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Article

Evaluation of the Role of 24- Epibrassinolide in Propagation of Gac (*Momordica cochinchinensis* Spreng) and its Content of β-carotene and lycopene *in vitro*

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Abstract: This study was conducted in order to estimate the effect of growth regulator 24-epibrassinolide (EBR) on leaves content of total carotenoids in general and beta-carotene and lycopene in particular by using the tissue culture technique of propagating *Momordica cochinchinensis* Spreng. The efficiency of NaClO in reducing the percentage of explant pollution appeared as its concentration increased; nodes gave a higher response rate than apical buds in the initiation stage due to the effect of different concentrations of benzyladenine (BA), for shoots multiplication, it was found that as concentration of EBR increases, the number of shoots decreases, (EBR 0.0+ GA3 0.1+ BA 1.5) mg/L was significantly superior with an average of 3.10 ± 0.27 in the number of shoots, while treatment (EBR 0.05+ GA3 0.1+ BA 1.5) mg/L gave highest rate of shoots length (4.51 ± 0.30 cm). For leaves the content of carotenoids, it was found that EBR raises level of it, where (EBR 0.1+ GA3 0.1+ BA 1.5) mg/L gave highest content of total carotenoids (9.5 %), compared to mother plant (8.9 %) using spectrophotometric method, same treatment gave the highest content of beta-carotene and lycopene (23.8, 5.3) ppm respectively compared to mother plant (4.7, 0.7) ppm respectively, using HPLC for both compounds.

Keywords: *Momordica cochinchinensis* Spreng; *in vitro* culture; 24-epibrassinolide (EBR); carotenoids.

Introduction

Momordica cochinchinensis Spreng a plant of cucurbitaceae family. Gac is the name given to this fruit in Vietnam[1]. Gac fruits are valuable for antiinflammatory[2] and anticancer activities [3–5]. In addition to seeds [6,7], roots [8–10], leaves have been traditionally used in many treatments, such as warts, fever, inflammation and hemorrhoids [11].Fruit possesses the highest amount of nutritionally essential carotenoids (lycopene and β–carotene) of all known fruits and vegetables, including tomatoes and carrots, by more than 200 and 54 times, respectively [12]. As for leaves of the plant, the content of carotenoids in them has not been studied, and the determination of its content of other phytochemicals compounds is limited; previous studies have proven that leaves contain a pentacyclic steroid as an antioxidant [13], Tri-terpenoids that have antimicrobial activity [14]. Recently, HPLC was used in the identification of phenolic and flavonoid compounds in leaves of Gac plant [15].

Carotenoids are tetraterpene pigments that appear in nature in purple, red, orange or yellow colors. These pigments are the most common [16], are essential compounds in photosynthetic bacteria, algae, and plants [17]. Carotenoid-derived phytohormones, including abscisic acid (ABA) and strigolactone [18]. In ordinary human foods, there are about 55 types of carotenoids, and among them, there are about 25 types in the blood that are eaten: β-carotene, α-carotene, lycopene, β-cryptoxanthin, lutein, and zeaxanthin make up more than 95% of the total carotenoids [19–21]. Lycopene gives many fruits and vegetables red color [22]. Lycopene ranks first among antioxidants, 100 times more effective than tocopherol and twice as effective as β-carotene. It is regarded to be safe and non-toxic, and there are usually no adverse side effects from using it[23], lycopene showed anti-proliferative activity against HeLa cancer cells at a concentration of 1.5 ppm, thus is a potential source of treatment for cervical cancer [24]. In addition to skin, colon and prostate cancers, as well as a reduction in the risk of cardiovascular disease. It shields cells from the harmful effects of reactive oxygen species [22]. Gac fruit contains at least five times the amount of lycopene found in other fruits [25,26]. β -carotene (yellow and orange colors) is the most common form of pro-vitamin A found in foods and helps

avoid insufficiency. β -carotene has also been identified as a possible anticancer agent [27] and have antioxidant properties [28].
Explants are grown on synthetic media, and plant growth regulators are used to support and manipulate growth. Numerous studies have shown the potential of *in vitro* culture of various medicinal plants and the efficient protocols that enhance the production of secondary metabolites and conservation of a wide range of medicinal plants [29] In addition, one of Gac plant problems is the decrease in the germination rate of seeds [30]. Brassinosteroids, such as 24-epibrassinolide (EBR), are some of the biologically most active growth regulators that specifically modulate plant responses to abiotic stress and increased carotenoids [31].
The method for determining low concentrations of compounds using UV spectra is simple and rapid; as for analyzing the active substances and estimating their content, HPLC technology is used, including the content of carotenoids in different parts of many plants, it was used to analyze the content of Gac from beta-carotene and lycopene [32].
The current study was conducted to propagate *Momordica cochinchinensis* using a tissue culture study on the effectiveness of 24-epibrassinolide on propagation and plant content of carotenoids.

Materials and Methods

Location and Plant Materials Source

Plant tissue culture experiments were carried out in the laboratories of Janet al-nakheel for Plant Tissue Culture Ltd., and the extraction and Estimation of active substances were carried out in laboratories of the Department of Environment and Water of the Ministry of Science and Technology, Iraq. The explants were taken from Gac vine planted in Baghdad, Iraq [5].

Sterilization of Equipment and Media

The tools used, which included instruments such as rest support, forceps, and blade holders, were sterilized in the oven at a temperature of 190-200 °C for 30 minutes. In addition to sterilizing the tweezers and blades after each cutting process using a glass bead sterilizer (Lab Associates, Netherlands). As for distilled water used in the process of sterilizing explants, kraft paper is used as a surface for cutting the explants and culture media in all stages, it is sterilized in an autoclave (Labtech, Korea) [33].

Sterilization of Explants

Explants (single node cuttings and apical buds 3 cm in length) were washed with streaming water for 30 min, then transferred to laminar flow cabinets (Labtech, Korea) to sterilized (concentrations of sodium hypochlorite NaClO (concentration of 6% in Clorox commercial solution, Saudi Arabia) used were (0, 1.5, 3%), explants immersed in it for (4, 8) minutes with continuous shaking after adding a few drops of tween 20.
The medium was prepared for all stages of propagation [33] with use (Murashige and Skoog medium [34] including vitamins 4.405 g/L and plant growth regulators (Duchefa, Netherlands); incubation conditions for all stages were at 26 ± 2 °C, light period 16 hours, dark period 8 hours, and light intensity was 1500-1800 lux.

Initiation Stage

The treatment that gave the least contamination rate with explants remained alive from the sterilization stage, explants planted on initiation medium equipped with (GA3 0.1+ IAA 0.5) mg/L overlapping with BA (0, 1, 1.5) mg/L. For each concentration, 10 replicates were used with nodes and apical buds; after 4 weeks of incubation, measurements were taken, which included the percentage of open buds%[33].

Multiplication Stage

Selected the treatment that gave the best response from the initiation stage and shoots resulting from the growth of nodes were used in multiplication experiment (were cut at a rate of 1.5 cm), planted on the medium prepared with (GA3 0.1+ BA 1.5), overlapping with EBR (0, 0.05, 0.1) mg/L. For each concentration, 10 replicates were used, and after 4 weeks of incubation, measurements were taken, including the number and length of shoots.

Estimation of Carotenoids

UV-VIS Spectrophotometer

Of fresh leaves, 40 mg were taken from each of the three treatments from the multiplication stage of tissue propagation, S1= (GA3 0.1, BA 1.5, EBR 0) mg/L, S2= (GA3 0.1, BA 1.5, EBR 0.05) mg/L, S3= (GA3 0.1, BA 1.5, EBR 0.1) mg/L and mother plant leaves (control)[35] with slight modifications (kept in the refrigerator for 1-2 days), such as a UV-VIS spectrophotometer from Shimadzu, Japan.

High-Performance Liquid Chromatography (HPLC)

Extraction and Estimation of β – carotene Compounds

An amount of 0.5 g fresh weight was taken from each of the four samples that were taken in the spectrophotometer experiment [36] with slight modifications (after removing petroleum ether at 40 °C using a rotary evaporator, the concentrate was reconstituted up to 0.5 mL with a mobile phase into a volumetric flask), HPLC instrument (Sykamn, German). Standard compounds of β – carotene (Sigma-Aldrich, USA) were used at the same conditions and concentrations of β carotene were quantified by comparing the area of standard with an area of each model by using equation[37]:

Concentration of compound µg/ml = (area of sample/area of standard) x concentration of standard µg/ml x dilution factor.

Extraction and Estimation of lycopene compounds

Amount 1 g of fresh weight from each sample of the four samples used in the previous experiment [38] with slight modifications (the organic phase was obtained and dried, then the residue was redissolved in 0.5 mL with a mobile phase before being injected). The standard compound of lycopene (Sigma-Aldrich, USA) was used at the same conditions and concentrations of lycopene were measured using the same equation as β-carotene.

Experimental Design and Statistical Analysis

Statistical analysis system (SAS)[39] The program was used to determine different factors in study parameters. Factorial experiment, two way and one way of other, applied in completely randomized design (CRD). The least significant difference (LSD) test (Analysis of variation-ANOVA) at ($P<0.05$) was used to compare between means in this study significantly.

Results

In vitro Culture

Effect of Sodium Hypochlorite Concentration and Time Periods in Percentage of Contamination of Nodes and Apical Buds

Tables (1) and (2) show the results of sodium hypochlorite (NaClO) used to sterilize nodes and apical buds, respectively; concentrations of NaClO had a significant effect in reducing the percentage of contamination also sterilization duration and interaction between concentrations of NaClO with periods.

Table 1. Effect of sodium hypochlorite (NaClO) concentration and periods in contamination percentage of nodes after 7 days for *M. cochinchinensis*.

NaClO Conc. (%)	Time (min)		Mean of Conc.
	4	8	
0.0	100.0 a	100.0 a	100.0 A
1.5	100.0 a	10.0 c	55.0 B
3.0	60.0 b	0.0 c	30.0 C
Mean of time	86.6 A	36.6 B	----
[1] L.S.D. * (P < 0.05)	Conc. = 15.6 *, Time =12.8 *, Conc. x Time= 22.1 * Means with different letters differed significantly		

[1] L.S.D. Least significant difference.

Table 2. Effect of sodium hypochlorite (NaClO) concentration and periods in contamination percentage of apical buds after 7 days for *M. cochinchinensis*.

NaClO Conc. (%)	**Time (min)**		**Mean of Conc.**
	4	**8**	
0.0	100.0 a	100.0 a	100.0 A
1.5	80.0 ab	0.0 c	40.0 B
3.0	60.0 b	0.0 c	30.0 B
Mean of time	80.0 A	33.0 B	----
[1] L.S.D. * (P < 0.05)	Conc. = 17.2 *, Time = 14.1 *, Conc. x Time= 24.4 * Means with different letters differed significantly		

[1] L.S.D. The least significant difference.

Effect of Benzyl adenine (BA) Levels on Response of Explant at Initiation Stage

Results of Table (3) show that for nodes (fig 1), 1.5 mg/L was significantly superior to the rest of the concentrations, as for the apical buds (fig 2), there were no significant differences between concentrations.

Table 3. Effect of BA levels on the response of explants at initiation stage after 4 weeks for *M. cochinchinensis*.

([1] GA3 0.1+ [2] IAA 0.5) + [3] BA (mg/L)	Nodes %	apical buds %
0.0	50.0 b	30.0
1.0	80.0 ab	40.0
1.5	100.0 a	40.0
LSD value	35.7*	[6] NS
[4] L.S.D. [5] * (P < 0.05)	Means with different letters in the same column differed significantly.	

[1] GA3= Gibberellic acid, [2] IAA= Indol-3-acitic acid, [3] BA= Benzyladenine, [4] L.S.D.= Least significant difference, [5] * = significant, [6] NS= No significant.

Figure 1. Effect of BA levels (1= 0.0, 2= 1.0, 3= 1.5) mg/L + (GA3 0.1+ IAA 0.5) mg/L on the response of nods at initiation stage after 4 weeks for *M. cochinchinensis.*

Figure 2. Effect of (GA3 0.1 + IAA 0.5 + BA 1.5) mg/L on the response of apical buds at initiation stage after 4 weeks for *M. cochinchinensis.*

Effect of 24-epibrassinolide (EBR) Levels in Number and Length of Shoots at Multiplication Stage
Results of Table (4) (fig 3) show significant differences between EBR concentrations concerning the number and length of shoots.

Table 4. Effect of EBR levels in number and length of shoots at multiplication stage for *M. cochinchinensis*.

([1] GA3 0.1+ [2] BA 1.5) + [3] EBR (mg/L)	**Mean ± SE**	
	Number	**Length (cm)**
0.0	3.10 ±0.27 a	2.30 ±0.17 b
0.05	2.20 ±0.29 b	4.51 ±0.30 a
0.1	1.50 ±0.22 b	4.05 ±0.32 a
LSD value	0.76 *	0.80 *
[4] L.S.D. [5] * (P < 0.05)	Means with different letters in the same column differed significantly.	

[1] GA3= Gibberellic acid, [2] BA= Benzyladenine, [3] EBR= 24-epibrassinolide, [4] L.S.D.= Least significant difference, [5] * = significant.

Figure 3. Effect of EBR levels (left to right: 0, 0.05, 0.1) mg/L + (GA3 0.1 + BA 1.5) mg/L in length and number of shoots at multiplication stage after 4 weeks for *M. cochinchinensis*.

Estimation of Carotenoids

Spectrophotometric Determination of Total Carotenoids (Quantitative Analysis)

Table (5) shows that the highest content of total carotenoids was 9.5 % at S3 and the control (mother plant leaves) was 8.9 %.

Table 5. Spectrophotometric determination of total carotenoids for *M. cochinchinensis* fresh leaves

Samples	Total carotenoids (%) fresh mass
[1] **Control**	8.9
[2] **S1**	6.8
[3] **S2**	8.5
[4] **S3**	9.5

[1] Control= mother plant, ([2] S1= (GA3 0.1+ BA 1.5+ EBR 0) mg/L, [3] S2= (GA3 0.1+ BA 1.5+ EBR 0.05) mg/L, [4] S3= (GA3 0.1+ BA 1.5+ EBR 0.1) mg/L) treatments the multiplication stage of tissue propagation.

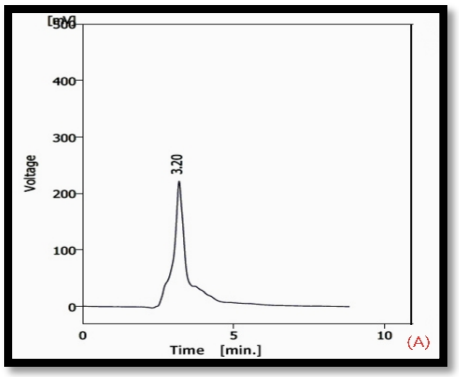
3.2.2. HPLC Analysis (Qualitative analysis)

According to Table (6) (fig 4 and 5), from retention times, it can be said that all extracts contain components of β- carotene and lycopene, but their β- carotene content was higher than lycopene, as for β- carotene content of extracts, the highest concentration was (23.825 ppm) at S3 (GA3 0.1+ BA 1.5+ EBR 0.1) mg/L. As for the lycopene content of extracts, the highest concentration was (5.308 ppm) at S3 (GA3 0.1, BA 1.5, EBR 0.1) mg/L from the multiplication stage of the tissue propagation process.

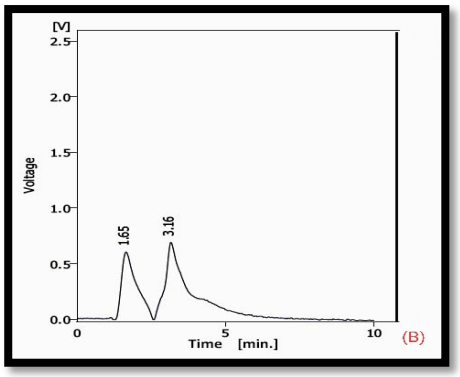
Table 6. HPLC analysis of β- carotene and lycopene, retention time (min), area, concentration (ppm), *M. cochinchinensis* fresh leaves

Active compounds	Standards and samples	Retention time (min)	Area	Concentration (ppm)
β- carotene	Standard	3.196	1537	20
	[2] S1	3.160	491	6.389
	[3] S2	3.160	1388	18.061
	[4] S3	3.160	1831	23.825
	[1] Control	3.160	359	4.671
Lycopene	Standard	6.040	2479	20
	[2] S1	6.170	304	1.225
	[3] S2	6.170	816	3.292
	[4] S3	6.170	1316	5.308
	[1] Control	6.170	185	0.746

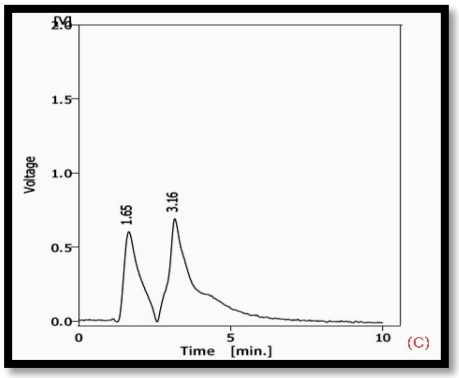
[1] Control= mother plant, ([2] S1= (GA3 0.1+ BA 1.5+ EBR 0) mg/L, [3] S2= (GA3 0.1+ BA 1.5+ EBR 0.05) mg/L, [4] S3= (GA3 0.1+ BA 1.5+ EBR 0.1) mg/L) treatments the multiplication stage of tissue propagation.



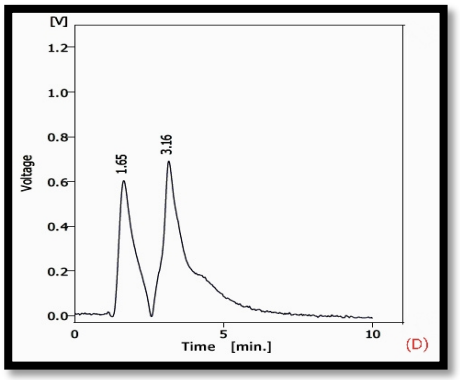
(**a**)



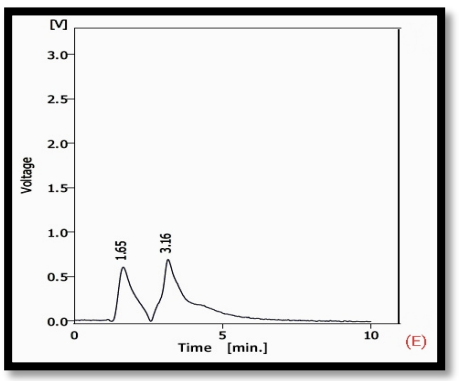
(**b**)



(**c**)



(**d**)



(**e**)

Figure 4. Chromatographic of β- carotene of (a) standard solution, (b) multiplication stage of tissue propagation (GA3 0.1+ BA 1.5+ EBR 0) mg/L, (c) multiplication stage of tissue propagation (GA3 0.1+ BA 1.5+ EBR 0.05) mg/L, (d) multiplication stage of tissue propagation (GA3 0.1+ BA 1.5+ EBR 0.1) mg/L, (e) mother plant (*M. cochinchinensis)* fresh leaves, isocratic RF-HPLC C18-ODS (250 mm * 4.6 mm, 3 µm), mobile phase: ethyl acetate: acetonitrile: acetic acid (30:68:2, v/v/v) with 0.22 mM BHT, flow rate: 0.7 mL/min, dilution factor: 1, column temperature: 35 °C, injection volume: 100 µL, UV detection: 472 nm.

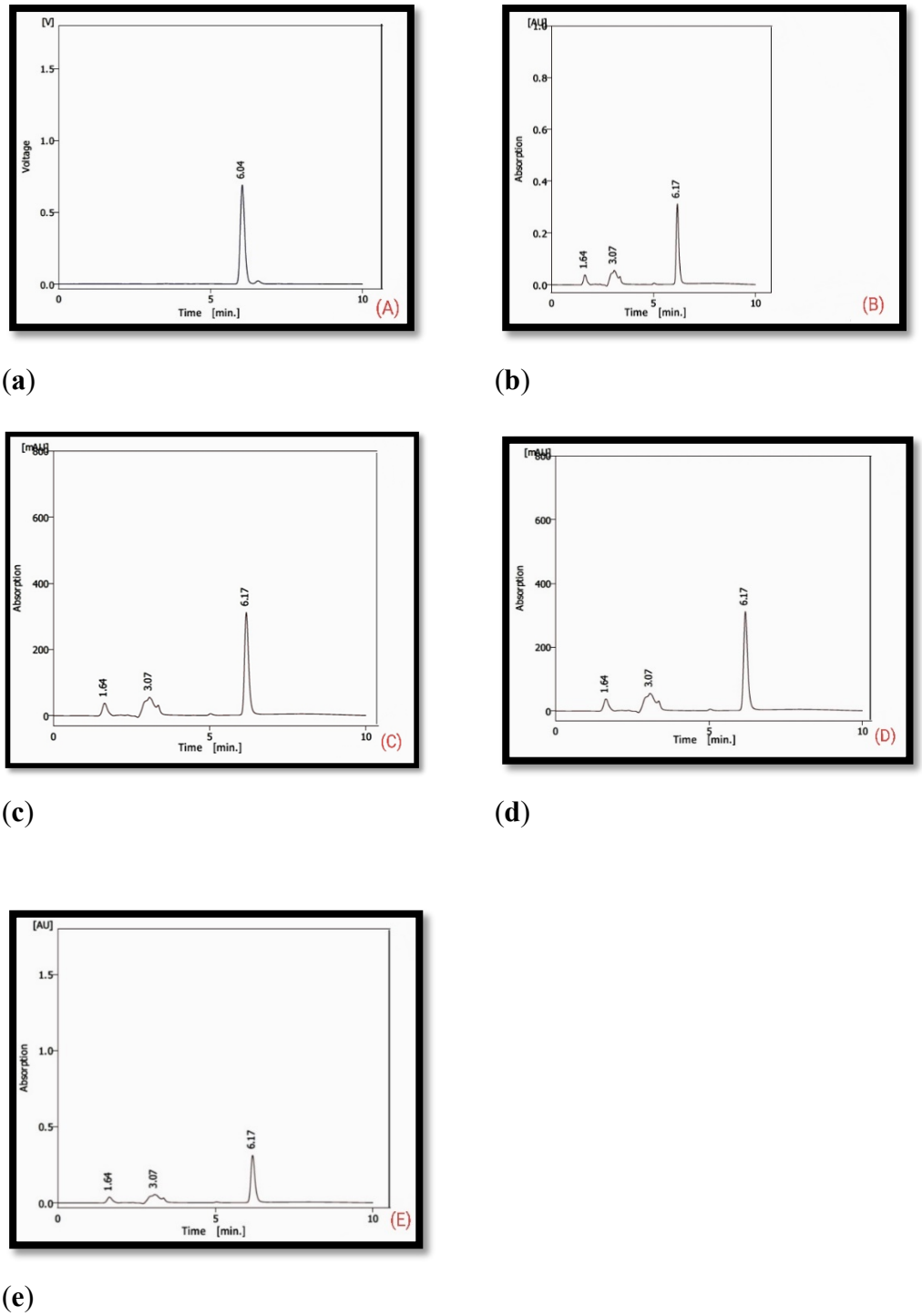


Figure 5. Chromatographic of lycopene of (a) standard solution, (b) multiplication stage of tissue propagation (GA3 0.1+ BA 1.5+ EBR 0) mg/L, (c) multiplication stage of tissue propagation (GA3 0.1+ BA 1.5+ EBR 0.05) mg/L, (d) multiplication stage of tissue propagation (GA3 0.1+ BA 1.5+ EBR 0.1) mg/L, (e) mother plant (*M. cochinchinensis*) fresh leaves, Isocratic RF-HPLC C18-ODS (250 mm * 4.6 mm, 3 µm), mobile Phase: MeOH / isopropyl alcohol / THF (30:30:35) containing 250 ppm BHT and 0.05% TEA, flow rate: 1 mL/min, dilution factor: 2. column temperature: 35 °C, injection volume: 100 µL, UV detection: 472 nm.

Discussion

The efficiency of NaClO in reducing the percentage of explant pollution appeared as its concentration increased; this is inconsistent with results in the sterilization of *Swingle Citrumelo* and *Troyer Citrange* plant[33]. A concentration of 1.5% gave the highest survival rate for explains at 8 minutes in this study, while a concentration of 3.0% at 10 minutes gave the highest survival rate for explant[33] And this does not agree with the results of the experiment, as it negatively affected the survival of explants and led to their death in

8 minutes. The mechanism of action of sodium hypochlorite and its effect as a sterilizer is due to Hypochlorous acid (HOCL), which is a solid oxidizing substance, as this acid is formed as a result of chlorine dissolving in water [40].

Shoots that were transferred from MS medium containing BAP (2.0 mg/L) + IAA (0.2 mg/L) to the same medium but with the addition of low concentrations of GA3 contributed to shoots elongation in *luffa acutangula* plant[41]. The results of the current experiment are inconsistent with the results of *Momordica dioicabud* plants, in which a fracture of nodal explants was observed at a deficient concentration of IAA (0.1 mg/L) with BAP (2.0 mg/L) [42]. Most research scientists reported that BAP in the concentration range of (1.0 - 3.0 mg/L) gave good results with apical buds as an explant in *Cucumis sativus*, *Cucumis melo*, *Cucurbita maxima* [43,44]Also, some scientists reported that using BAP in combination with IAA helps initiate shoots in *Citrullus lanatus* and *Cucumis melo L.* plants [45,46]These results did not agree with the results of the current experiment on apical buds, so apical buds were excluded from the following experiments.

The effect of GA3 at low concentrations with BAP was better for shoot multiplication than the effect of BAP singly in *Benincasa Hispida L.*[47]. To evaluate the potential effects of adding EBR on *Prunus armeniaca* grown *in vitro*, the number and length of shoots were significantly increased in MS medium supplemented with 0.2 mg/L EBR and 2 mg/L BAP [48]. This does not agree with the results of this experiment, as the increase in EBR concentrations did not lead to an increase in the number of shoots but only an increase in the lengths of shoots. Results also agree with the results of the experiment on two *citrus cultivars*, where the medium free of BL (one of the brassinosteroids) and containing 2 mg/L BA gave the highest rate in a number of shoots[33]. The reason for increase in number of shoots in presence of BA and absence of EBR is that cytokinins encourage vegetative growths and multiply it significantly [49]. As for the effect of EBR on increasing the length of shoots, these results are also in agreement with was reached by in studying effect of levels of BL which led to emergence of significant differences between lengths at BA 1.5mg/L[33].Also proved when two cultivars of sweet pepper were treated *in vitro* by EBR, it stimulated stem elongation[50]. Reason for effect of EBR in increasing length of shoots may be due to fact that in most cases, brassinosteroids show an effect similar to effect of auxins, gibberellins and cytokinins, also have a clear effect on some physiological activities of plant, including their effect on cell elongation and maintaining apical dominance [51].

Content of carotenoids (µg/ ml) in leaves of *Adiantum sp*. (24 hr 1.75, 48 hr 1.60, 72 hr 1.70), *Crystiella sp.* (24 hr 1.33, 48 hr 0.70, 72 hr 1.03), *Drypteris sp*. (24 hr 1.80, 48 hr 1.66, 72 hr 1.37)[52]. Also in leaves of *Armoracia rusticana L.* plant (mg/g) (horseradish leaves and by-products extracts 0.09, 0.13 respectively)[53]. Also (µg/ ml) for both septate and aseptate leaves for *Oreopanax Xalepensis*, (80% acetone to extract the sample: not determined, 0.538) respectively, (acetone absolute to extract the sample: 1.089, 1.047), respectively [54]. As for study the effect of growth regulators on content of carotenoids, in a study to measure the effect of foliar application of GA3 and BA on photosynthetic pigments of *Dizigotheeca Elegantissima* plant after 60 days of spraying, the highest content of carotenoids (2.53 mg/ml fresh weight) was at GA3 100 mg/L + BA 200 mg/L compared to the control (1.95 mg/ml fresh weight) [55], also in a similar study on the *Ficus benjamina*, the highest content of carotenoids (3.85 µg/ml fresh weight) was at GA3 100 mg/L + BA 100 mg/L, compared to the control (2.73 µg/ml fresh weight) [56]. All concentrations of gibberellic acid (GA3), 6-benzylaminopurine (6-BA) and four concentrations of 24-epibrassinolide (EBR) caused significant increases in total carotenoid contents (mg/g) in *C. pyrenoidosa* ZF strain biomass when compared to controls after 12 days of treatment [57], this is in agreement with the results for EBR, effect of sprayed the EBR (0.1 µM) on contents of carotenoids were studied on leaves of *Solanum lycopersicum L.*, (0.30 mg/g fresh mass) compared to control (0.29 mg/g fresh mass)[58]. The salt stress caused a significant decrease in carotenoid contents in leaves of two varieties of Capsicum annuum (hot pepper, sweet pepper) compared with control. The data showed that application of EBR 0.5 ppm enhanced the carotenoid content (mg/100gm f.w.) with salinity [31].

HPLC performed the separation and quantification of β-carotene (18.84 µg/g) on a reversed-phase C18 column for leaves of *Inula helenium L.* after solvent extraction and saponification[59]. Lycopene by isocratic HPLC analysis for *Barringtonia racemosa* leaves was analyzed, where the result showed that the retention time of standard lycopene was 6.28, while the retention time of lycopene content of leaf extract was 6.20, chloroform extract of *B. racemosa* leaf may have the potential to be used as anti-inflammation and antioxidant agents and it was revealed that active compound in *B. racemosa* is lycopene [60].

The effect of 24-epibrassinolide (EBR) on carotenoid accumulation and quality attributes of tomato (*Solanum lycopersicum*) fruit, EBR-treated pericarp discs of ethylene insensitive mutant, never ripe, accumulated significantly more carotenoid than those of the control. The results suggest that BR seems to be involved in

modulating pigments accumulation[61]. An alternative strategy is the genetic manipulation of BR biosynthesis or signaling is to alter BR content or its signaling intensity in crop plants [62].
Overexpression of *DWARF* gene was reported to cause an increase in BR levels, thereby enhancing carotenoid accumulation in tomato fruit[63]. Results indicated that carotenoid accumulation in tomato fruits correlated with BR signaling strength in *SLBRI1* overexpression lines [64].

Conclusions

In conclusion, the findings of this study regarding the effectiveness of various substances on plant growth and carotenoid content are inconsistent with previous research. For instance, the optimal concentration of NaClO for explant sterilization and the influence of BAP and IAA on shoot multiplication in luffa acutangula differed from prior studies. Similarly, the effects of EBR on shoot proliferation and elongation in Benincasa Hispida and Prunus armeniaca, respectively, contradicted past observations.
These discrepancies highlight the need for further investigation into the influence of these substances on various plant species and cultivars. Future research should explore the underlying mechanisms behind these variations and establish optimal protocols for specific plant growth and metabolite production.

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