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IN VITRO PROPAGATION OF *Eucalyptus citriodora* PLANT

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ABSTRACT

This work was carried out in Plant Tissue Culture Laboratory of Prof. Dr. Abd El-Fatah Helmy Belal, Faculty of Environmental Agricultural Sciences, Arish University, North Sinai, Egypt during the period from 2016 to 2018. This study was conducted to study the effect of medium type, explant source and growth regulator type and concentration on micropropagation of *E. citriodora* plant which is grown in Sinai Peninsula. Results showed that full strength of Murashige and Skoog's medium (MS) was the most suitable medium for seed germination and shoot growth. Meanwhile, addition of BA at 1.00 mg l^{-1} was effective for improving shoot growth and development. Also, multiplying of shoots was enhanced by using nodal cutting explants cultured on MS medium supplemented with 1.00 mg l^{-1} BA plus 1.00 mg l^{-1} NAA. On the other hand, shoot rooting was achieved by using MS medium supplemented with 2.50 mg l^{-1} IBA. It is worth to mention that obtained plantlets were successfully acclimatized (80% survivability) in a combination of peatmoss, vermiculite and washed sand or peatmoss and vermiculite at equal volumes.

Keywords: *Eucalyptus citriodora – micropropagation - MS media - acclimatization.*

INTRODUCTION

The Eucalypts genus is a member of the *Myrtaceae* family, it include three genera; *Eucalyptus*, *Corymbia* and *Angophora* **(Pandey, 1987; Trueman and Richardson, 2007).** *Eucalyptus citriodora* is a large, evergreen tree, 24-40 m (max. 50 m) in height; leaves are strongly lemon-scented and fruits are urn-shaped **(Orwa *et al.*, 2009)**. The economic importance of *E. citriodora* plant was producing pulp, producing wood for timber, oil distillation (which used in perfumery, antiseptic for relieving coughs and colds, insect repellents especially against mosquitoes), planted for reforestation and for ornamental garden **(Hill and Johnson, 1995)**. The genus of *Eucalyptus* is generally propagated by seeds at present; however seedlings did not exceed 50% successful establishment and self-incompatibility found between each of them. Whilst it is very difficult or/and failure to propagate by vegetative propagation such as budding, grafting and layering due to problem of incompatibility between scion and rootstocks **(Girouard, 1974; Zobel, 1993; Verma *et al.*, 2013; Bryant and Trueman, 2015).**

In order to increase the efficiency of *in vitro* techniques, tissue cultures have made use of purified chemicals and sophisticated physical facilities where by the temperature, humidity, lighting conditions and aeration are controlled. It is also important to improve total production of ornamental, medicinal and aromatic plants or increasing the yield per feddan by using tissue cultural techniques **(Nizar, 2001)**. Also, **Ho *et al.* (1998) and Prakash and Gurumurthi (2010)** on genus of *Eucalyptus* found that using full MS media was the best germination medium of *Eucalyptus* seeds. While, **Blomstedt *et al.* (1991), Cheng *et al.* (1992) and Dibax *et al.* (2005)** observed that using half strength MS mineral salts was the best germination medium.

On the other hand, **Koriesh and Al-Manie (2000) and Koriesh *et al.* (2003)** obtained multiple

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shoots for *Eucalyptus citriodora* from nodal and shoot-tip explants on MS medium. Shoot tip explants recorded the highest mean shoot length and mean number of shoots. Similarly, nodal explants showed relatively good mean shoot length and number of shoot **(Qader *et al.*, 2014)**. In the same line, **Thirunavoukkarasu *et al.* (2010)** reported that the cultures grown on MS medium exhibited better response than the cultures grown in WP Medium.

Pasha and Irfan (2011) reported that the highest percentage of bud break and initiation of *Eucalyptus citriodora* shoots was reported in the medium containing benzyladenine and kinetin at 0.5-0.2 mg l^{-1} after 10- 15 days of culture. It is known that the function of rooting stage is to prepare the plantlets for transplanting and establishment outside the artificial closed environment of culture vessels **(Murashige, 1974).** Thus, **Koriesh *et al.* (2003)** found that *Eucalyptus citriodora* shoots rooted when using full strength MS medium fortified with 1 or 2 mg l^{-1} IBA. While, **Rahman *et al.* (2013**) mentioned that the highest percentage of rooting of the shoots of *Paulownia tomentosa* occurred with addition of IBA (0.5 mg l^{-1}) compared with culture medium without growth regulators. Meanwhile, acclimatization stage is considered one of the very important stages for *in vitro* micropropagated plants which affect plant survival. Also, they recorded the maximum percentage of acclimatized plantlets survival with the mixture of compost, soil and sand (1:1:1, v/v) for *Paulownia tomentosa* Steud.

Thus, the main objective of this experiment was to study the effects of *in vitro* culture media type and explant source as well as growth regulator type and concentration on micropropagation of *Eucalyptus citriodora* trees which grown in Sinai Peninsula.

MATERIALS AND METHODS

This study was carried out at Prof. Dr. Abd El-Fatah H. Belal Tissue Culture Laboratory, Faculty of Environmental Agricultural Sciences, Arish University, North Sinai, Egypt, during the period from 2016 to 2018 to study the effects of *in vitro* culture media type and explant source as well as growth regulator type and concentration on micropropagation of *Eucalyptus citriodora* trees which grown in Sinai Peninsula.

1. Establishment stage

1.1. Plant material

Eucalyptus citriodora L. seeds were obtained from Gene-Bank at Desert Research Center (DRC), El-Sheikh Zuwyed, North Sinai, Egytpt.

1.2. Seeds sterilization

Seeds were shaked with a few drops of liquid soap for 5 minutes then rinsed under running tap water for 60 minutes. After that seeds were soaked for 30 sec. in 70% ethyl alcohol followed by soaking for 5 minutes in 15% Clorox (containing 5.25% sodium hypochlorite). Seeds were thoroughly rinsed three times with sterile distilled water after each previous step.

1.3. Effect of culture media strength

Seeds were cultured aseptically on free MS-medium **(Murashige and Skoog, 1962)** at different five strengths namely; full strength MS salts (MS), half strength MS salts (1/2MS), quarter strength MS salts (1/4MS), eighth strength MS salts (1/8MS) and tenth strength MS salts (1/10MS). Media supplemented with 100 mg l^{-1} myo-inositol and 3% sucrose. Medium pH was adjusted at 5.7 – 5.8 before gelling with 7.00 g l^{-1} agar. Each treatment was consisted of 12 jars and each one contained 50 ml of medium.

1.4. Recorded data

After 28 days from inoculation date, seed germination percentage (SGP), seedling vigor index (SVI), shoot length (cm) and number of leaves/shoot

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were measured. Seed germination percentage (SGP) was evaluated according to the following equation **(ISTA, 1999)**: (No. of germinated seeds / No. of cultivated seeds) × 100.Seedling vigor index (SVI) was estimated according to the following equation **(Hangarter, 1997)**: (Seedling length (cm) × Germination %) / 100

2. Multiplication stage

This stage aimed to increase the number of shoots, so that shoots obtained from establishment stage was used as explant source during multiplication experiments to study the following factors:

2.1. Effect of explant source and media type

Shoot-tips or one-node cuttings of germinated plants of *E. citriodora* were cultured on different media type i.e.; **Murashige and Skoog (1962)**, Woody Plant Media **(Llyod and McCown, 1980)** and Schenk and Hildebrandt **(Schenk and Hildebrandt, 1972)** to select the best explants source which encouraged the highest growth development and best medium type that induce the highest explant development.

2.2. Effect of explant source and cytokinin type

Aseptic seedlings of 4 weeks old of *E. citriodora* were used. Every shoot was divided into two explant parts; shoot-tip and nodal cutting. Each explant was about 1-1.5 cm length. These explants were cultured on MS media which supplemented with three cytokinin types; Kinetin (Kin), 6-benzyladenine (BA) and 2-isopentenyl adenine (2iP). Each one was studied at the rate of 1.00 mg l^{-1} compared with free MS medium (control) to detect the best cytokinin type which could induce the highest multiplication.

2.3. Effect of explant source and different BA and NAA concentrations

Shoot-tips or one-node cuttings of *E. citriodora* were cultured on MS media which supplemented with two concentrations of benzyladenine (0.50 and 1.00 mg l^{-1}) in combination with four (concentrations 0.10, 0.50, 1.00 and 1.50 mg l^{-1}) of α-naphthaleneacetic acid (NAA). Also, free MS medium (without growth regulators) was tested. Each treatment consisted of 12 jars and each one contained 30 ml of medium. This study conducted to investigate the most suitable combination between Kin and NAA that induce the highest multiplication rate.

2.4. Recorded data

Shoot length (cm), number of leaves/shoot, number of shoots/explant and number of leaves/shoot were recorded after 30 days from inoculation date.

3. Rooting stage

The proliferated shoots obtained from the multiplication treatment (nodal cutting on medium supplemented with BA and NAA at 1.0 mg/l) were used as explant source and cultured on Murashige and Skoog **(MS, 1962)** supplemented with 100 mg l^{-1} myo-inositol, 30.0 g l^{-1} sucrose and 7.00 g l^{-1} agar. These shoots were grown on MS medium free from growth regulators for 4 weeks before using as explant source for the following experiment to eliminate any residual effect of PGRs that might inhibit or reduce rooting.

3.1. Effect of auxins type

Shoots (3-4 cm length) were excised from the proliferated shoots and cultured on MS media supplemented with 1.00 mg l^{-1} of indole-3-butyric acid (IBA), α-naphthaleneacetic acid NAA or indole acetic acid (IAA) to determine the best auxin type that could enhance the best root formation in *E. citriodora*.

3.2. Effect of IBA concentration

Shoots (3-4 cm length) were cultured on MS medium supplemented with different concentrations (0.00, 0.50, 1.00, 1.50 and 2.00 mg l^{-1}) of IBA to investigate the suitable concentration which could encourage the highest root formation rate.

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3.2. Recorded data

Shoot length (cm), number of leaves, number of shoots/shoot, number of leaves/shoot, number of main roots/ Shoot and root length (cm) were recorded after 30 days from inoculation date.

4. Acclimatization stage

Rooted plantlets (about 8-9 cm length) of *E. citriodora* were acclimatized by transferring them to plastic pots (9 x 7 cm) containing peatmoss, vermiculite and washed sand or peatmoss and vermiculite at equal volumes. After 30 days survival percentage was recorded.

5. Statistical Analysis

Each experiment set up in a completely randomized design (CRD) with four replicates and each replicate consisted of three jars each one contained four explants. Data were statistical analyzed with analysis of variance (ANOVA) procedure using SAS statistical software package **(SAS, 2004).** Differences between means were compared by using Duncan's multiple range test **(Duncan, 1955)** at 0.05 level of probability.

RESULTS AND DISCCUSION

1. Establishment stage

Data in Table 1 show that the highest germination percentages were obtained when seeds were cultured on full, half or quarter MS strengths medium without significant differences among these treatments, while tenth strength of MS media achieved the lowest germination percentage, seedling height, number of leaves and the seedling vigor index (72.50 %, 2.86 cm, 5.87 and 2.07, respectively). There were a little variations among different medium strengths (especially full, half and quarter strengths) concerning seedling length, number of leaves/shoot and seedling vigor index. This result was in agreement with that of **Pandey *et al.* (2014)** who stated that the highest percentage of seed germination (95%) was recorded with full MS basal medium followed by half MS basal media (86%) in *Psoralea corylifolia*. Also, this result was in agreement with **Mishra *et al.* (2013)** on *Pterocarpus marsupium* and **Silva *et al.* (2016)** on *Dipteyx alata* where they concluded that a 25% concentration of MS salts was the best option for the *in vitro* establishment of these plants.

2. Multiplication stage

2.1. Effect of explants and media type

Data presented in Table 2 clear that the tallest shoots were recorded when MS medium was used regardless explant source (nodal cutting or shoot tip). While, the maximum number of leaves/shoot was gained when nodal cuttings or shoot tips were cultured on MS medium or as nodal cutting was cultured on SH medium without significant differences among these treatments. There were a little variations among different investigated treatments concerning No. of shoots/explant.

These results are in agreement with those obtained by **Brondani *et al.* (2012)** on *Eucalyptus benthamii*, **Thirunavoukkarasu *et al.* (2010)** on *Dalbergia sissoo* Roxb. and **Koriesh *et al.* (2003)** on *E. citriodora* who stated that the induction of shoots grown on MS medium exhibited better response than WP medium and SH medium. This result may be explained by that the type and concentration of nutrients present in MS media play a vital role in improving development and growth of shoots **(Niedz and Evens, 2007)**.

2.2. Effect of explant and cytokinin type

Data in Table 3 show that the highest value of shoot length (1.52 cm) was belonged to nodal cutting which cultured on MS medium supplemented with 1 mg l^{-1} BA. While, the lowest value of shoot length was belonged to shoot tip explant which inoculated in MS medium enriched with 1 mg l^{-1} 2ip. Concerning

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No. of shoots/explant, it is clear that the ultimate number of shoots were obtained as BA was used regardless explant source used. Regarding No. of leaves/shoot, it is obvious that medium fortified with BA gave the maximum No. of leaves/shoot regardless explant source. This result is in agreement with those mentioned by **Koriesh *et al.* (2003)** on *E. citriodora*, **Glocke *et al*. (2006)** on *E. erythronema* **and Girijashanka, (2012)** on *E. camaldulensis*.

2.3. Effect of explant type and different BA and NAA concentrations

Data in Table 4 show that the highest shoot length (cm) and number of leaves /shoot (2.45 cm and 2.45 leaves/shoot, respectively) were recorded when nodal cutting was cultured on medium supplemented with BA and NAA at 1.0 mg/l for each one. Also, using the same explant source combined with 0.5 mg/l BA and 1.0 mg/l NAA gave almost the same number of leaves/shoot. There were no wide variations among different tested treatments concerning number of shoots/explant. This result was in accordance with those obtained by **Sharma and Kalia (2005)** on *Eucalyptus* and **Lobna *et al.* (2008)** on *Paulownia kowakamii*.

3. Rooting stage

3.1. Effect of auxin type

Data presented in Table 5 indicate that growth regulator type had a significant effect on shoot growth and rooting, since shoot length the highest shoot length, number of shoots/explant, number of roots/shoot and root length of *E. citriodora* were gained when IBA was used. On the other side, the maximum number of leaves/shoot was recorded as IBA at 1.00 mg l^{-1} was used. It is worth to mention that there were no significant differences among different auxin types concerning number of roots/shoot. These results are in a harmony with those obtained by **De Fossard *et al.* (1973)** and **Das and Mitra (1990)** since they indicated that culturing the nodes of *Eucalyptus* in nutrient media supplemented with IBA enhanced the roots development and formation. On the same line, **Koriesh *et al.* (2003)** indicated that the root number and root length were increased by using medium supplemented with IBA compared with non-treated medium (control).

3.2. Effect of IBA concentration

Results presented in Figures 1 and 2 as well as Photo 1 show that addition of IBA at any applied concentration significantly improved shoot and root growth. The highest value of number of shoots was gained with the highest concentration of IBA. No. of leaves/shoot did not significantly affect by increasing IBA concentration in the range of 0.5 to 2.0 mg/l while higher concentration (2.5 mg/l) had depressive effect in this regard. It is obvious that high concentrations (1.5, 2.0 and 2.5 mg/l) were more effective than low concentration for increasing No. of initiated roots. Shoot and root length reached the maximum values when high concentrations (2.0 and 2.5 mg/l) were used without significant difference between both concentrations. These results are in a harmony with the findings of **Koriesh *et al.* (2003)** since they indicated that *Eucalyptus* cultured on MS media and supplemented with the highest concentrations (2- 3 mg l^{-1}) of IBA resulted in enhancing the root number compared with the lowest concentrations (0.5- 1 mg l^{-1}).

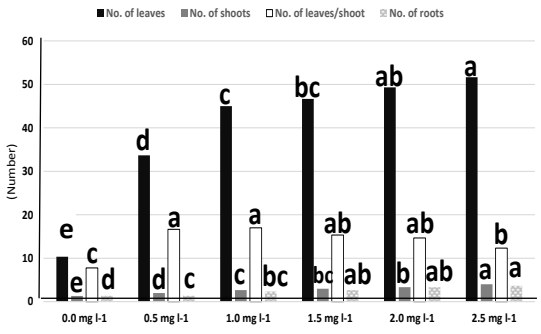

Fig. 1. Effect of IBA concentrations on number of leaves, number of shoots, number of leaves/shoots and number of roots of *E. citriodora* plant

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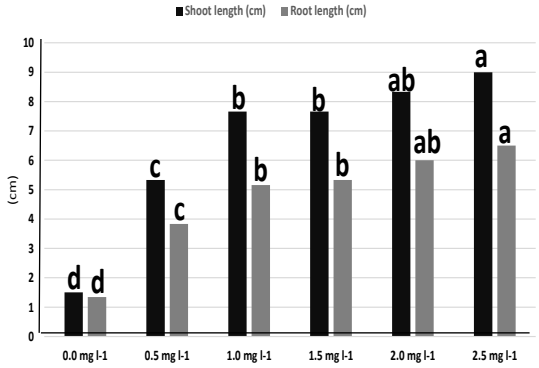

Fig. 2. Effect of IBA concentrations on shoot length and root length (cm) of *E. citriodora* plant

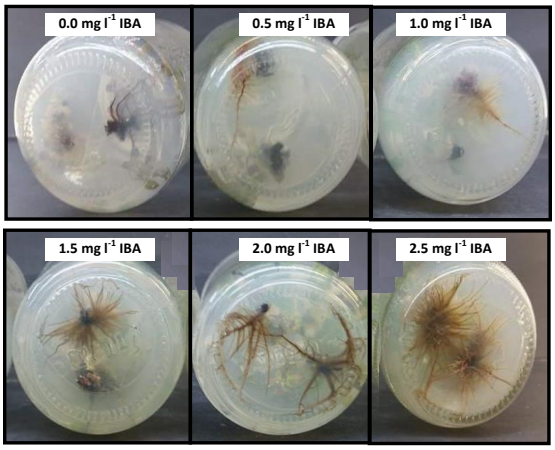

Photo (1): Effect of IBA concentration on rooting of Eucalyptus citriodora shoots

4. Acclimatization stage

Eucalyptus citriodora plantlets were successfully acclimatized by using a combination of peatmoss, sand and vermiculite or peatmoss and vermiculite at equal volumes where 80% of the tested plants were survived.

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Table 1. Effect of MS media strength on germination percentage, seedling length, number of leaves/ seedling and the seedling vigor index (SVI) of *E. citriodora* plant

Treatments	Germination (%)	Seedling length (cm)	No. of leaves/seedling	Seedling vigor index
Full strength	90.00 a	3.54 ab	5.93 ab	3.19 ab
Half strength	77.50 bc	3.89 a	6.20 ab	3.01 ab
Quarter strength	87.50 ab	4.04 a	6.53 a	3.54 a
Eighth strength	67.50 c	3.25 ab	5.90 b	2.19 b
Tenth strength	72.50 c	2.86 b	5.87 b	2.07 b

•Means followed by the same letter within each column are not significantly different at 0.05 level of probability according to Duncan's multiple range test.

Table 2. Interaction effect of media type and explant type on shoot length (cm), number of shoots and number of leaves/shoots of *E. citriodora* plant

Treatments		Shoot length (cm)	No. of shoots/explant	No. of leaves/shoots
MS	**Nodal cutting**	8.86 a	2.66 a	7.99 a
	Shoot-tip	8.65 a	2.33 ab	7.27 ab
WPM	**Nodal cutting**	7.63 b	2.33 ab	4.72 b
	Shoot-tip	7.21 bc	2.33 ab	4.69 b
SH	**Nodal cutting**	6.83 bc	1.66 ab	5.33 ab
	Shoot-tip	6.63 c	1.33 b	4.50 b

Table 3. Interaction effect of cytokinins type on shoot length (cm), number of shoots and number of leaves/shoots of *E. citriodora* plant

Treatments		Shoot length (cm)	No. of shoots/explant	No. of leaves/shoots
Control	**Nodal cutting**	2.4 c	1.68 b	7.67 b
	Shoot-tip	2.0 d	2.53 b	6.92 b
BA	**Nodal cutting**	4.0 a	6.15 a	11.11 a
	Shoot-tip	3.6 b	5.71 a	11.21 a
Kin	**Nodal cutting**	2.8 c	1.86 b	8.45 b
	Shoot-tip	2.5 c	1.86 b	7.53 b
2ip	**Nodal cutting**	2.5 c	1.50 b	7.44 b
	Shoot-tip	1.9 d	2.25 b	6.68 b

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Table 4. Interaction effect of explants type and different BA and NAA concentrations on shoot length (cm), number of shoots and number of leaves/shoots of *Eucalyptus citriodora* plant

Treatments			Shoot length (cm)	No. of shoots/explant	No. ofleaves/shoots
Explants source	BA conc.	NAA conc.			
Nodal	Control (0.0 mg l^{-1})		1.47 d	1.70 b	6.80 fg
	0.5 mg l^{-1}	0.1 mg l^{-1}	1.37 de	1.60 b	6.40 fg
		0.5 mg l^{-1}	1.83 c	2.25 a	7.85 cd
		1.0 mg l^{-1}	2.15 b	2.58 a	14.11 a
		1.5 mg l^{-1}	1.52 d	1.63 b	6.83 ef
	1.0 mg l^{-1}	0.1 mg l^{-1}	1.37 de	1.60 b	6.40 fg
		0.5 mg l^{-1}	1.84 c	2.26 a	8.25 c
		1.0 mg l^{-1}	2.45 a	2.63 a	14.77 a
		1.5 mg l^{-1}	1.54 d	1.66 b	7.04 def
Shoot-tip	Control (0.0 mg l^{-1})		1.55 d	1.33 b	6.82 ef
	0.5 mg l^{-1}	0.1 mg l^{-1}	1.30 e	1.65 b	6.33 fg
		0.5 mg l^{-1}	1.27 e	1.55 b	5.63 g
		1.0 mg l^{-1}	1.79 c	2.20 a	7.60 cde
		1.5 mg l^{-1}	1.85 c	2.33 a	9.46 b
	1.0 mg l^{-1}	0.1 mg l^{-1}	1.38 de	1.63 b	6.08 fg
		0.5 mg l^{-1}	1.30 e	1.56 b	6.06 fg
		1.0 mg l^{-1}	1.82 c	2.20 a	7.73 cde
		1.5 mg l^{-1}	2.08 b	2.49 a	9.73 b

- Means followed by the same letter within each column are not significantly different at 0.05 level of probability according to Duncan's multiple range test.
- Nodal (N) and Shoot-tip (ST).

Table 5. Effect of auxins type on shoot length (cm), number of shoots, number of leaves/shoot, number of roots and root length (cm) of *E. citriodora* plant

Treatments	Shoot length (cm)	No. of shoots/explant	No. of leaves/shoot	No. of roots/shoot	Root length (cm)
Control	1.00 c	1.00 d	8.00 c	1.00 b	1.00 c
IBA	8.33 a	3.33 a	13.66 b	2.66 a	5.33 a
IAA	6.66 b	2.33 b	13.00 b	2.33 a	3.83 b
NAA	6.33 b	1.00 d	20.00 a	2.33 a	1.54 c