

In vitro propagation of citrus rootstock *Citrumelo swingle*

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ABSTRACT

This study was conducted to propagate *Citrumelo swingle* rootstock *in vitro*. At the initiation experiments, single nodes were cultured in MS or MT medium supplemented with five concentrations of BA (0, 1, 2, 3, and 4 mg l⁻¹). After four weeks of the initiation, the MS medium showed a significant response increase in percentage (92%). While the MT medium gave 56%, as soon as, the highest significant No. of shoots obtained from MT medium (2.04 shoot). During the multiplication experiments, shoots in 1 cm length were cultured in the best medium from the initiation stage (MT + 2 mg l⁻¹ BA) supplemented with five concentrations of Kin (0, 2, 4, 6, and 8 mg l⁻¹). After 8 weeks the results of the multiplication stage showed that the MT medium overcame significantly in comparison with the MS medium in the shoot length (1.12 and 0.69 cm respectively), Furthermore, the media supplemented with 2 mg l⁻¹ of BA and Kin achieved the highest significant value in all multiplication characters (5.15 shoots, 1.13 cm, and 19.80 leaves) respectively. For the rooting stage, MT medium in ½ strength of salts supplemented with IBA (0, 0.5, 1, 1.5, and 2 mg l⁻¹) was used. After 6 weeks, The results of the rooting experiment showed that 1.5 mg l⁻¹ IBA resulted in the highest value in the rooting percentage (90%), the highest No. of roots (4.67 roots shoot⁻¹) has overcome significantly in comparison with 0, 0.5, and 1 mg l⁻¹ treatments, although.

Keywords: *Citrumelo swingle* rootstocks, BA, Kin, IBA, MS, MT media.

INTRODUCTION

Citrus fruits belonging to the family Rutaceae are one of the most widespread fruit crops in the world and are very desirable to consumers because they are a rich source of vitamins, minerals, and dietary fiber necessary for human health as well as their high marketing value [5]. Citrus trees have been planted in Iraq since a long time ago till now, to provide suitable environmental conditions for their cultivation They are a type of fruit with nutritional and therapeutic value for many diseases [5]. Iraq produced 1,397,000 tons of citrus fruits domestically in 2019 [16]. *Citrumelo swingle* rootstock resulted from hybridization between the *citrus paradise* Macf grapefruit and the triple orange *Poncirus trifoliata* L. Raft [19]. It is

considered an active strong rootstock and the trees grafted on, it have good productivity, strong growth, and medium to copious yield. Its fruit is medium in size and of good quality, resistant to many diseases, including gum disease “Gummosis” and rapid deterioration “Tristeza”. In addition to root rot, cracking, and wood pecking viral “Xyloporosis” as well as to nematodes, tolerating extreme cold, and its resistance to drought conditions is good because it has a large and deep root aggregate with soil [9];[8], It is well compatible with Eureka lemon and grapefruit varieties, but its compatibility is weak with some orange and mandarin varieties, and it is not recommended to use it in poorly drained soils [19].

Cytokinins are growth regulators for plants that break apical dominance, encourage cell division, and stimulate the formation of adventitious shoots and axillary buds in plants [26].

[14] investigated the effects of 1.5 mg l⁻¹ BA or 1 mg l⁻¹ Kinetin as well as their interactions on the growth of shoots from shoot tips and epicotyl for Citrus rootstocks, including Volkamer lemon (*Citrus volkmeriana*, Ten.), sour orange (*Citrus aurantium*, L.), Rangpur lime, *Troyer citrange*, and Cleopatra mandarin (*Citrus reshni*, Blanco) on MS media. They discovered that the rootstocks are modified to respond to multiplication, and the interaction between BA and Kin resulted in a considerable superiority in the number of shoots. A study by [25] examined the effect of five concentrations of BA (0, 0.5, 1, 2, and 4 mg l⁻¹) added to MS medium to initiate and multiply of apical shoots that were taken from seedlings of 3 years old of six different citrus rootstocks, (*Citrus volkmeriana*, *Citrumelo* 'Swingle', Citrange 'Carrizo', *Poncirus trifoliata* 'Serra', *Poncirus trifoliata* 'Rubidoux', and *Poncirus trifoliata* 'Flying Dragon'). They found that all rootstocks had a 100% percentage response on MS medium with 2 mg l⁻¹ BA. The best number of shoots (2.11 shoots) and leaves (7.22 leaves) were produced by the *Citrus volkmeriana* rootstock, which was significantly superior to other rootstocks when grown on media containing 1 mg l⁻¹ BA. The maximum percentage of rooting and length of roots were produced by the media containing 2 mg l⁻¹ NAA. [15] studied the single node of the orange *Citrus sinensis* (L.) Osbeck produced the best response in the initiation stage on MS and MT media, (64 and 62%, respectively), which differed significantly from WPM [20] and B5 media, given 44 and 54% respectively. This was achieved on culture media (MS, MT, WPM woody Plant Medium, and B5) supplemented with (0, 0.5, 1, 2, 3, and 4 mg l⁻¹ of BA Or Kin. The nodes that were cultured on the MS and MT media, which each produced 2.2 shoots and were significantly different from the other two media, provided the most shoots. the

best multiplication shoots were in MT media supplemented with 3 mg l⁻¹ of BA interacted with 0.1 and 0.5 NAA, resulting in 3.3 and 2.9 shoots respectively. [11] studied different BA concentrations (0.01, 0.1, 1, and 2 mg l⁻¹) affect the growth of *Citrus latifolia* nodes in MS media, as well as different NAA, IBA, or IAA concentrations (0.01, 1, 1.5, and 2 mg l⁻¹) on roots. During the multiplication stage, the medium containing 2 mg l⁻¹ BA and 1 mg l⁻¹ BA produced the most shoots with the best length, Moreover, 1.5 mg l⁻¹ NAA produced the highest rooting percentage (75%).

This study aimed to determine the effects of salt concentrations difference of (MS and MT) media (Table 1) and PGRs (BA, Kin, and IBA) concentrations and their interaction on citrus rootstock (*Citrumelo swingle*) *in vitro*.

MATERIAL AND METHODS

The study was conducted at the Laboratory of Tissue Culture/ Department of Forestry Science/ College of Agriculture and Forestry/the University of Mosul, Iraq. Soft fresh shoots, 5 cm long of *Citrumelo swingle* rootstock were used from plants source grown in the Lath House to investigate the viability of *in vitro* reproduction. The leaves were taken off from the shoots and the explants were immersed in sodium hypochlorite (NaOCl) for 20 minutes. Then, they were rinsed in tap water for 30 minutes and transferred to a laminar-flow cabinet to be sterilized. The shoots have been washed four times 10 minutes for each using distilled sterile water with shaking. The shoots were transferred to sterile Petri dishes and cut into 1 cm length pieces, each explant included a single node for culture. The single nodes were cultured in a solid MS or MT media (Table 1) supplemented with five concentrations of BA (Benzyl Adenine (0, 1, 2, 3, and 4 mg l⁻¹) [23]; [22]. The percentage of response, number of shoots, shoot length (cm), and number of leaves were all recorded after four weeks.

The best Concentration of BA from the initiation stage (2 mg l^{-1}) was added to the medium during the multiplication stage, with Kin at five concentrations (0, 2, 4, 6, and 8 mg l^{-1}). After 8 weeks of multiplication, the number of shoots, shoot lengths (cm), and the number of leaves were counted. The multiplied shoots were grown on MS or MT media (half-strength of salts) during the rooting stage supplemented with (0, 0.5, 1, 1.5, and 2 mg l^{-1}) of IBA. The percentage

of rooting, number of roots, root length (cm) and rooting duration were all measured after six weeks.

The rooting experiment, was carried out as a simple experiment. The experiments were carried out as factorial experiments by using a complete randomized Design (CRD) with 10 replicates for each treatment. The SAS (V. 9) system was used [27] to analyze the data, and the means were compared with the Duncan multiple ranges test at a 0.05 level [6].

Tabel (1): Differences between Basic salt of MS medium [23] and MT medium [22]

Formula	MS Salt	MT Salt
	Concentration (mg l^{-1})	Concentration (mg l^{-1})
Macro Nutrient		
NH_4NO_3	1650	1650
KNO_3	1900	1900
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440	332.2
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370	180.69
KH_2PO_4	170	170
Micro Nutrient		
H_3BO_3	6.2	6.2
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3	16.9
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	8.6
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	0.213
KI	0.83	0.83
$\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$	0.025	0.025
$\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$	0.025	0.025
$\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$	27.8	27.8
Na_2EDTA	37.3	37.3
Vitamins and Amino Acids		
Thiamine-HCl	0.1	10
Nicotinic acid	0.5	5
Pyridoxine-HCl	0.5	10
Glycine	2	2
Myo - Inositol	100	100
Sucrose (Carbon source)	30000	50000

RESULTS

Initiation stage: The MS medium showed a significant increase in the node's response percentage which achieved 92% in comparison with the MT medium which achieved 56% (table,1), while there are no significant differences between the BA Conc. treatments, On the other hand, the interaction between MS or MT media and

BA Conc. significantly affected on this traits, shows in MS medium supplemented with 1 and 2 mg l^{-1} BA gave 100% response which it overcomes significantly over MT medium interacted with 1, 3 and 4 mg l^{-1} , whereas for MT medium the best treatment was MT supplemented with 2 mg l^{-1} which achieved 70% of the response.

Table (1): Effect of medium type, BA concentration, and their interaction on response percentage (%) of a single node of *Citrumelo swingle* rootstock after 4 weeks.

BA Conc. (mg l ⁻¹)	Medium type		Means of BA Conc.
	MS (%)	MT	
0	90.00 ab	60.00 ab	75.00 a
1	100.00 a	50.00 b	75.00 a
2	100.00 a	70.00 ab	85.00 a
3	80.00 ab	50.00 b	65.00 a
4	90.00 ab	50.00 b	70.00 a
Means of media	92.00 a	56.00 b	

*Means of each factor and their interaction followed by the same letters are not significantly different from each other according to Duncan's multiple ranges test at 0.05 level.

In comparison to the MS medium (1.20 shoots), the MT medium showed the best significant results (2.04 shoots) in the number of shoots (Table 2). However, BA Conc., in the same table, demonstrates that 3 mg l⁻¹ BA produced the highest number of shoots (1.83 shoots), which

differs significantly from the control treatment (BA-free). The interaction between 1 mg l⁻¹ BA and MT medium produced the highest shoots number (3.20 shoots) and differed significantly from other treatments, Except the interaction between MT and 3 mg l⁻¹ BA (2.75 shoots).

Table (2): Effect of medium type, BA Conc. and their interaction on shoot number grown from a single node of *Citrumelo swingle* rootstock after 4 weeks.

BA Conc. (mg l ⁻¹)	Medium type		Means of BA Conc.
	MS	MT	
0	1.00 d	1.00 d	1.00 b
1	1.00 d	3.20 a	1.73 a
2	1.40 d	1.57 cd	1.47 ab
3	1.38 d	2.75 ab	1.83 a
4	1.22 d	2.20 bc	1.57 a
Means of media	1.20 b	2.04 a	

*Means of each factor and their interaction followed by the same letters are not significantly different from each other according to Duncan's multiple ranges test at 0.05 level.

Results in (Table 3) indicated that the best significant length of shoots (1.35 cm) was obtained in the MS medium, although, the longest shoots (1.67 cm), which were superior significantly from other BA concentrations, were obtained in 1 mg l⁻¹ BA. The interaction between MS and 1 mg l⁻¹ BA is significantly overcome in comparison with other BA concentrations supplemented with MT medium in the Length of shoots except for the interaction between MT medium and 1 mg l⁻¹ BA that achieved 1.20 cm. while the lowest significant value of shoot length was achieved in the interaction between 3 mg l⁻¹ BA and MT medium (0.48 cm).

Table (3): Effect of medium type, BA Conc. and their interaction on shoot length (cm) grown from a single node of *Citrumelo swingle* rootstock after 4 weeks.

BA Conc. (mg l ⁻¹)	Medium type		Means of BA Conc.
	MS	MT	
0	0.98 bc	0.78 bc	0.90 b
1	1.91 a	1.20 a-c	1.67 a
2	1.42 ab	0.66 bc	1.11 b
3	1.13 bc	0.48 c	0.91 b
4	1.23 ab	0.80 bc	1.08 b
Means of media	1.35 a	0.79 b	

*Means of each factor and their interaction followed by the same letters are not significantly different from each other according to Duncan's multiple ranges test at 0.05 level.

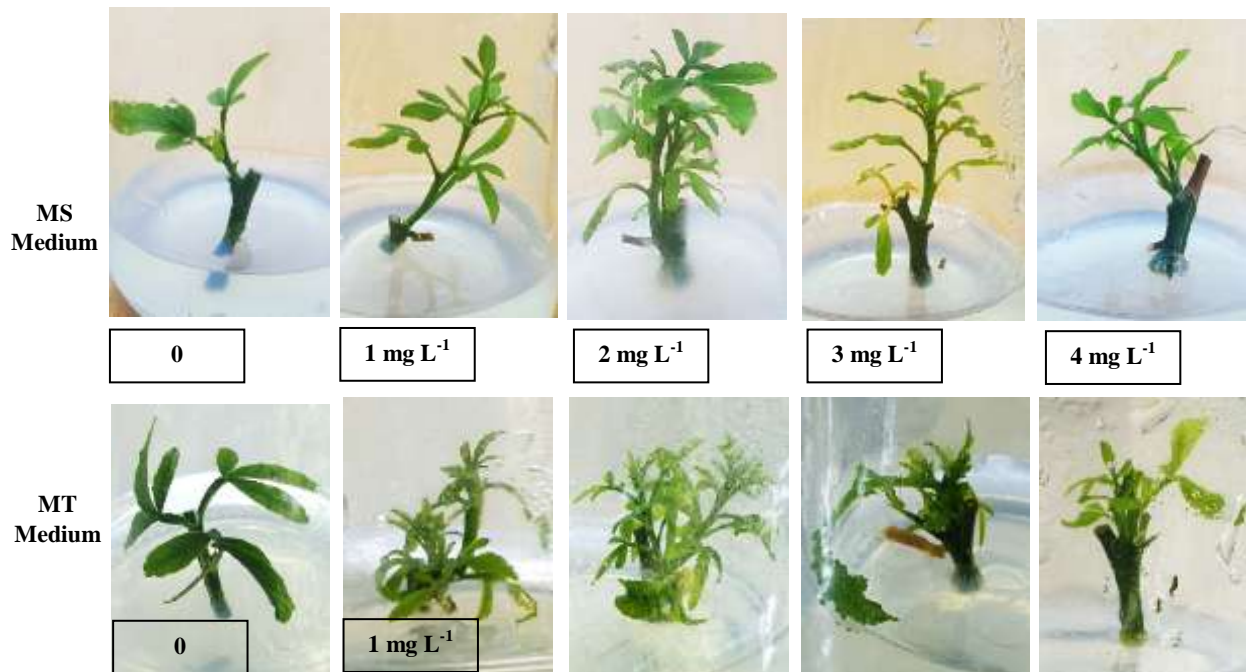
According to the results in Table 4, the MT medium produced the highest significant number of leaves (8.11 leaves) when compared to the MS medium (4.78 leaves), also, the results showed that 1 mg l⁻¹ BA enhanced the number of leaves (8.67 leaves) compared to the other

treatments of BA, However, 1 mg l⁻¹ BA and MT medium interaction produced the highest significant increase in the number of leaves (15.20 leaves) compared to all other BA concentrations that interacted with MS or MT media.

Table (4): Effect of medium type, BA Conc. and their interaction on leaf number grown from a single node of *Citrumelo swingle* rootstock after 4 weeks.

BA Conc. (mg l ⁻¹)	Medium type		Means of BA Conc.
	MS	MT	
0	2.89 d	3.00 d	2.93 c
1	5.40 b-d	15.20 a	8.67 a
2	5.30 b-d	7.29 bc	6.12 b
3	4.63 cd	8.25 b	5.83 b
4	5.56 b-d	8.20 b	6.50 b
Means of media	4.78 b	8.11 a	

*Means of each factor and their interaction followed by the same letters are not significantly different from each other according to Duncan's multiple ranges test at 0.05 level.



Picture (1) the effect of MS and MT media supplemented with BA Conc. on shoots initiation of *Citrumelo swingle* nodes after 4 weeks.

Multiplication stage: From the date of the initiation stage the best treatment of BA was 2 mg l⁻¹, so this concentration of BA was added to MS or Mt Media and interacted with different Conc. of Kin. The data values in Table (5) indicate that neither the MS nor MT medium insignificantly affected the number of shoots, which reached 4.22 and 4.02 shoots, respectively. In comparison to the

Kin Concentrations, the highest number of shoots (5.15 shoots) was in the treatment of 2 mg l⁻¹ Kin. which were significantly dominated over other treatments, In contrast to most other interactions, the MT medium interacted with 2 mg of l⁻¹ Kin resulting in the highest number of shoots (5.20 shoots).

Table (5): Effect of medium type, Kin Conc. and their interaction on shoots number of *Citrumelo swingle* rootstock shoot tips after 8 weeks.

Kin. Conc. (mg l ⁻¹)	Medium type		Means of Kin.
	MS	MT	
0	3.80 cd	4.30 a-d	4.05 b
2	5.10 ab	5.20 a	5.15 a
4	3.50 cd	3.90 b-d	3.70 b
6	4.40 a-c	3.60 cd	4.00 b
8	4.30 a-d	3.10 d	3.70 b
Means of Medium	4.22 a	4.02 a	

*Means of each factor and their interaction followed by the same letters are not significantly different from each other according to Duncan's multiple ranges test at 0.05 level.

According to the results in Table (6), the MT medium significantly increased shoot length (1.12 cm), in comparison with the MS medium (0.69 cm). The 2 mg l⁻¹ Kin. concentration produced the longest shoots (1.13 cm), which significantly dominated over 8 mg l⁻¹ Kin. (0.65cm) only. By the way, all interactions of Kin

concentration with MS media are notably different from each other, but the interaction between MT medium and 2 mg l⁻¹ Kin gave the highest significant shoot length (1.43 cm) as compared with the interactions between MS medium and all concentrations of Kin and MT medium with 8 mg l⁻¹ Kin.

Table (6): Effect of medium type, Kin Conc. and their interaction on shoot length (cm) of *Citrumelo swingle* rootstock shoot tips after 8 weeks.

Kin conc. (mg l ⁻¹)	Medium type		Means of Kin
	MS	MT	
0	0.72 b	1.42 a	1.07 a
2	0.83 b	1.43 a	1.13 a
4	0.75 b	1.03 ab	0.89 ab
6	0.58 b	0.99 ab	0.79 ab
8	0.57 b	0.73 b	0.65 b
Means of Media	0.69 b	1.12 a	

*Means of each factor and their interaction followed by the same letters are not significantly different from each other according to Duncan's multiple ranges test at 0.05 level.

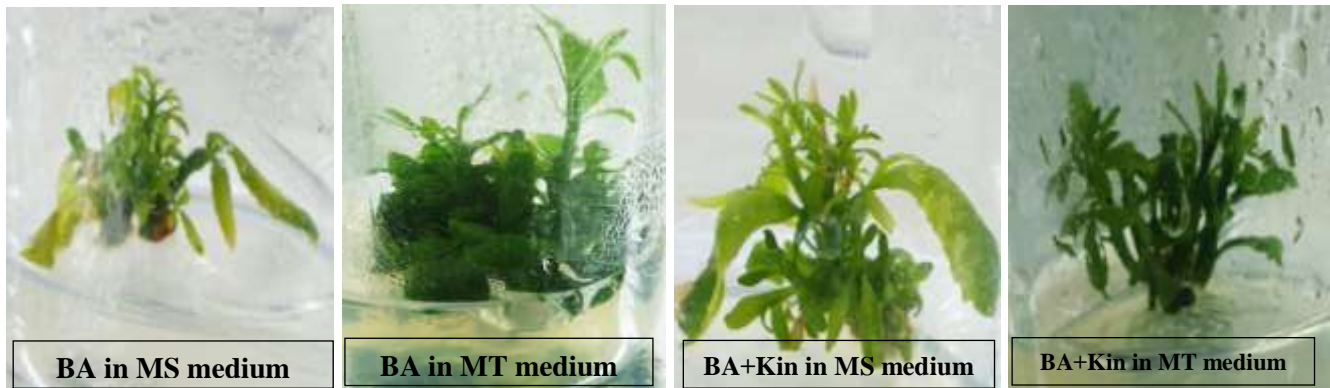
Data in Table (7) indicated that MT medium didn't differ significantly in the No. of leaves (17.12 leaves) in comparison with MS medium (14.98 leaves), but Kin and its interaction with the media significantly affected this parameter, the treatment of 2 mg l⁻¹ Kin achieved the highest number of leaves (19.80 leaves) and

significantly overcame in comparison with 6 and 8 mg l⁻¹ Kin treatments which achieved 15.05 and 11.40 leaves respectively. The interaction between MT medium and 0 and 2 mg l⁻¹ Kin gave the best No. of leaves (21.20 and 21.50 leaves respectively), and it differs significantly from the most of other interactions.

Table (7): Effect of medium type, Kin Conc. and their interaction on leaves number of *Citrumelo swingle* rootstock shoot tips after 8 weeks.

Kin conc. (mg l ⁻¹)	Medium type		Means of Kin
	MS	MT	
0	13.40 b-d	21.20 a	17.30 ab
2	18.10 ab	21.50 a	19.80 a
4	16.60 a-c	16.80 a-c	16.70 ab
6	15.60 b-d	14.50 b-d	15.05 b
8	11.20 d	11.60 cd	11.40 c
Means of Medium	14.98 a	17.12 a	

*Means of each factor and their interaction followed by the same letters are not significantly different from each other according to Duncan's multiple ranges test at 0.05 level.



Picture (2) the effect of 2 mg L^{-1} BA + 2 mg L^{-1} Kin on shoots multiplication of *Citrumelo swingle* after 8 weeks.

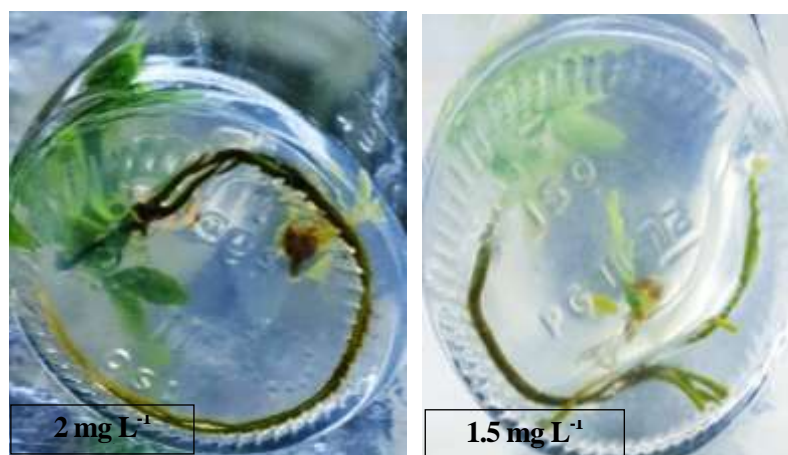
Rooting stage: After 6 weeks of cultured shoots in Rooting solid media $\frac{1}{2}$ MT supplemented with 0, 0.5, 1, 1.5, and 2 mg l^{-1} of IBA. Table (8) shows the best response of rooting was obtained in MT medium $\frac{1}{2}$ strength supplemented with 1.5 mg l^{-1} IBA (90%), While the treatment of 1.5 mg l^{-1} IBA resulted in the highest number and length of roots

($4.67 \text{ roots shoot}^{-1}$ and 9.78 cm respectively) and it overcome significantly in comparison with control treatment (without IBA), although, the treatment of 1.5 mg l^{-1} IBA achieved the best significant results in rooting duration (15.11 days) in comparison with other IBA treatments.

Table (8): Effect of IBA Conc. on rooting percentage of *Citrumelo swingle* rootstock shoot tips cultured on MT medium after 6weeks.

IBA Conc. (mg l^{-1})	Rooting perc.	No. of Roots	Root Length (cm)	rooting duration (days)
0.0	30.00 b	2.00 b	3.67 c	34.33 a
0.5	30.00 b	1.67 b	5.33 bc	28.67 b
1.0	50.00 ab	2.00 b	8.20 ab	21.60 c
1.5	90.00 a	4.67 a	9.78 a	15.11 d
2.0	50.00 ab	3.80 ab	6.40 a-c	21.20 c

*Means of each parameter followed by the same letters are not significantly different from each other according to Duncan's multiple ranges test at 0.05 level.



Picture (3) the effect of IBA Conc. on rooting of *Citrumelo swingle* shoots in MT medium $\frac{1}{2}$ strength of salts after 6 weeks.

DISCUSSION

The significant effect of MT medium at the initiation stage in the number of shoots and No. of leaves and in the multiplication stage in the No. of shoots, shoots length, and No. of leaves possibly causes by higher sucrose concentration in MT medium (50 gm l^{-1}) compared with MS medium (30 gm l^{-1}), higher vitamin concentrations (Thiamin-HCl, Nicotinic Acid, and Pyridoxin-HCl content in MT ($10, 5$ and 10 mg l^{-1} , respectively), or a combination of these factors. [26] stated that vitamins play important roles as cofactors of enzymes work and are added in very small quantities, especially vitamin B1 (Thiamine-HCl), which is supposed to be the only one that should be added to the medium (Table 1). On the other hand, it was found that the addition of Nicotinic acid (B_3) and Pyridoxin (B_6) to the medium stimulated the growth of the planted parts, especially if the concentrations of cytokinins added to the medium were low. Because citrus plant's growth and response are weak in tissue culture. [22] developed a new mixture of the medium that stimulates the multiplication of citrus plants by increasing the concentrations of vitamins, and this is what was observed from the results of Tables (2, 4, and 6) compared to the MS medium. Furthermore, the addition of sugars to the medium is considered an energy and carbon source, as the type and concentration of sugar used depends mainly on the quality of the cultivated explants as well as the purpose of its cultivation [26]. [24] found that the differentiation that occurs in the cultured plant tissue, which leads to the formation of vasculature, is affected to a large extent mainly by the quality and concentration of carbohydrates used in the medium.

As compared with the control treatment (without BA), BA provided the best response during the initiation and multiplication stages and increased the number of shoots, which may be because of cytokinin's ability to reduce the effectiveness of apical dominance as well as their ability to differentiate lateral buds vascular, which makes them more likely to grow and branches

[18]. Additionally, its role in attracting and accumulating metabolites at the sites of lateral buds, as well as its role in promoting the synthesis of RNA, protein, and chlorophyll [13], as well as its role in stimulating the growth of lateral buds, as well as its potential role in a hormonal balance between the internal content of plant tissues and what was added to the BA nutrient medium, which resulted in the multiplication of the shoots. According to [29], the balance between growth from the lateral and apical meristems is reflected in the plant's branching, and this balance is influenced by a variety of environmental factors, physiological, and morphological, including the quantity, arrangement, and integration of active meristems, as well as the development and movement of hormones [7], although BA is the phytohormone that is most frequently utilized in trials to induce shoot growth in citrus since it is thought to be the best cytokinin for direct shoot regeneration [10]; [12]; [17] and [30] and because there were fewer shoots in these treatments (1 mg l^{-1}) than in the other treatments, the opportunity for the plants to absorb the nutrient from the medium was greater than in the treatments with high concentrations (3 and 4 mg l^{-1}) of BA, which produced more shoots [2]; [28]. This difference can be seen in the superiority of the lengths of the shoots in these treatments. The stimulant may induce freshly formed leaves to grow on the shoots, which would explain BA's strongest effect, which increases the number of shoots (Tables 5 and 7).

The positive effect of some Kin concentrations on causing the enhanced response of the plant part compared to the comparison treatment, maybe due to the role of Kin in blocking protein and chlorophyll catabolism as well as stimulation of photosynthetic enzymes. The extent of the response to cytokines depends on the concentrations used and the physiological state of the cells receiving the cytokines [3]. By increasing the concentration, the response intensifies until it reaches the optimal limit for hormonal balance, which leads to cell activation and accelerated growth, Furthermore, increasing

Kin concentration decreases growth response rates, and this decrease does not always mean cell death but is usually a result of hormonal inhibition [4].

The significant effect of cytokines (BA + Kin) can be attributed to their role in the construction of RNA and their direct role in the processes of translation, reproduction, cell multiplication, and consequently, plant growth, or it can be attributed to the different chemical compositions of each of them. In addition, to the different effectiveness of reaction centers, BA is characterized by having 3 double bonds in addition to the Benzin ring, while Kin has one double bond [21].

The beneficial effects of IBA on the emergence and development of citrus rootstocks' roots may be attributable to its role in promoting cell division and elongation, which are the initial stages of the development of any plant organ [1], as well as its role in xylem differentiation, which is crucial for moving nutrients from the nutrient medium to the leaves, where the nutrients are produced and mineral elements are transformed into organic compounds (carbohydrates) [21].

CONCLUSION

We deduced from the results that the MS medium produced the best results for the features of the initiation stage, with the exception that the MT medium had more shoots and leaves, but the multiplication stage data showed that the interaction of 2 mg l^{-1} of both BA and Kin in MT medium considerably increased all features, on the other hand, the optimum outcome of rooting percentage, root number, root length, and root duration was in the MT medium supplemented with 1.5 mg l^{-1} IBA.

RECOMMENDATION

It is advised to carry out more field investigations because *Citrumelo swingle* rootstock was micro-propagated in vitro under study circumstances and because of their cold resilience.

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