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In vitro regeneration and improving kaempferol accumulation in blackberry (*Rubus fruticosus* L.) callus and suspension cultures

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Abstract

Blackberry (*Rubus fruticosus* L.) is a multipurpose crop that can be used for herbal medicinal purpose, culinary, cosmetics, pharmaceutical and medicinal purposes. This fruit's high quantity and variety of anthocyanins, polyphenols and antioxidant compounds exactly reflect well for its health benefits. Plant polyphenols have been found to influence molecular activities in a positive way.

The aim of this study is to preserve blackberry (*Rubus fruticosus* L.) by *in vitro* propagation using shoot tips, direct organogenesis, indirect organogenesis, production of kaempferol, one of the plant's main active constituents, and increasing their quantity using precursor feeding.

A maximum growth and development occurred only in the BA of the multiplication, but at 2ip or TDZ, a reduction in mean number of axillary shoots/explant was caused. At 0.75 mg/l TDZ, the maximum mean number of adventitious shoots/explant and organogenesis frequency was produced. Best callus induction was achieved on MS medium with 1.0 mg/l TDZ under total darkness. The amount of cytokinin in the culture media has a big impact on the quantity and frequency of shoot inductions. MS medium supplemented with 0.50 mg/l TDZ formed the most adventitious buds (13.1) and had the highest adventitious buds formation percentage (90 %). The highest rooting percentage of *Rubus fruticosus* L. (91%) was obtained on MS medium with 1.00 mg/l IBA plus 0.50 mg/l NAA. In addition, it was found that suspension culture containing MS medium with 1.0 mg/l TDZ along with **t**yrosine at a concentration of 66.07 µM enhanced production of kaempferol (5.7-fold), compared to control.

Keywords: Acclimatization, callus, *in vitro*, kaempferol, organogenesis, *Rubus fruticosus*, Thidiazuron.

1. Introduction:

Rubus fruticosus L. (Rosaceae) is a shrub known for its blackberry-like fruit, which is traded all over the world for its delectable taste, agreeable flavour, and nutritious profile. It belongs to the Rosaceae family (**Hummer and Janick, 2007**).

Plants have always played an important part in medicine, both traditional and modern. Plant-derived components are used by about 80% of the world's population for their essential health and fitness (**Winter and Tang, 2012 ; Yuan *et al.*, 2016**). They are high in phytochemicals, which have a magical capacity to treat ailments and can be used in a variety of industries, including pharmaceuticals, cosmetics, and nutraceuticals (**Nasri *et al.*, 2014**). Because of their affordability, accessibility, environmental friendliness, and potential efficacy equivalent to high-cost synthetic pharmacological agents, they are receiving more attention among the growing population. Thus, pharmacognosy, phytochemistry, and horticulture have received the majority of study attention in the field of medicinal plants to date. The growing demand for such products has necessitated the development of strategies to increase production while reducing environmental impact (**Atanasov *et al.*, 2015**).

Plants are thought to be safe for humans. Since the dawn of time, they have been utilized to cure and prevent specific maladies and diseases in humans. Medicinal plants are those that have therapeutic metabolites with favourable pharmacological effects (**Schulz *et al.*, 2001**). The therapeutic benefits of these plants are due to the presence of secondary

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metabolites, a varied group of natural metabolic products with differing structures and metabolic processes. These metabolites aren't required for plant growth and development (**Rosenthal, 1991**), but they serve a variety of functions as signalling molecules and defence agents (**Ncube and Van Staden, 2015**). They're considered economically essential because they're utilized in medications, tastes, scents, pesticides, , etc (**Guerriero *et al*., 2018**).

However, only a limited portion of the plant biodiversity has been investigated for potential therapeutic applications. The pharmaceutical industry has focused the last few decades concentrating on synthetic compound libraries as a means of drug research (**Atanasov *et al*., 2015**). The resurgence of interest in medicinal plants, as well as an increase in interest in bioactive natural goods, began in the last decade, owing to common perception that green drugs are better and safer than their conventional chemical counterparts. Very frequently, bioactive natural products have an array of lead constructs, which are starting points for chemical manipulation to derive an ideal drug for therapeutics (**Newman and Cragg, 2016**). Traditional medicinal plants prove to be a rich and extraordinary source of new anticancer medicines.

Berry fruits are high in phenolic compounds like phenolic acids, anthocyanins, flavonols, ellagitannins, gallotannins, and proanthocyanidins, which have shown to have significant antioxidant properties (**Reyes-Carmona *et al*., 2005**). The berries' flavonoids and phenolic compounds are anti-carcinogens with anti-neurodegenerative and anti-inflammatory properties (**Zia-Ul-Haq *et al*., 2014; Barbieri *et al*., 2017)**. Kaempferol and quercetin (a polyphenol cluster) are both active anticancer compounds that accumulate in large quantities in *Rubus fruticosus*. These polyphenols have a number of biochemical and pharmacological functions, including antioxidant, anti-inflammatory, anticancer, and cardioprotective effects (**Maaliki *et al*., 2019**). As a result, pharmaceutical firms rely extensively on *Rubus fruticosus* plants in the wild for anticancer compounds and medicinal properties.

Blackberry propagation through hard wood or stem cuttings has proved to be difficult and complex **(Bray *et al*., 2003).** Asexual and sexual propagation of *Rubus fruticosus* pose certain difficulties in fulfilling the pharmaceutical industry's commercial demand in these circumstances. As a result, a callogenesis method may be a viable option for generating significant amounts of plant cell biomass from which to extract essential secondary metabolites. Furthermore, due to environmental conditions and harvesting stage the composition of secondary metabolites is liable to change (**Yang *et al*., 2018**). *In vitro* callogenesis of *Rubus fruticosus* for the development of its therapeutically essential secondary compounds could regulate these improvements. The generation of secondary metabolites can be boosted by adding a small amount of some to the culture. These additives can activate genes in the secondary metabolic pathway (**George *et al*., 2008**). As a result, biotechnological processing of kaempferol and quercetin, from *Rubus fruticosus* plant cell culture could be an appealing option.

Rubus *in vitro* propagation was first documented in the 1970s. Many reports on *in vitro* propagation of raspberry and blackberry cultivars have been published since then (**Sobczykiewicz, 1992; Zawadzka and Orlikowska [(2006a); Zawadzka and Orlikowska (2009**); **Lepse and Lauge (2009)**]. The in vitro response is hampered by poor explant disinfection and low propagation rates (**Pelto and Clark, 1999**), which makes micropropagation of new cultivars difficult [**Donnelly and Daubeny (1986); Bobrowski *et al*., (1996**); **Zawadzka and Orlikowska (2006b)**]. Acclimatization to ex vitro conditions is an essential phase in Rubus micropropagation, therefore improving this stage of the process is crucial (**Fira and Clapa, 2009**). callus formation can be caused from *Rubus fruticosus* young leaves (**Badr-Elden *et al*., 2016**).

The aim of this research is to establish a consistent micropropagation protocol that will result in a high rate of multiplication and high quantities of high-quality planting material and secondary products. However, no research has been done on improving callus biomass quality or analyzing the content of therapeutically relevant secondary components. As a result, the goal of this study was to increase callus biomass and kaempferol levelin *Rubus fruticosus* callus and suspension culture under optimal conditions.

Experimental

These experiments were carried out on *Rubus fruticosus* L. at Tissue Culture Laboratories, Desert Research Center (DRC), Cairo, Egypt, from 2018 to

2021, to investigate the impact of various factors on the behaviours of *in vitro* consecutive micropropagation stages such as culture establishment, multiplication, direct and indirect organogenesis, rooting, acclimatization, callus induction, callus growth, and some biomass production of in vitro shootlets and callogenesis.

Explants source:

Actively shoots growing were separated from field-grown plants. Explant (shoot tip) was immersed in ethanol (70 %v/v) for 1 minute and then in a solution containing 20 % Clorox (5.25 % NaOCl) for 10 minutes before being rinsed several times in sterile double distilled water and then in 0.1 % (m/v) mercuric chloride for 5 minutes followed by rinsing 3 times with sterile water to get rid of traces of mercury chloride inside the Laminar Air Flow Chamber, then cultured on free-growth regulators salt media supplemented with 3% sucrose and 2,7g/l phytagel, pH was adjusted at 5.7. All cultures were kept at a temperature of 25±2 °C under 16 h photoperiod at 3000 lux.

1.Effect of three different types of media on *in vitro* growth and shoot regeneration of blackberry

Sterilized shoot tips were cultured without growth regulators on three types of media: **Murashige and Skoog, 1962** (MS) basal medium, **Murashige and Tucker** (MT) medium, and **Llyod and McCown, 1981 (**WPM) . Under controlled conditions, cultured explants were incubated for four weeks. The percentage of explants that survived, the number of shootlets, the length of shootlets (cm), and the number of leaves per explant were all determined.

2-Effect of three different types of cytokinins BA, 2ip and TDZ on growth and development of blackberry

The shoots resulting after the initiation stage were transferred to the multiplication stage to study the effect of various concentrations of Benzyl adenine (BA), 2- isobentenyl-adenin (2-ip) and Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) TDZ at 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l each alone on shootlets multiplication. The percentage of growth induction, mean number of axillary shoots/ explant and mean length of axillary shoots were recorded after four weeks.

3-*In vitro* direct and indirect organogenesis of Black berry

3-1- Direct organogenesis of blackberry

The aim of this experiment was to see how different concentrations of TDZ and 6-(4-Hydroxy-3-methylbut-2-enylamino) purine) Zeatin at (0.0, 0.25, 0.5, 0.75, and 1.0 mg/l) affected shootlets developed from *in vitro* young leaves (fully expanded). After 12 weeks, mean number of axillary shoots/explant and organogenesis frequency were noted.

3-2- Indirect organogenesis of Black berry

3.2.1- callus induction of blackberry

In vitro younger leaves from the second and third position in the plagiotropic axis were cultured on MS medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2, 4-D) at (0.0, 0.5, 0.75, 1.0,1.5, and 2.0 mg/l) and TDZ at (0.0, 0.5,0.75, 1.0,1.5, and 2.0 mg/l) alone or in combination with (0.0 and 0.5 mg/l) BA for callus induction. Explants capable of inducing callus value, callus percentage, and adventitious buds were used to collect data after 6 weeks.

3-2-2-Effect of TDZ at different concentrations on callus differentiation

When callus was transferred to MS medium containing TDZ at (0.0, 0.1, 0.25, 0.5, 0.75, 1.0, and 1.25 mg/l), an experiment was conducted to investigate the effect of different concentrations of TDZ on shoot differentiation (indirect organogenesis). Following an 8-week culture period, mean number of adventitious buds /explant and adventitious buds percentage were counted.

4- Rooting stage

In addition to the control (free from plant growth regulators), elongated shoots of blackberry were evaluated for rooting on root induction media of MS salts at half strengths with different treatments of auxins; IBA at (0.00, 0.10, 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mg/l) in combination with NAA at (0.00 and 0.5 mg/l). After 4 weeks of culture, rooting percentage (%), mean number of roots/explant, mean length of roots and shoot height (cm) were recorded.

5- Acclimatization stage

Rooted shoots were washed to extract medium residues and fungicide-treated with a 0.2 % topsin (w/v) solution before being transplanted into pots filled with a sand and peat moss (1:1 v/v) soil mixture. Transparent polyethylene bags were used to cover the pots, which were then placed in a greenhouse. After a week, the covers were progressively removed over the course of a month.

The percentage of transplants that survived (%) was registered.

6- Active constituent (kaempferol)

6-1-Application of the precursor

To increase the amount of the active constituent (kaempferol), the derived calli from the best medium formed callus were cultured on MS medium supplemented with different amounts of the precursor phenylalanine (33.03, .66.07 and 132.15 µM) and tyrosine (33.03, .66.07 and 132.15 µM), in addition to the control (free of plant growth regulators) and (free of precursor). The cultures were incubated in the dark at 25±2 °C until more calli were produced.

6-2-Establishment of suspension culture and precursor feeding

Suspension cultures were started by inserting 2.0g of fresh homogenous friable callus in 250 ml Erlenmeyer flasks containing 100 ml liquid MS medium augmented with 3 % (w/v) sucrose, 0.01 %(w/v) myo-inositol, 1.0 mg/l TDZ (phytagel free). As a precursor of kaempferol, different amounts of phenylalanine and tyrosine (Alpha Chemika India) were applied to the medium (33.03, .66.07 and 132.15 µM). The precursor was prepared as a condensed stock solution and applied to the cultures at the required concentrations after filter sterilization with a Millex syringe powered filter unit (0.22 m).

As previously stated, the pH of the media was balanced to 5.7-5.8 before autoclaving. As a control, phenylalanine and the MS medium without tyrosine was used. Flasks were sealed with cotton plugs for gas exchange and incubated at 110 rpm and 25±2ºC in the dark on a rotary shaker (Daihan Scientific, Korea).

The callus suspension cultures were harvested, filtered with filter paper, and liquid nitrogen freeze-dried. The samples were held in the freezer until the kaempferol were extracted and determined.

6-3- Kaempferol quantitative analysis

High Performance Liquid Chromatography (HPLC) was used to determine the amount of concentrated kaempferol in blackberry callus and suspension cultures.

6-3-1-Kaempferol extraction

The calli were collected after four weeks of culture in the dark without disrupting the culture media in the culture vessels, and their fresh weight was registered. After that, the calli were freeze-dried to achieve dry weight. After that, a kitchen blender was used to grind the dried calli into a fine powder. For each sample, one gram of powdered calli was correctly weighed and ultrasonicated for 15 minutes, followed by three extractions with 10 ml of ethanol (30 ml total). After filtration, a vacuum rotary evaporator was used to concentrate the mixed ethanolic callus extract, and each residue was balanced to 10 mL using methanol. Each sample was purified *via* a polytetraflage filter prior to HPLC analysis. Each sample was passed through a 0.45 m porosity polytetrafluoroethylene membrane (Nalgene®, New York, USA) before HPLC analysis.

Separation of kaempferol by HPLC

Samples of kaempferol were injected in HPLC (Ultimate 3000) according to the method of **Biswas *et al.*, (2013)**.

The system Thermo (Ultimate 3000) consisted of: pump, automatic sample injector, and associated DELL-compatible computer supported with Cromelion7 interpretation program. A diode array detector DAD-3000 was used. The Thermo-hypersil reversed phase C18 column 2.5× 30cm was operated at 25º C. Mobile phase consists of distilled water (solvent A) and methanol (solvent B). The UV absorption spectra of the standards as well as the samples were recorded in the range of 230–400 nm. Samples and standards solutions as well as the mobile phase were degassed and filtered through 0.45 µm membrane filter (Millipore). Identification of the compounds was done by comparison of their retention's time and UV absorption spectrum with those of the standards.

Injected volume 20 µl
Column: RP- C18
Column size: 2.5× 30cm
Mobile phase: H_2O (solvent A) and methanol (solvent B) = 75: 25 (v/v)
Flow rate: 1.0 ml/min
Temperature: 25°C
Detection: diode array

Calculation of kaempferol

Using a DELL-compatible computer and the Cromelion7 interpretation software, the peak area and concentration of kaempferol in blackberry samples were calculated by comparing their relative retention time (min) with the normal. The kaempferol

concentration was measured as mg/100g fresh weight.

Identification of the compounds was done by comparison of their retention's time with those of the standards.

Data analysis

Anova, a statistical analysis programme, was used to perform data variance analysis. Duncan's multiple range tests were used to see if the differences between means for all treatments were significant at the 5% level. At $P \leq 0.05$, means preceded by the same letter are not substantially different.

Results:

1.Effect of three different types of media on *in vitro* growth and shoot regeneration of blackberry (*Rubus fruticosus* L.)

Table (1) demonstrates the influence of three separate media forms on the establishment of *Rubus fruticosus* explant. When explants cultured on WPM medium, the maximum significant value of explant survival (100%) was obtained.

As compared to the other two media under examination, explant cultured on MS medium Fig. (1) produced the most shootlets (2.33), the longest shootlest (3.33) cm, and the largest number of leaves (6.33/ shootlets).

Table (1): Effect of three media types (MS, MT and WPM) on shoot initiation during establishment stage of Blackberry

Medium types	% of survived explants	Mean number of axillary shoots/ explant	Mean length of axillary shoots (cm)	Mean number of Leaf / explant
MS	88.33^{c}	2.33^{a}	3.33^{a}	6.33^{a}
MT	95^{b}	1.33^{b}	2.00^{ab}	5.33^{b}
WPM	100^{a}	1.00^{b}	1.33^{b}	4.00^{c}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Fig. (1): Initiation stage of *Rubus fruticosus* shoot tips on MS medium.

2-Effect of three different types of cytokinins BA, 2ip and TDZ on growth and development of blackberry *Rubus fruticosus*

Table (2) and Fig. (2) display the *in vitro* multiplication of *Rubus fruticosus* .The effects of various concentrations of BA, 2ip, and TDZ, as well as a control medium without plant growth regulators, on the multiplication as seen in the data. In all of the media examined, the percentage of growth shootlets had a highly significant impact.

Regardless of the culture media supplemented with different cytokinins, 1.5 mg/l BA produced more shootlets (17) than 2ip or TDZ.

In comparison to the other treatments, the MS medium without plant growth regulators (control) produced the longest shootlets (4.5cm).

Table (2): Effect of cytokinins BA, Kin and TDZ on growth and development of *Rubus fruticosus*.

Growth regulators conc. (mg/L)		% of growth induction	Mean number of axillary shoots/explant	Mean length of axillary shoot (cm)
Control		70.66^{i}	4^{j}	4.50^{a}
BA	**0.25**	75.00^{h}	5^{i}	4.00^{b}
	0.50	80.66^{f}	7^{h}	3.50^{c}
	1.00	90.66^{e}	11^{e}	3.06^{d}
	1.50	98.00^{b}	17^{a}	2.70^{e}
	2.00	92.00^{d}	12^{d}	2.50^{g}
2iP	**0.25**	77.00^{g}	7^{h}	3.50^{c}
	0.50	90.66^{e}	9^{f}	3.06^{d}
	1.00	95.00^{c}	13^{c}	2.70^{e}
	1.50	99.00^{a}	15^{b}	2.60^{f}
	2.00	80.66^{f}	8^{g}	2.40^{h}
TDZ	**0.25**	55.00^{l}	2^{l}	3.06^{d}
	0.50	60.00^{k}	3^{k}	2.50^{g}
	1.00	65.00^{j}	5^{i}	2.06^{i}
	1.50	30.66^{m}	1^{m}	1.90^{j}
	2.00	25.00^{n}	1^{m}	1.50^{k}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Fig. (2): Shoot proliferation of *Rubus fruticosus* on MS medium with 1.50 mg/l BA.

3-*In vitro* direct and indirect organogenesis of Black berry

3-1- Direct organogenesis of blackberry

Leaf explants were used to regenerate shoots. Shoot regeneration was observed for the first time in the second week and continued until the end of the trial (12^{th} week).

The effect of two cytokinin groups (TDZ and Zeatin) on organogenesis development (shootlets) from *Rubus fruticosus* L. leaves was specifically shown in Table (3). Inducing adventitious organogenesis from *in vitro* leaves was substantially more successful with TDZ. MS medium combined with 0.75 mg/l TDZ developed 8 shootlets/leaf with a 95 % regeneration rate compared to other treatments.

Table (3):Effect of cytokinin concentration on adventitious shoot formation (direct organogenesis) from leaf explants of blackberry.

Growth regulators conc. (mg/l)		Mean number of axillary shoots/explant	Organogenesis frequency
Control (free from growth regulators)		0.1^{g}	0.1^{h}
TDZ	0.25	2.33^{d}	15^{e}
	0.50	6.0^{b}	50^{b}
	0.75	8.0^{a}	95^{a}
	1.00	4.0^{c}	40^{c}
Zeatin	0.25	0.1^{g}	0.1^{h}
	0.50	1.0^{f}	5^{g}
	0.75	2.0^{e}	20^{d}
	1.00	1.0^{f}	10^{f}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

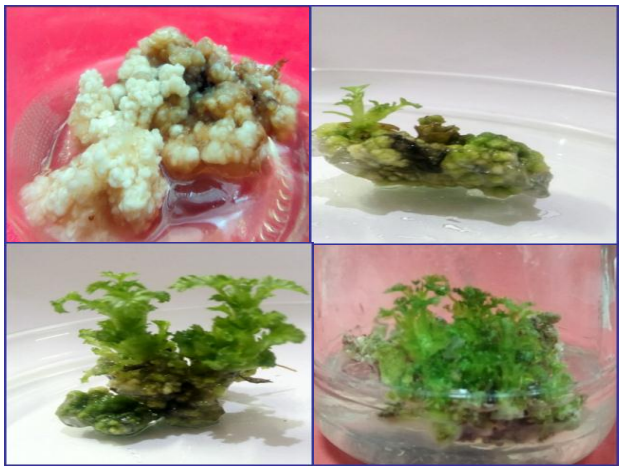


Fig. (3): Different steps of *Rubus fruticosus* adventitious buds formed on MS medium supplemented with 0.75 mg/l TDZ.

3-2- Indirect organogenesis of Black berry

3-2-1-Effect of (2,4-D) and cytokinins (BA and TDZ) on callus induction of blackberry

Effect of (2, 4-D) concentration with cytokinins (BA and TDZ) on callus induction of blackberry

In comparison to the control medium (without plant growth regulators), callus induction was observed on MS medium containing varying concentrations of 2,4-D and TDZ at (0.5, 0.75, 1.0, 1.5, and 2.0 mg/l) with or without BA at (0.5 mg/l).

Table (4) and Fig. (4) demonstrate that on all MS media containing either 2,4-D and TDZ separately or in conjunction with BA, all leaf sections produced yellowish white friable callus.

The highest callus value (6.83) was seen on MS medium containing 1.0 mg/l TDZ, which also generated the highest callus percentage (100%) and globular embryo (7.0), compared to the other treatments in this sample. There was no callus induction in the control medium, which was devoid of plant growth regulators.

Table (4):Effect of 2, 4-D or TDZ with BA on callus induction of blackberry.

Growth regulators conc. (mg/l)			Callus value	Callus percentage	Mean number of shoots/ explant
2, 4-D	TDZ	BA			
0.00	0.0	0.00	0.1^{m}	1.00^{r}	0.10^{e}
0.00	0.0	0.50	0.2^{m}	5.00^{q}	0.10^{e}
0.50	0.0	0.00	0.5^{l}	15.00^{p}	0.10^{e}
0.50	0.0	0.50	0.73^{l}	20.00^{o}	0.10^{e}
0.75	0.0	0.00	1.0^{k}	30.00^{n}	0.10^{e}
0.75	0.0	0.50	1.6^{i}	33.66^{m}	0.10^{e}
1.00	0.0	0.00	2.1^{fg}	50.00^{l}	0.10^{e}
1.00	0.0	0.50	2.76^{de}	55.00^{k}	0.10^{e}
1.50	0.0	0.00	5.0^{c}	80.00^{e}	0.10^{e}
1.50	0.0	0.50	6.2^{b}	82.66^{d}	0.10^{e}
2.00	0.0	0.00	2.0^{g}	60.00^{j}	0.10^{e}
2.00	0.0	0.50	2.63^{e}	63.66^{i}	0.10^{e}
0.0	0.50	0.00	2.0^{g}	650^{h}	2.66^{c}
0.0	0.50	0.50	1.66^{hi}	60.00^{j}	1.66^{d}
0.0	0.75	0.00	2.33^{f}	75.00^{f}	5.00^{b}
0.0	0.75	0.50	1.9^{gh}	70.00^{g}	3.00^{c}
0.0	1.00	0.00	6.83^{a}	100.00^{a}	7.00^{a}
0.0	1.00	0.50	5.0^{c}	95.00^{b}	4.66^{b}
0.0	1.50	0.00	2.9^{d}	85.00^{c}	0.10^{e}
0.0	1.50	0.50	1.9^{gh}	80.00^{e}	0.10^{e}
0.0	2.00	0.00	2.0^{g}	60.00^{j}	0.10^{e}
0.0	2.00	0.50	1.26^{j}	55.00^{k}	0.10^{e}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

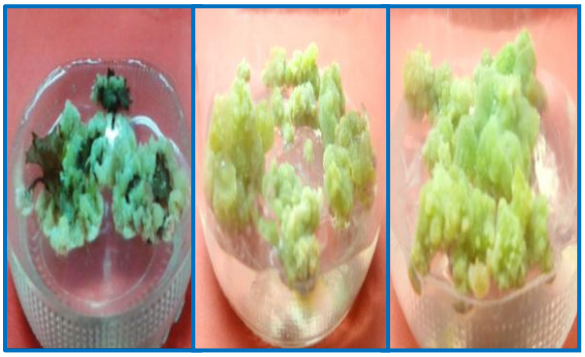


Fig. (4): Callus of *Rubus fruticosus* induced on MS medium supplemented with 1.0 mg/l TDZ.

3-2-2-Effect of TDZ at different concentrations on callus differentiation

The MS medium fortified with TDZ had the greatest significant impact on callus differentiation of (*Rubus fruticosus* L.) as seen in Table (5) and Fig. (5). When compared to the other treatments in this treatment, callus on MS medium containing 0.50 mg/l TDZ developed the most adventitious buds (13.1) and gave the greatest adventitious buds formation (90%).

Table (5):Effect of different TDZ concentrations on differentiation callus from *in-vitro* leaves of blackberry.

TDZ conc. (mg/l)	Mean number of adventitious buds /explant	adventitious buds percentage
0.00	0.10^{g}	1.33^{g}
0.10	2.06^{f}	15.00^{f}
0.25	4.10^{e}	55.00^{e}
0.50	13.10^{a}	90.00^{a}
0.75	6.06^{c}	65.00^{d}
1.00	7.10^{b}	85.00^{b}
1.25	5.10^{d}	70.00^{c}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

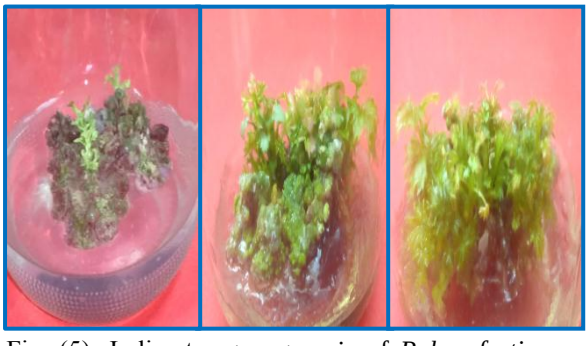


Fig. (5): Indirect organogenesis of *Rubus fruticosus* induced on MS medium supplemented with 1 mg/l TDZ.

4- Rooting stage

Table (6) shows that on MS medium combined with 1.00 mg/l IBA plus 0.50 mg/l NAA, the highest rooting percentage of *Rubus fruticosus* (91%) was obtained. In addition, this treatment achieved the highest mean root number (9) and length (8cm), as well as the highest median shoot height of 6.6cm Fig.(6).

Table (6):Effect of MS medium containing different treatments of auxins; IBA and NAA, on the rooting of *Rubus fruticosus* shoots.

Auxin conc. (mg/l)		Mean number of roots/ explant	Mean length of roots (cm)	Mean shoot height (cm)	Rooting %
IBA	NAA				
0.00	0.00	0.1^{i}	0.1^{i}	5.0^{k}	0.1^{p}
0.00	0.50	1.0^{h}	0.5^{i}	5.0^{k}	5.0^{o}
0.10	0.00	1.0^{h}	2.0^{h}	5.1^{j}	10^{n}
0.10	0.50	2.0^{g}	2.5^{g}	5.2^{i}	13^{m}
0.25	0.00	2.0^{g}	2.5^{g}	5.2^{i}	15^{l}
0.25	0.50	4.0^{f}	3.0^{f}	5.3^{h}	19^{k}
0.50	0.00	4.0^{f}	3.0^{f}	5.5^{g}	20^{j}
0.50	0.50	5.0^{e}	3.0^{f}	5.5^{g}	25^{i}
0.75	0.00	5.0^{e}	4.0^{e}	5.6^{f}	50^{h}
0.75	0.50	7.0^{c}	4.5^{d}	5.7^{e}	55^{g}
1.00	0.00	8.0^{b}	6.5^{b}	6.5^{b}	90^{b}
1.00	0.50	9.0^{a}	8.0^{a}	6.6^{a}	91^{a}
1.25	0.00	6.0^{d}	4.0^{e}	6.1^{d}	80^{d}
1.25	0.50	8.0^{b}	5.0^{c}	6.2^{c}	85^{c}
1.50	0.00	4.0^{f}	4.0^{e}	6.1^{d}	70^{f}
1.50	0.50	6.0^{d}	5.0^{c}	6.2^{c}	75^{e}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Fig. (6): Rooting of *Rubus fruticosus* on MS medium supplemented with 1.0 mg/l IBA plus 0.50 mg/l NAA after 30 days of incubation.

5- Acclimatization stage

After four weeks of being transferred into the peatmoss: sand mixture (1:1 v/v), an average of 90% of the acclimatized plantlets survived (Fig.7, 8).

Fig. (7): Hardened *in vitro* derived plantlets of blackberry (*Rubus fruticosus* L.).

Fig. (8): plants formed a fruit after two years of

acclimatization.

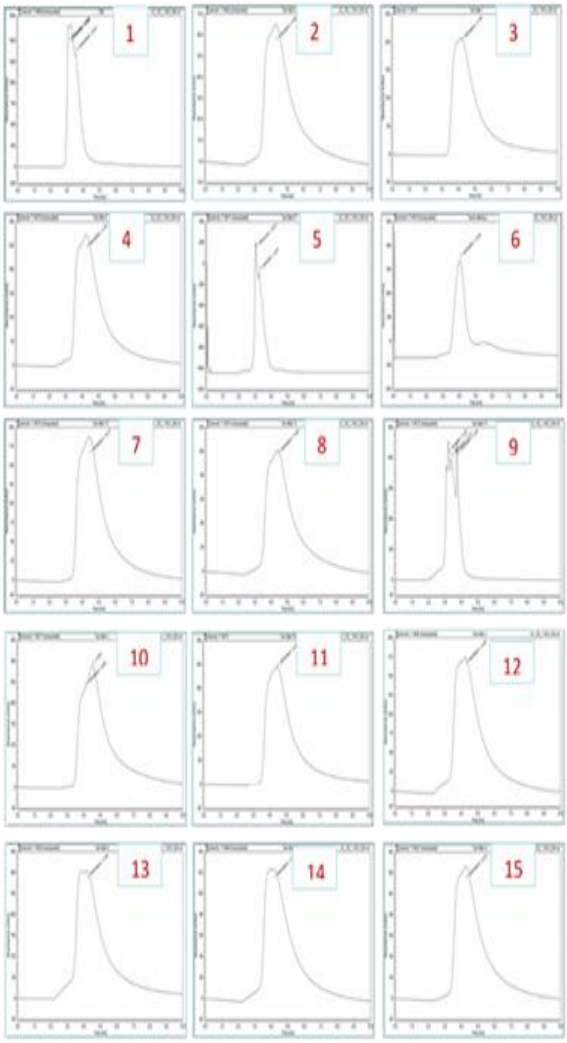
6- Active constituent (kaempferol)

In the callus and suspension culture experiment, the plant growth regulators combination that caused the highest callus induction percentage and fresh weight was chosen. In suspension cultures of blackberry, the impact of adding phenylalanine and tyrosine at different concentrations (33.03, .66.07 and 132.15 µM) on kaempferol accumulation percentage (g/100 g fresh weight of callus) was investigated as represented in Table (7). Just after 30 day of culture yielded a rise in kaempferol percentage. Suspension culture with tyrosine at a concentration of 66.07 µM resulted in the largest kaempferol aggregation (2.45 %), a 5.7-fold improvement over the control procedure (medium without tyrosine at zero time), followed by callus culture containing phenylalanine at 66.07 µM, which resulted in a 5.14-fold increase over the control treatment. The amount of kaempferol was reduced after repeated exposure of cultures to media with or without tyrosine at all concentrations.

Table (7):Effect of different concentrations of phenylalanine and tyrosine on the accumulation of kaempferol and quercetin (g/100 g fresh weight of callus) in callus and suspension culture of blackberry on MS medium supplemented with 1 mg/l TDZ.

Type		Conc	Conc. (%)		Increase (fold)
			Kaem-pferol	Que-rcetin	Kaem-pferol
Control			0.43	0.00	0.00
Callus culture	Phenyl-alanine	33.03	2.00	0.00	4.65
		66.07	2.21	0.00	5.14
		132.15	0.00	2.08	0.00
	Tyrosine	33.03	1.51	0.00	3.51
		66.07	1.46	0.00	3.39
		132.15	0.67	0.00	1.55
Sus-pension culture	Control		0.52	0.62	0.00
	Phenyl-alanine	33.03	2.28	0.00	4.56
		66.07	0.97	0.00	1.94
		132.15	0.93	0.00	1.86
	Tyrosine	33.03	0.62	0.00	1.24
		66.07	2.45	0.00	5.7
		132.15	1.42	0.00	2.84

Fig. (9): HPLC chromatograph shows the peak of Kaempferol from leaf callus of blackberry (*Rubus fruticosus* L.) cultured on MS callus proliferation medium containing 1 mg/l TDZ under total darkness.
1= Mother plant (Grow in open field).
2= Callus culture without precursor (Control).
3= Callus culture with 33.03 µM Phenylalanine.
4= Callus culture with 66.07µM Phenylalanine.
5= Callus culture with 132.15 µM Phenylalanine.
6= Callus culture with 33.03 µM Tyrosine.
7= Callus culture with 66.07µM Tyrosine.
8= Callus culture with 132.15µM Tyrosine.
9=Suspension culture without precursor(Control).
10=Suspension culture with 33.03 µM Phenylalanine.
11=Suspension culture with 66.07µM Phenylalanine.
12=Suspension culture with 132.15 µM Phenylalanine.
13= Suspension culture with 33.03 µM Tyrosine.
14= Suspension culture with 66.07µM Tyrosine.
15= Suspension culture with 132.15µM Tyrosine.



Discussion

This study's primary goal was to improve the blackberry micropropagation procedure (*Rubus fruticosus* L.), studying the effects of various factors on the behaviours of in vitro successive micropropagation stages such as culture establishment, multiplication, direct and indirect organogenesis, rooting, acclimatization, callus induction, callus growth, and some biomass production of in vitro shootlets and callogenesis within the broad framework of cell biology and plant science.

The main benefit of micropropagation is that it ensures a continuous supply of healthy, genetically identical, and pathogen-free plants throughout the year (**Rani and Raina, 2000**). It's a life saver when it comes to multiplying male sterile, fertility maintainer, and restorer lines. Micropropagation can speed up the breeding process in perennial breeding projects by allowing for in vitro selection and a duplicated trial of new releases (**Debnath, 2007**).

Due to known recalcitrance of blackberry (*Rubus fruticosus* L.) to micropropagation, expectations were that production of phenolic compounds might be in the way of efficient regeneration. **Batista *et al*., (2018)** described that cultures kept under light produced more phenols than those kept under darkness.

The composition of the growth media has a significant impact on plant tissue growth and morphogenesis. Macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, carbon sources, organic supplements, stabilising agents, and growth regulators are all found in plant tissue culture media. In plant tissue culture, Murashige and Skoog (**Murashige and Skoog, 1962**) is the most often used media. For several plant species, the B5 (**Gamborg et *al*., 1968**), N6 (**Chu, 1978**), and Nitsch and Nitsch (**Nitsch and Nitsch, 1969**) have been widely employed. Furthermore, the DKW (Driver and Kuniyuki, 1984) and WPM medium (**Lloyd and McCown, 1980**) are utilized in the culture of woody species. The plant species and tissue culture medium are both taken into consideration when choosing a growth medium (**Gamborg and Phillips, 1995**).

In our study, when explants were cultured on WPM medium, the maximum significant value of explant survival (100%) was obtained. Our findings are consistent with those of **Fathy *et al*., (2018)**, who demonstrated that MS medium is the best medium for culture initiation of *Rubus fruticosus* L., producing the most shootlets, shootlets length, and leaves/ shootlets. Also, **Benmahioul (2017)** on *Pistacia vera*, who found that MS medium was the best medium for culture initiation, and **Darwesh *et al*., (2017)** on *Khaya senegalensis*, who found that culturing explants on MS medium increased shootlet proliferation rate faster than WPM or B5 medium.

Plant growth regulators have been shown to affect plant regeneration in Rubus species in the past (**McNicol and Graham 1990; Mezzetti *et al*.,1997; Tsao 1999; Pelto and Clark 2001**). These investigations, on the other hand, were limited to a small number of cultivars, yielding a variety of outcomes. **Graham *et al*., (1997)**, for example, found a wide range of *Rubus idaeus* shoot regeneration frequencies (6–90 percent) when TDZ was applied. TDZ caused direct organogenesis in Rubus species, according to **Turk *et al*., (1994)**; **Millan-Mendoza and Graham (1999)**. Another study on the purple raspberry 'Amethyst' discovered that TDZ was more effective than BA at regenerating shoots (**Lenz *et al*., 2016)**.

In our research, we found that shoot regeneration significantly increased with an increase of BA. Differential responses to TDZ and/or BA varied in different red raspberry cultivars, which included 'Ruby,' 'Autumn Bliss,' 'Heritage,' and 'Latham' (**Turk *et al.*, 1994; Mezzetti *et al.*, 1997; Millan-Mendoza and Graham 1999; Tsao 1999; Zawadzka and Orlikowska 2006**). **Darwesh *et al.*, (2017)** reported positive effects of BA augmented multiplication medium on shootlet proliferation characteristics in *Khaya senegalensis*. This positive finding may be explained by the hypothesis that an externally added cytokinin (BA) altered the internal hormonal equilibrium, causing the highest cell differentiation.

This positive outcome may be clarified by the hypothesis that the externally added cytokinin (BA) altered the internal hormonal balance, causing the maximum cell division through vegetative organ. According to **Isac *et al.*, (2014)** cytokinins such as BAP are very successful in fostering blackberry shoot initiation, whether direct or indirect. Using 2 mg/l BAP amounts, **Jadán *et al.*, (2015)** were able to produce a higher number of internodes and plants. According to **Badr-Elden *et al.*, (2016)**, the BA concentration of 1.5 mg/l is the most effective for Blackberry multiplication. On the contrary, **Kefayeti *et al.*, (2019)** used 2.0 mg/l BAP and 0.02 mg/l IBA to stimulate the most number of shoots.

Direct organogenesis is most typically used in woody plants for shoot multiplication since both the apical (shot tips) and axillary (nodes) buds have the ability to grow into a shoot. The quantity and frequency of shoot inductions are influenced by the amount of cytokinin in the culture media.

Some research on the effects of cytokinins on adventitious organogenesis from the leaf in different blackberry cultivars found that BAP and TDZ (substituted urea with cytokinin like activity) are the most commonly used cytokinins for induction of regeneration of different Rubus genotypes (**Cousineau and Donelly, 1991; Mcnicol and Graham, 1990; Fiola *et al.*, 1990., Graham *et al.*, 1997; Mezzetti *et al.*, 1997; Meng *et al.*, 2004**). The results obtained imply that TDZ, applied individually at 0.75 mg/l, is more efficient in induction of regeneration than Zeatin.

Applied concentration of TDZ (1.0 mg/l) in this experiment resulted in pretty high percentage of regeneration in cv Čačanska Bestrna, as compared to the results of **Fiola *et al.*, (1990)** who, with similar concentration of TDZ (5µM) in blackberry cvs Loch Ness and Shawnee, obtained only 8% of regeneration. Increase in TDZ concentration up to the 50 µM did not significantly change the percentage of regeneration from leaves in the stated cultivars

In this study, MS medium combined with 0.75 mg/l TDZ developed 8 shootlets/leaf with a 95 % regeneration rate compared to other treatments. Mean while, the maximum shoot proliferation (13.1 shoots/explant) ability by organogenesis from the callus was obtained in the current investigation at 0.75mg/l TDZ. These findings are close to those reported by **Yucesan *et al.*, 2015**, who found that low concentrations of TDZ promote Gooseberry proliferation while higher concentrations induce callus in woody plants.

Kim and Dai (2020) showed that the regeneration rates were 70% for 'Joan J' and 82.2% for 'Polana,' when 7-d-old leaf tissues of Raspberry were pulsed on WPM with 2.5 µM BA + 1.0 µM TDZ and WPM with 2.5 µM BA plus 0.1 µM TDZ, respectively.

Different types of auxins are commonly used to stimulate rooting in woody plants. In the present study, two auxins (IBA and NAA) were tested for root formation. 1.0 mg/l IBA and 0.5 mg/l NAA gave 91% root formation and maximum number of roots (9 roots/shoot). These results are consistent with those of **Najaf-Abadi and Hamidoghli, (2009)** showed that *in vitro* rooting of Black berry was produced on medium contaning 2 mg/l IBA. For certain cultivars of Thornless blackberry, **Kefayeti *et al.*, (2019)** stated that the best rooting medium was 0.4 mg/l IBA and 0.4 mg/l NAA use (Rubus sp). Mean while, rooting of Raspberry (cv. Joan J) was quickly accomplished (100%) in half (MS) medium supplemented with 10 M (NAA), according to **Kim and Dai (2020).**

They demonstrated that an acclimatisation mixture of peat moss and sand in a ratio of (1:1) improved acclimatization of plantlets growth behaviour, especially survival rates. Peatmoss has both high water and nutrient retention characteristics, while sand has a high penetration effect and strong aeration for roots.

Plant cell, tissue, and organ cultures seem to be effective biotechnological techniques for increasing bioactive metabolite levels in higher plant species. To meet the increased pharmaceutical needs, the benefits of micropropagation in numerous medicinal plants to create antioxidant metabolites are accessible

(**Chattopadhyay *et al*.,2002 ; Giri and Zaheer, 2016; Dias *et al*.,2015; Karuppusamy, 2009; Chandran *et al*.,2020**).

The first stage consists in the development of the callus culture, which is obtained from the growth and maintenance of an unorganized cell mass, formed from segments of tissue, organs or cells that have been previously cultured (**Chattopadhyay *et al*., 2002; George, 2008**). In the present study, we used different concentrations of 2, 4-D or TDZ with BA to callus induction of blackberry. The highest callus value (6.83) was seen on MS medium containing 1.0 mg/l TDZ, which also generated the highest callus percentage (100%) and globular embryo (7.0), compared to the other treatments.

Sarkar & Banerjee, (2020) observed similar results in *Solanum erianthum*, finding that callus cultured on a medium containing 0.5 mg/l TDZ produced the most shoot buds.

Generally, auxins are required for the callus induction from plant explants. When they are applied to the culture medium, they have the ability to alter the programmed physiology in the complete tissue of the plant. The cells that respond to the auxin are reverted to a dedifferentiation stage and start to divide (**George, 2008**). For the callus formation, different concentrations of 2,4-D were used, since according to **González *et al*., (2003)**, the auxins produce elongation and formation of adventitious roots at low concentrations, while at higher concentrations, they induce callus formation, being 2,4-D the most frequently used auxin (**George, 2008**).

Several experiments on cell cultures revealed that a variety of factors affect the formation of secondary metabolites, including precursor specificity, concentration, exposure time, culture conditions, and cell growth level (**Wiktorowska *et al*.,2 010**). The ideal and effective method is cell suspension culture, which produces a homogeneous, fast-growing material that is simple to scale up. For the development of plant biomass and secondary metabolites, a large-scale cell and tissue culture method using a bioreactor is promising. It has many benefits, including large-scale dissemination, a controlled atmosphere, and product continuity. It has many benefits, including large-scale propagation, a managed system, product accuracy, and lower micropropagation costs (**Paek *et al*., 2005**).

In this investigation, phenylalanine and tyrosine were added to callus and cell suspension cultures media to induce the endogenous level of kaempferol and quercetin. Suspension culture with tyrosine at a concentration of 66.07 µM resulted in the largest kaempferol aggregation (2.45 %), a 5.7-fold.

These results are close to the results of **Bong *et al*., (2021)** observed that suspension cells had the highest overall phenolic content (55.35 mg GAE/g DW), while leaf had the highest flavonoid content (25.13 mg QE/g DW). Also, (**Karuppaiya and Tsay, 2019)** showed that in comparison to control, B5 medium with 1 mg/l 2,4D and 2 g/ peptone provided more leaf callus biomass and increased podophyllotoxin (16.3 fold), kaempferol (12.39 fold), and quercetin (5.03 fold).

Conclusion

Blackberry propagation through hard wood or stem cuttings has proved to be difficult and complex **(Bray *et al*., 2003).** Asexual and sexual propagation of *Rubus fruticosus* pose certain difficulties in fulfilling the pharmaceutical industry's commercial demand in these circumstances. As a result, a callogenesis method may be a viable option for generating significant amounts of plant cell biomass from which to extract essential secondary metabolites.

Mostly in the BA of the multiplication did the maximal growth and production occur, but at 2ip or TDZ, the mean number of axillary shoots/explant was reduced. The maximum mean number of adventitious buds/explant and organogenesis frequency were obtained at 0.75 mg/l TDZ. Under total darkness, the best callus induction was obtained in MS medium with 1 mg/l TDZ.

MS medium with 0.50 mg/l TDZ produced the most adventitious buds from callus (13.1) and had the highest adventitious buds forming rate (90 %). On MS medium containing 1.00 mg/l IBA and 0.50 mg/l NAA, the highest rooting percentage of *Rubus fruticosus* L. (91%) was achieved.

Decreasing the strength of medium had a promoting effect on rooting plantlets. The optimum number of acclimatized plants can be obtained on peat moss mixture.

Furthermore, compared to control, suspension culture containing MS medium with 1 mg/l TDZ and **t**yrosine at 66.07 µM resulted in the largest kaempferol aggregation (2.45 %), a 5.7-fold improvement over the control procedure. As a result, independent of the exploitation of its natural plant population, *Rubus fruticosus* callogenesis may provide an alternate outlet for increased secondary compound production.

The current study can be used to develop an appropriate protocol for the synthesis of kaempferol in blackberry (*Rubus fruticosus* L.), a plant with high pharmaceutical demand.

To our knowledge, this is the first study to show improved callus biomass quality and content of kaempferol in blackberry (*Rubus fruticosus* L.).

Conflicts of interest

"There are no conflicts to declare".

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الإكثار المعملى وتحسين تراكم الكامفيرول في الكالس ومزارع المعلقات الخلوية لنبات البلاك بييري

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يعتبر نبات البلاك بيري محصولًا متعدد الأغراض يمكن استخدامه للأغراض الطبية العشبية والطهي ومستحضرات التجميل والأغراض الصيدلانية والطبية. هذه الفاكهة تحتوي علي كمية عالية وأنواع مختلفة من الأنثوسيانين والبوليفينول والمركبات المضادة للأكسدة والتي تعكس فوائدها الصحية . وجد أن البوليفينول النباتي يؤثر على الأنشطة الجزيئية بطريقة إيجابية.

الهدف من هذه الدراسة هو الحفاظ على نبات البلاك بيري عن طريق الإكثار المعملي بإستخدام القمة النامية ، تكوين الأعضاء بطريقة مباشرة وغير مباشرة عن طريق تكوين الكالس وإنتاج الكامفيرول، والذي يعتبر واحد من أحد المواد الفعالة في هذا النبات وكذلك زيادة كميته باستخدام التغذية ببادئ.

تم الحصول علي أعلى معدل لتضاعف المجاميع الخضرية على بيئة موراشيج وسكوج المزودة بالسيتوكينين BA، ولكن عند إستخدام كلًا من 2ip أو TDZ حدث انخفاض في متوسط عدد البراعم الإبطية / جزء نباتي. عند إستخدام TDZ بتركيز ٠٫٧٥مجم / لتر تم الحصول علي أعلي معدل لتكوين البراعم العرضية / جزء نباتي بصورة مباشرة وكذلك أعلي نسبة مئوية لتكوين الأعضاء النباتية. تم الحصول علي أعلي نسبة لتكوين الكالس علي بيئة موراشيجي وسكوج المضاف إليها ١٫٠ ملجم/لتر TDZ وتم تحضين الاجزاء النباتية في ظلام كامل.

تلعب كمية السيتوكينين في بيئة الزراعة دور كبير في كمية وكذلك في النسبة المئوية لتكوين المجاميع الخضرية. وجد أن وسط الزراعة المكون من بيئة موراشيجي وسكوج والمضاف أليها ٠٫٥٠ملجم/لتر TDZ أدي إلي الحصول علي أعلي معدل لتكوين الأعضاء بصورة غير مباشرة وكذلك أعلي نسبة مئوية لتكوين الأعضاء ٩٠٪. تم الحصول علي أعلي نسبة مئوية لتكوين الجذور ٩١٪ بإستخدام بيئة موراشيجي وسكوج والمضاف إليها ١٫٠ ملجم/لتر أندول حمض البيوتريك و ٠٫٥٠ ملجم/لتر أندول حمض الخليك. بالإضافة إلى ذلك ، وجد أن مزرعة المعلق الخلوي والتي تحتوي على بيئة موراشيجي وسكوج كوسط للزراعة و المضاف إليها ١٫٠ ملجم/لتر TDZ مع ٦٦٫٠٧ ميكرومول من التيروزين أدي إلي تحسين إنتاج الكامفيرول بمقدار (٥٫٧ مرة) مقارنة بالكنترول.