS+0.1 2iP	100.0	65.24	2.02	0.00	0.00	0.00
3/4 MS+0.2 2iP	100.0	71.49	3.17	0.00	0.00	0.00
$LSD_{0.05}$	0.87	5.31	0.89	4.39	0.40	2.73

Table 2: Photosynthetic pigments contents in shootlets of *Sequoia sempervirens* (mg/100 g F.W.) effecting by MS media power and various cytokinin types and concentrations

Treatments (mg L^{-1})	Chl. a	Chl. b	Carotenoids
Control (full MS free of hormones)	119.30	47.77	118.43
1/2 MS+0.1 BA	220.37	81.70	266.57
1/2 MS+0.2 BA	225.57	87.90	258.73
1/2 MS+0.1 2iP	300.37	125.87	261.97
1/2 MS+0.2 2iP	198.97	67.10	210.37
3/4 MS+0.1 BA	178.83	58.57	208.07
3/4 MS+0.2 BA	180.03	62.80	163.40
3/4 MS+0.1 2iP	292.37	102.70	270.77
3/4 MS+0.2 2iP	260.40	89.80	237.27
$LSD_{0.05}$	6.52	13.84	8.65

MS: Murashige and skoog, 2ip: N6-(2-Isopentenyl) adenine, BA: Benzyl adenine

was positively represented in the heaviest callus fresh weight (6.90 g/jar) as compared to other treatments. Noticeably, there is a negative relation between callus growth and increasing the concentration of 2, 4-D. The opposite trend was found when p-CPA was used to induce callus tissues where its high concentration (3 mg L^{-1}) caused the heaviest callus fresh weight (5.97 g/jar) comparing with other concentrations (1 and 2 mg L^{-1}) of this auxin.

Photosynthetic pigments: According to the effect of MS media power and various cytokinin types and concentrations on photosynthetic pigments content, the tabulated data in Table 2 recorded the highest shootlets contents of both chlorophyll a and b (300.37 and 125.87 mg/100 g F.W., respectively) that were estimated when the power of MS medium was lowered to half strength and added with 0.1 mg L^{-1} of 2ip as compared to control and other treatments. While the highest value of

Table 3: Effect of different types of cytokinins on antioxidant activity, total phenols and flavonoids contents in shootlets of *Sequoia sempervirens*

Treatments (mg L^{-1})	Antioxidant activity (%)	Total phenols content (mg of GA g^{-1} DW)	Total flavonoids content (mg of quercetin equivalent g^{-1} DW)
Mother plant	83.27	19.34	3.78
Full MS	62.82	6.88	1.16
1/2 MS+BA 0.1	72.42	11.77	1.34
1/2 MS+BA 0.2	76.13	9.38	1.84
1/2 MS+2ip 0.1	65.44	8.85	1.44
1/2 MS+2ip 0.2	70.57	10.77	1.91
3/4 MS+BA 0.1	79.22	13.78	2.71
3/4 MS+BA 0.2	82.57	15.38	3.28
3/4 MS+2ip 0.1	74.01	12.68	2.52
3/4 MS+2ip 0.2	78.67	13.27	2.69
$LSD_{0.05}$	3.32	1.13	0.39

MS: Murashige and skoog, 2ip: N6-(2-Isopentenyl) adenine, BA: Benzyl adenine

Table 4: Effect of various auxin types and concentrations in MS culture media contained BA (0.2 mg L^{-1}) on antioxidant activity, total phenolics and flavonoids content in calli of *Sequoia sempervirens*

Treatments (mg L^{-1})	Antioxidant activity (%)	Total phenols content (mg of GA g^{-1} DW)	Total flavonoids content (mg of quercetin equivalent g^{-1} DW)
p-CPA 1.0+BA 0.2	41.69	6.30	0.94
p-CPA 2.0+BA 0.2	45.28	7.08	1.00
p-CPA 3.0+BA 0.2	46.16	8.23	1.08
2, 4-D 2.5+BA 0.2	48.15	9.54	1.32
2, 4-D 5.0+BA 0.2	49.92	9.95	1.54
2, 4-D 10.0+BA 0.2	44.96	7.93	1.13
$LSD_{0.05}$	2.37	1.83	0.15

p-CPA: 4-Chlorophenoxy-acetic acid; BA: Benzyl adenine, 2, 4-D: 2, 4-dichlorophenoxy acetic acid

carotenoid (270.77 mg/100 g F.W.) were recorded with ¾ strength of MS with the same cytokinin.

Secondary metabolites

Active compounds

In shootlets cultures: Data in Table 3 revealed that all shootlets cultured on different concentrations of 2iP and BA produced significant effects on antioxidant activity, total phenols and flavonoids contents as compared to MS-free of hormones treatment.

Antioxidant activity was in the highest value (82.57%) in shootlets that were cultured on MS (3/4 strength) supplemented with BA at 0.2 mg L^{-1} with no significant difference between this value and that of the mother plant (83.27%).

The same treatment (3/4 MS+0.2 mg L^{-1} BA) was noticed to be close to the mother plant for both total phenols and flavonoids contents that were in the highest values (15.38 mg of GA/g DW and 3.28 mg of Quercetin equivalent/g DW, respectively) as compared to other treatments.

In callus culture: Data in Table 4 revealed that 2, 4-D (5 mg L^{-1})+BA (0.2 mg L^{-1}) increased all active compounds (antioxidant activity, total phenols and flavonoids) in the calli samples in highest values (49.92%, 9.95 and 1.54 mg g^{-1} D.W., respectively) followed by 2, 4-D (2.5 mg L^{-1}) +BA (0.2 mg L^{-1}) which resulted from 48.15%, 9.54 and 1.32 mg g^{-1} D.W., respectively as compared to other treatments.

Essential oil components (%)

In shootlets cultures: The sample was taken from the mother plant during February to compare its oil ingredients with those fractionated from the oil of *in vitro* shootlets treatments. Eighteen constituents in the essential oil of *S. sempervirens* leaves were identified corresponding to 95.1% of the total oil. Results of the chromatographic analysis of oil samples extracted from *Sequoia sempervirens* shootlets presented in Table 5 showed that Terpinene-4-ol was the most important essential oil component (with contents of 35.90-47.58%), followed by Eugenol (with contents of 14.64-18.77%), ɣ-Terpinene (with contents of 9.15-18.45%). The effect of different types of cytokinins treatments on the Terpinene-4-ol content in oil extracted from *Sequoia sempervirens* shootlet. The highest mean content (47.58%) was recorded in the oil of shootlets cultured on MS (3/4 strength)+BA 0.2 mg L^{-1} followed by shootlets cultured on MS (3/4 strength)+BA 0.1 mg L^{-1} which gave a mean Terpinene-4-ol content of 45.66% whereas, the lowest content (35.90%) was obtained from MS-medium free of hormones.

Table 5 highlights the difference between essential oil components from obtained shootlets *in vitro* and mother

Table 5: Chemical composition of leaf essential oil of *S. sempervirens* (mother plant) and *in vitro* shootlet

Components (%) of the essential oil	Treatments									
	Mother plant	Full MS 4.4 g L^{-1} (free)	1/2 MS+BA (0.1 mg L^{-1})	1/2 MS+BA (0.2 mg L^{-1})	1/2 MS+2ip (0.1 mg L^{-1})	1/2 MS+2ip (0.2 mg L^{-1})	3/4 MS+BA (0.1 mg L^{-1})	3/4 MS+BA (0.2 mg L^{-1})	3/4 MS+2ip (0.1 mg L^{-1})	3/4 MS+2ip (0.2 mg L^{-1})
α-Pinene	4.64	2.58	2.94	3.31	3.39	3.17	3.11	1.32	3.13	3.53
Sabinene	5.59	5.58	5.34	4.37	3.70	5.84	5.30	4.72	4.77	3.19
Myrcene	4.42	-	-	-	-	-	-	-	-	-
α-Phellandrene	10.07	5.60	2.55	2.33	2.42	5.13	4.39	3.48	4.40	3.10
limonene	11.61	3.56	1.34	1.15	1.39	1.23	1.05	1.62	1.83	1.63
cis-β-Ocimene	5.41	-	-	-	-	-	-	-	-	-
m-Cymene	2.30	-	-	-	-	-	-	-	-	-
γ-Terpinene	5.31	9.15	16.20	16.84	18.45	9.59	10.41	10.95	12.10	17.43
Terpinolene	1.91	-	-	-	-	-	-	-	-	-
Terpinene-4-ol	10.47	35.90	39.94	41.00	39.22	42.69	45.66	47.58	44.55	43.75
p-Cymen-8-ol	2.90	-	-	-	-	-	-	-	-	-
α-Terpineol	2.97	-	-	-	-	-	-	-	-	-
cis-Piperitol	3.70	3.30	2.12	0.83	1.11	1.67	1.49	1.43	1.90	0.80
Geraniol	5.49	-	-	-	-	-	-	-	-	-
Thymol	5.01	8.43	3.18	2.67	1.61	5.32	2.86	3.13	3.44	2.70
Carvacrol	2.17	4.25	2.04	2.25	2.80	2.36	1.70	1.81	3.09	1.12
Eugenol	5.53	14.98	14.64	15.05	15.31	16.22	18.77	18.25	16.07	15.02
β-Caryophyllene	5.61	3.16	0.63	0.82	1.08	1.75	1.57	1.28	0.42	1.00
Identified (%)	95.10	96.49	90.91	90.61	90.48	94.96	96.29	95.57	95.70	93.27
Unidentified (%)	4.90	3.51	9.10	9.39	9.52	5.04	3.71	4.43	4.30	6.73

MS: Murashige and skoog, 2ip: N6-(2-Isopentenyl) adenine, BA: Benzyl adenine

Table 6: Effect of various auxin types and concentrations in MS media that contained BA (0.2 mg L^{-1}) on components (%) of the essential oil of *Sequoia sempervirens* calli

Components (%) of the essential oil	Treatments (mg L^{-1})					
	P-CPA 1.0+BA 0.2	P-CPA 2.0+BA 0.2	P-CPA 3.0+BA 0.2	2, 4-D 2.5+BA 0.2	2, 4-D 5.0+BA 0.2	2, 4-D 10.0+BA 0.2
α-Pinene	2.314	2.152	1.478	2.547	1.965	2.065
Sabinene	2.154	1.852	1.207	1.347	1.478	2.968
α-Phellandrene	25.124	24.698	23.478	20.145	22.417	19.348
limonene	29.524	33.658	34.210	36.458	38.987	30.527
γ-Terpinene	5.364	6.781	6.310	7.204	9.330	5.110
Fenchone	2.145	2.254	2.634	3.154	2.365	2.541
Terpinene-4-ol	1.025	1.147	1.236	1.365	1.104	3.204
Broneol	11.201	12.021	13.247	12.364	14.782	11.254
Citronellol	1.025	1.632	1.756	1.587	1.891	3.250
Thymol acetate	0.256	0.324	0.324	0.896	0.457	0.352
Eugenol	0.213	0.365	0.412	0.236	0.301	0.215
β-Caryophyllene	2.0654	2.9687	19.348	30.527	5.11	2.541

p-CPA: 4-Chlorophenoxy-acetic acid, BA: Benzyl adenine, 2, 4-D: 2, 4-dichlorophenoxy acetic acid

plant. The Terpinene-4-ol (with content 47.58%), Eugenol (with content 18.77%) and ɣ-Terpinene (with content 18.45%) in oil extracted from shootlet were increased as compared to essential oil extracted from the mother plant (10.47, 5.53 and 5.31%, respectively). While, several components disappeared in oil extracted from *in vitro* shootlets such as Myrcene, cis-β-Ocimene, m-Cymene, Terpinolene, p-Cymen-8-ol, α-Terpineol and Geraniol.

In callus culture: The chromatographic analysis showed that Limonene was the most important essential oil component in callus tissues (with contents of 29.524-38.987%), followed by α-Phellandrene (with contents of 19.348-25.124%), Broneol (with contents of 11.201-14.782%).

The data presented in Table 6 showed the effect of different types of auxin treatments on the Limonene content in the oil extracted from *Sequoia sempervirens* calli. The highest main content (38.987%) was recorded in the oil of calli cultured on 2, 4 D (5 mg)+BA 0.2 mg L^{-1} followed by calli cultured on 2, 4 D (2.5 mg)+BA 0.2 mg L^{-1} (which gave a mean Limonene content of 36.458%), whereas the lowest content (29.524%) was obtained from P-CPA (1)+BA (0.2) mg L^{-1}.

DISCUSSION

In the current study, the type and concentration of the used disinfectant have a positive or negative effect on the survival ability of the explant. Similarly, Taha *et al*.[21] observed the free contaminated and highest survived plants that were obtained when the surface sterilization with Clorox and MC were applied. However, Rice *et al*.[22] reported the specification of Clorox as a powerful antimicrobial agent. The plant toxicity for survived explants was attributed to the sensitivity of plant tissues to excessive sterilization with heavy metals from mercury[23].

The power of MS culture media and various cytokinin types and concentration had noticeable effects on both shooting and rooting formed *in vitro*. The results were confirmed by Abou Dahab *et al*.[24] stated that the highest number of shootlets/explant of *Taxodium distichum* was recorded with ½ MS supplemented with BA but full-strength MS medium gave minimal shootlets number. Hashish *et al*.[25] observed that the ability of *Hibiscus sinensis* explants to produce multiple shoots was highest on ¾ strength of MS medium and BA. Concerning the role of cytokinin, Ziv[26] revealed that using cytokinin at a high level was an effective method to reduce shoot growth and promote meristematic cluster formation. Aloufa *et al*.[27] Showed that the shoots number/ explant was increased with an increase in the cytokinin level. The BA was used to increase the number of shoots[28]. Kozak and Saaata[29] observed the highest number of axillary shoots was obtained with BA while using 2ip stimulated strong growth expressed in the length of the Rhubarb plant. Shahriai *et al*.[30] noticed that BA resulted in the highest shoot number and the shortest heights of Caralis' Alstroemeria Cultivar regeneration, while 2iP resulted in a lower number of shoots and more significant heights. Using 2ip plus IBA produced the longest shootlets of *Antigonon leptopus* plant by Youssef *et al*.[31].

For callus growth, generally, callus development was best on MS medium supplemented with 2.4-D comparing with those grown with p-CPA. This result was confirmed by Abd El- Kadder *et al*.[32] on *Dillenia indica* who recommended that the addition of 2, 4-D to the culture medium was favoured to obtain good quality of callus mass and high production percentage. Lestari *et al*.[33] indicated that a combination of growth regulators from a group of auxins and cytokinin in a balanced amount can initiate cell enlargement and induction callus. The promoted effect of auxins on callus induction and growth might be attributed to that auxins promote the biosynthesis of ethylene by increasing the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthesis as was suggested by Kende[34].

The obtained data in the present work led to the suggestion that pigments contents in shootlets were influenced by MS power and cytokinin type irrespective of its concentration. In this share, Marino and Bertazzo[35] mentioned that chlorophylls a and b were influenced by cytokinin in the culture media and this might attribute to the enhancement of pigments accumulation in the shootlets tissues. Szweykowska[36] reported that cytokinins are known to affect photosynthesis, promote chloroplast biogenesis and chlorophyll biosynthesis.

Sequoia sempervirens represents a good choice for a novel source of bioactive compounds as was revealed by Pasqua *et al*.[37], who noticed that the accumulation of flavonoids is usually correlated to morphogenesis during plant development. In this study, the significant variations in some active compounds of obtained shootlets were found as a result of different media strengths and cytokinins concentrations. These results are supported by the findings of Kuhlmann and Röhl[38], who pointed out that the contents of phenolics and flavonoids are still lower than in *ex vitro* plants in comparison with *in vitro* plants. Different types and concentrations of PGRs have been reported to regulate developmental processes and modify the concentration of secondary metabolites in plants differently. Baskaran *et al*.[39] also mentioned that the concentration of secondary metabolites in *in vitro* cultures is influenced by the composition of the medium. Total phenolic and flavonoid contents were upregulated in *in vitro* cultures treated with BA[40]. A similar stimulatory effect of cytokinins on flavonoid biosynthesis in shoot cultures was also recorded for *Scutellaria alpina*[41].

Callus has been widely used in both basic research and industrial applications[42]. It could be noticed from the present results that the above-mentioned estimated compounds in callus tissues were in negative relation with increasing 2, 4-D concentration to 10 mg L^{-1} in MS culture medium while the opposite trend was observed with increasing P-CPA concentration. A similar effect has been reported in several other plant species[43]. Moreover, there was a strong relationship between total phenolic and antioxidant activity indicated that phenolic compounds were a major contributor to antioxidant activity in *Sequoia sempervirens*. These data are in agreement with Bendini *et al*.[44] and Wojdylo *et al*.[45], who reported the same relationship as phenolics are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolics content of plants may be contributed directly to their antioxidant action, phenylpropanoid and flavonoid biosynthesis. The great enhancement of total phenolics and

flavonoids content in both shootlet and calli may be due to the presence of growth regulators.

Terpenoids are commonly used as chemical markers for systematic investigations of conifers. Several sesqui and diterpenoids occur only in certain clusters of families or are restricted to some species of one conifer family. Plants of the Cupressaceae family are characterized by certain classes of terpenes and several sesquiterpenoids that are only known in this family[46].

The present investigation showed that terpenoids (including saturated hydrocarbon, monoterpenoids, sesquiterpenoids and oxygenated ones) in the oil were predominant. The main constituents of the essential oil from mother plant were limonene (11.61%), Terpinene-4-ol (10.47%) and α-phellandrene (10.07%). This agreed with the previous results submitted by Sefidkon *et al.*[47], who reported that the main components of oil from *S. sempervirens* leaves were α-phellandrene, limonene (13.30%) and terpinene-4-o1 (6.47%). While the oil samples extracted from *Sequoia sempervirens* calli showed that Limonene was the most important essential oil component followed by α-Phellandrene and Broneol. These results are in agreement with Hossain *et al.*[48] indicated that there is a considerable difference among volatile constituents profile of calli and the plant leaves. Whereas, the essential oil obtained from the plant leaves was dominated by monoterpenes and sesquiterpenes, the oil from calli tissue induced from leaf segments composed of diterpenes, triterpenes and hydrocarbons. It is consistent with the fact that plant metabolites produced in callus culture may be different from the plant organs. It is also worthy to mention that there are considerable differences in the essential oil profile of calli induced from different hormonal combinations. It is thought that various hormonal treatments may tend to change metabolic pathways. Differentiation stage influences as another factor on metabolic process and phytochemicals in callus culture[49].

CONCLUSION

A rapid and reliable protocol for micropropagation of *Sequoia sempervirens* is necessary where, this species is only one *S. sempervirens* tree in Egypt which had been threatened, never produces seeds. Once the problem of eradicating contamination was circumvented, this species could be easily propagated and properly rooted with the addition of growth regulators *in vitro*. Micropropagated plantlets retained their typical *S. sempervirens* characteristics, although with some differences in the essential oil profile.

SIGNIFICANCE STATEMENTS

This study discovered the optimum factors that can be beneficial to enhance the micropropagation of shootlets and induce callus of *Sequoia sempervirens*. This study will help the researchers to uncover the critical areas of *Sequoia sempervirens* that had been threatened and their active compounds production that can help to reduce the micropropagated plants losses that many researchers were not able to explore.

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REFERENCES

1. Ma, Q.W., F.L. Li and C.S. Li, 2005. The coast redwoods (*Sequoia*, taxodiaceae) from the eocene of heilongjiang and the miocene of Yunnan, China. Rev. Palaeobot. Palynol., 135: 117-129.
2. Busing, R.T. and T. Fujimori, 2005. Biomass, production and woody detritus in an old coast redwood (*Sequoia sempervirens*) forest. Plant Ecol., 177: 177-188.
3. Korban, S.S. and I.W. Sul, 2007. Micropropagation of Coast Redwood (*Sequoia sempervirens*). In: Protocols for Micropropagation of Woody Trees and Fruits, Jain, S.M. and H. Häggman (Eds.)., Springer, Dordrecht, Netherlands, ISBN: 978-1-4020-6352-7, pp: 23-32.
4. Giri, C.C., B. Shyamkumar and C. Anjaneyulu, 2004. Progress in tissue culture, genetic transformation and applications of biotechnology to trees: An overview. Trees, 18: 115-135.
5. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Planta., 15: 473-497.
6. Cohen, D., 1995. The culture medium. Acta Hortic., 393: 15-24.
7. Saad, A.I.M. and A.M. Elshahed, 2012. Plant Tissue Culture Media. In: Recent Advances in Plant *in vitro* Culture, Leva, A. and L.M.R. Rinaldi (Eds.)., InTech, China, pp: 219.
8. Ioio, R.D., F.S. Linhares, E. Scacchi, E. Casamitjana-Martinez, R. Heidstra, P. Costantino and S. Sabatini, 2007. Cytokinins determine *Arabidopsis* root-meristem size by controlling cell differentiation. Curr. Biol., 17: 678-682.
9. Yew, C.K., B. Balakrishnan, J. Sundasekaran and S. Subramaniam, 2010. The effect of cytokinins on *in vitro* shoot length and multiplication of *Hymenocallis littoralis*. J. Med. Plants Res., 4: 2641-2646.

10. Nobre, J., C. Santos and A. Romano, 2000. Micropropagation of the mediterranean species *Viburnum tinus*. Plant Cell Tissue Org. Cult., 60: 75-78.
11. Mulabagal, V. and H.S. Tsay, 2004. Plant cell cultures-an alternative and efficient source for the production of biologically important secondary metabolites. Int. J. Appl. Sci. Eng., 2: 29-48.
12. Ahmed, M.B., 2012. Forest genetic resources country report Egypt. Thesis Forests & Wood Technology Department, Faculty of Agriculture, University of Alexandria. Egypt. 116 p. Available from: http://www.fao.org/3/i3825e/i382 5e21.pdf
13. Gad, M.M.A., M.A. Salem and E.F.M. Hewida, 2006. Clonal propagation and mass production of *Sequoia sempervirens* (D. Don) Endl. through tissue culture techniques. The First International Conference on "Strategy of Botanic Gardens", October 12, 2006, Agriculture Museum, Dokki, Giza, Egypt, pp: 54-69.
14. Taha, K.F. and Z.T.A. Shakour, 2016. Chemical composition and antibacterial activity of volatile oil of *Sequoia sempervirens* (Lamb.) grown in Egypt. Med. Aromat. Plants, Vol. 5. 10.4172/2167-0412.1000245.
15. Yang, C.M., K.W. Chang, M.H. Yin and H.M. Huang, 1998. Methods for the determination of the chlorophylls and their derivatives. Taiwania, 43: 116-122.
16. Brand-Williams, W., M.E. Cuvelier and C. Berset, 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci. Technol., 28: 25-30.
17. Singleton, V.L., R. Orthofer and R.M. Lamuela-Raventos, 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol., 299: 152-178.
18. Ordonez, A.A.L., J.D. Gomez, M.A. Vattuone and M.I. Lsla, 2006. Antioxidant activities of *Sechium edule* (Jacq.) Swartz extracts. Food Chem., 97: 452-458.
19. Marco-Medina, A. and J.L. Casas, 2013. RAPD and phytochemical analysis of *Thymus moroderi* plantlets after cryopreservation. Cryo Lett., 34: 119-127.
20. Steel, R.G.D. and J.H. Torrie, 1980. Principles and Procedures of Statistics a Biometrical Approach. 2nd Edn., Auckland: McGraw-Hill International, Kogakusha, ISBN: 0-07-060926-8, Pages: 633.
21. Taha, L.S., S.S. Sayed, M.M. Farahat and I.M. El-Sayed, 2018. *In vitro* culture and bulblets induction of asiatic hybrid lily 'red alert'. J. Biol. Sci., 18: 84-91.
22. Rice, R.D., P.G. Alderson, J. Hall and A. Ranchhod, 1992. Micropropagation: Principles and Commercial Practice. In: Plant Biotechnology, Fowler, M.W. and G.S. Warren (Eds.)., Pergamon Press, Oxford, pp: 129-149.
23. Singh, R. and J.P. Tiwari, 1998. *In-vitro* clonal propagation of jackfruit (*Artocarpus heterophyllus* Lamk.). Indian J. Hortic., 55: 213-217.
24. Abou Dahab, A.M., A.M. Habib, M.K. El-Bahr and A.M.M. Gabr, 2010. Establishment of an *in vitro* propagation protocol for *Taxodium distichum* and *Taxodium distichum* var. 'distichum'. Nat. Sci., 8: 216-227.
25. Hashish, K.I., L.S. Taha and S.M.M. Ibrahim, 2015. Micropropagation potentiality and pigments content of *Hibiscus rosa-sinensis* L. as affected by γ radiation. Int. J. ChemTech. Res., 8: 131-136.
26. Ziv, M., 1991. Quality of micropropagated plants-vitrification. *In vitro* Cell. Dev. Biol. Plant, 27: 64-69.
27. Aloufa, M.A.I., S.M.L. Bezerra and G.P.T. Jordao, 2003. *In vitro* clonal mass propagation of *Ximenia americana* L. Fruits, 53: 175-178.
28. Khaleghi, A., A. Khalighi, A. Sahraroo, M. Karimi, A. Rasoulnia, I.N. Ghafoori and R. Ataei, 2008. *In vitro* propagation of *Alstromeria* cv. 'Fuego'. Am. Eurasian J. Agric. Environ. Sci., 3: 492-497.
29. Kozak, D. and A. Salata, 2011. Effect of cytokinins on *in vitro* multiplication of rhubarb (*Rheum rhaponticum* L.) 'Karpow Lipskiego' shoots and *ex vitro* acclimatization and growth. Acta Sci. Pol. Hortor. Cult., 10: 75-87.
30. Shahriari, A.G., A. Bagheri, A. Sharifi and N. Moshtaghi, 2012. Efficient regeneration of 'Caralis' *Alstroemeria* cultivar from rhizome explants. Notulae Sci. Biol., 4: 86-90.
31. Youssef, N.M., L.S.A. Tahaa and S.N. Abd El-Khalek, 2021. Secondary metabolites characterization of *in vitro* propagated *Antigonon leptopus* cultures. Egypt. J. Chem., 64: 923-932.
32. Abd El-Kadder, E.M, I.I. Lashin, M.S. Aref, E.A. Hussian and E.A. Ewais, 2014. Physical elicitation of *Dillenia indica* callus for production of secondary metabolites. N. Y. Sci. J., 10: 48-57.
33. Lestari, N.K.D., N.W. Deswiniyanti, I.A. Astarini and L.M. Arpiwi, 2020. Morphogenesis *in vitro* flower pedicel of *Lilium longiflorum* with NAA and BAP. The International Conference on Biotechnology and Life Sciences, Feberaury 11, 2020, KnE Life Sciences, pp: 18-31.
34. Kende, H., 1989. Enzymes of ethylene biosynthesis. Plant Physiol., Vol. 91. 10.1104/pp.91.1.1.
35. Marino, G. and G. Bertazza, 1990. Micropropagation of *Actinidia deliciosa* cvs. 'Hayward' and 'Tomuri'. Sci. Hortic., 45: 65-74.
36. Szweykowska, A.M., 1992. Regulation of the Light Harvesting Chlorophyll a/b Biosynthesis by Cytokinin. In: Physiology and Biochemistry of Cytokinin in Plants, Kaminek, M. (Ed.)., Balogh Scientific Books, UK, pp: 137-139.
37. Pasqua, G., P. Avato, B. Monacelli, A.R. Santamaria and M.P. Argentieri, 2003. Metabolites in cell suspension cultures, calli and *in vitro* regenerated organs of *Hypericum perforatum* cv. Topas. Plant Sci., 165: 977-982.

38. Kuhlmann, A. and C. Röhl, 2006. Phenolic antioxidant compounds produced by *in vitro*. Cultures of rosemary (*Rosmarinus officinalis*) and their anti-inflammatory effect on lipopolysaccharide activated microglia. Pharm. Biol., 44: 401-410.
39. Baskaran, P., B. Ncube and J. Van Staden, 2012. *In vitro* propagation and secondary product production by *Merwilla plumbea* (Lindl.) Speta. Plant Growth Regul., 67: 235-245.
40. Baskaran, P., M. Moyo and J. Van Staden, 2014. *In vitro* plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. South Afr. J. Bot., 90: 74-79.
41. Grzegorczyk-Karolak, I., P. Rytczak, S. Bielecki and H. Wysokińska, 2017. The influence of liquid systems for shoot multiplication, secondary metabolite production and plant regeneration of *Scutellaria alpina*. Plant Cell Tissue Organ Cult., 128: 479-486.
42. Xie, D. and Y. Hong, 2001. *In-vitro* regeneration of *Acacia mangium* via organogenesis. Plant Cell Tissue Organ Cult., 66: 167-173.
43. Sen, M.K., S. Nasrin, S. Rahman and A.H.M. Jamal, 2014. *In vitro* callus induction and plantlet regeneration of *Achyranthes aspera* L., a high value medicinal plant. Asian Pac. J. Trop. Biomed., 4: 40-46.
44. Bendini, A., L. Cerretani, L. Pizzolante, T.G. Toschi and F. Guzzo *et al*., 2006. Phenol content related to antioxidant and antimicrobial activities of *Passiflora* spp. extracts. Eur. Food Res. Technol., 223: 102-109.
45. Wojdylo, A., J. Oszmianski and R. Czemerys, 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem., 105: 940-949.
46. Otto, A. and V. Wilde, 2001. Sesqui-, di- and triterpenoids as chemosystematic markers in extant conifers-A review. Bot. Rev., 67: 141-238.
47. Sefidkon, F., S. Meshkizadeh and S. Shahrzad, 2002. Comparison between oil composition of *Sequoia sempervirens* from tissue culture and main sample. Iran. J. Med. Aromat. Plants, 18: 23-42.
48. Hossain, M.A., A.B. Siddique, S.M.M. Rahman and M.A. Hossain, 2010. Chemical composition of the essential oils of *Stevia rebaudiana* Bertoni leaves. Asian J. Traditional Med., 5: 56-61.
49. Singh, N., K. Yadav, S. Kumari and Renu, 2011. Metabolic changes during differentiation in callus cultures of *Stevia rebaudiana* (Bertoni). J. Phytol., 3: 63-67.