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In Vitro Propagation of *Dillenia indica*

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Abstract: An efficient protocol was established for *in vitro* shoot multiplication of *Dillenia indica* using nodal explants. *Dillenia indica* (Family: Dilleniaceae) is classified according to its status in Egypt as a very rare and not propagated to day. For explant sterilization, Clorox was used at 15% for 10 min which gave a decontamination rate 85% with survival percentage 95%. The maximum shoot multiplication rate (7.1 shhots per explant) was achieved with MS medium enriched with 2.0 mgl^{-1} benzyl amino purine (BAP). Shootlets which were produced from multiplication stage were successfully rooted on half strength MS medium supplemented with 10 mgl^{-1} silver nitrate in combination with all indole butyric acid (IBA) concentration used (0.5, 0.75 and 1.0 mgl^{-1}) and silver nitrate at 5mgl^{-1} plus IBA at 0.75 and 1.0 mgl^{-1}. All plantlets produced *in vitro* have been successfully acclimatized.

Key words: *Dillenia indica*, *in vitro* culture, Plant growth regulators, silver nitrate.

INTRODUCTION

The genus *Dillenia* has 60 species, of which *Dillenia indica* Linnaeus (Family: Dilleniaceae) is the most common edible species. Originally from Indonesia, this evergreen tropical tree is now found from India to China. The common names include Chulta (Bengali, Hindi), Bhavya (Sanskrit) and Elephant apple (English). It is a spreading tree and has beautiful white fragrant flowers, toothed leaves, and globose fruits with small brown seeds (Janick and Poull, 2008). The leaf, bark, and fruit of this plant are used as traditional medicine. The juice of *D. indica* leaves, bark, and fruits are mixed and given orally (5-15 ml, two to five times daily) in the treatment of cancer and diarrhea (Sahrama *et al*., 2001). The fruit juice of this plant has cardiotonic effect, used as cooling beverage in fever and also employed in cough mixture (Shome *et al*., 1980). The solvent extracts of fruits of *D. indica* are reported to have antioxidant activity (Abdille *et al*., 2005). CNS depressant activities in mice were found from the alcoholic extract of the leaves of *D. indica* (Bhakuni *et al*, 1969) and the mucoadhesive agent obtained from fruits have been used in gel formulation (Kuotsu and Bandyopadhyay 2007). The methanolic extract of *Dillenia indica* L. fruits showed significant anti-leukemic activity in human leukemic cell lines U937, HL60 and K562. (Deepak *et al*. 2010).

Compared to traditional propagation with in vitro propagation procedure, *in vitro* propagation has several potential advantages over the traditional procedure: For large-scale *in vitro* plant production the important attributes are the quality, cost effectiveness, maintenance of genetic fidelity, and long-term storage. Moreover, micropropagation may be utilized, in basic research, in production of virus-free planting material, cryopreservation of endangered and elite woody species, applications in tree breeding and reforestation (Mohan Jain and Ha ggman, 2007).

Micropropagation has become a reliable and routine approach for large-scale rapid plant multiplication, which is based on plant cell, tissue and organ culture on well-defined tissue culture media under aseptic conditions. Up till now, micropropagation of many fruit trees including apricot (Perez-Tornero *et al*., 2000), mulberry (Anis *et al*., 2003), chestnut (Osterc *et al*., 2005), *khinjuk pistachio* was investigated.

Plant growth regulators have different effect on plants *in vitro*. In this concern, Rahman and Blake (1988) showed that a successful vegetative propagation of seedling jackfruit (*Artocarpus heterophyllus* Lam.) has been achieved by in vitro methods. Proliferation from nodal explants was greater than from shoot tips. Of the cytokinins tested, benzylaminopurine (BAP) was more effective than either 2-isopentenyladenine (2iP) or kinetin (Kin) and produced maximum proliferation when used at 5×10^{-6}M. BA is widely used in micropropagation of stone fruits and has given good results (Ružić *et al*., 2001) 2iP and Kin. are rarely used in micropropagation of fruit varieties (Jaakola *et al*, 2001).

Induction of rooting is an important step in micropropagation and genetic transformation protocols; however, it has been often proved difficult, particularly in the case of woody species (Bais *et al*., 2001). Effects of $AgNO_3$ on *in vitro* root formation of *Decalepis hamiltonii* were studied. Addition of 40 µM AgNO3 resulted in root initiation and elongation (Bais *et al*. 2000; Reddy *et al*. 2001).

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As reported by Khalifa and Loutfy (2006) *Dillenia indica* is classified according to its availability or status in Egypt as very rare and not propagated to day so, this work aims to conduct a protocol for *in vitro* propagation of this rare tree in Egypt (*Dillenia idica*).

MATERIALS AND METHODS

This study has been carried out in the Tissue Culture Res. Lab., Hort. Res. Institute, Agri. Res. Center (A.R.C.), Egypt, during the period from 2010 to 2012.

Plant Material:

Nodal explants were taken from *Dillenia indica* unique tree at Giza zoo Garden, Giza.

Explant Disinfection:

Explants were washed in soapy water using septol soap, then agitated in disinfectant solution of savlon (3%) for 40 min and rinsed with running tap water for one hour. Thereafter, explants were surface disinfected under aseptic condition in safety cabinet using ethanol 70% for one min. followed by a further sterilization:

a. Clorox (NaOCl 5.25%) 10, 15 or 20% (v/v) with a few drops of Tween-20 as emulsifier for 10 min.
b. Mercuric chloride at 0.1, 0.2 or 0.3 % % (w/v) with a few drops of Tween-20 as emulsifier for 10 min.

Three rinses with distilled water were adopted after each disinfection.

Culture Media and Incubation Condition:

Explants were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.7% agar (W/V). Cultures were incubated at 24±2 °C and illumination condition of 1500Lux using fluorescent lamb and 16/8 photoperiod.

1- Establishment Stage:

Explants were cultured on MS basal medium plus 3% w/v sucrose and solidified with 0.7 (w/v) agar without plant growth regulators for four weeks.

2- Shoot Multiplication Stage:

At this stage, each explant has expanded into a cluster of small shoots. Multiple shoots were separated and transplanted to new culture medium (Hartmann *et al*., 2002). Shoots were subcultured every 4 weeks. Material may be subcultured several times to new medium to maximise the quantity of shoots produced**.** MS medium was used in this stage, it was supplemented with the following plant growth regulators: BAP, Kin or 2-iP at 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mgl^{-1}.

3- Pre Rooting Stage:

In this stage unique shoots were transferred to MS free medium for three weeks to be strong enough to be rooted.

4- Rooting Stage:

Half strength of MS basal medium was used in this stage. It was supplemented with one of the following: IBA at 0.25, 0.50, 0.75 or 1.0 mgl^{-1} alone or in combination with 5 or 10 mgl^{-1} silver nitrate. Cultures were incubated for 8 weeks in this stage.

5- Acclimatization Stage:

Rooted plantlets were transferred to greenhouse in peat moss and covered with polyethylene bags for 2 weeks, then one pore was made in every bag for two another weeks. After that the bags were removed.

Statistical Analysis:

Mean separation was made using least significant differences (L.S.D.) at 5% level of significance. Test was applied for the comparison among means as described by Steel and Torrie (1980).

RESULTS AND DISCUSSION

1. Establishment Stage:

Effect of Disinfectant Types and Concentrations on Decontamination and Survival Percentages of Dillenia indica Explants in vitro:

Data in Table 1 and Fig. (1A) shows the sterilization efficiency of Clorox and mercuric chloride at different concentrations. Data clearly indicated that the two used disinfectants have a significant effect on

decontamination and survival percentage. As for the decontamination, using Clorox at 20% whereas using mercuric chloride at 0.1% gave the lowest significant one (20%). Data also indicated that decontamination rates were significantly increased with increasing disinfectant concentrations. As for survival rate, the maximum survival percentage (100%) was recorded for Clorox 10% while the lowest one (30%) was obtained when mercuric chloride was used at 0.3%. Finally, the best disinfectant treatment was obtained when Clorox was used at 15% for 10 min which gave a decontamination rate 85% with survival percentage 95%. These finding is in agreement with those reported by Abou Dahab *et al*., (2010) who reported that the best results in sterilization of nodal explants of *Taxodium distichum* and *Taxodium distichum* var. 'distichum' were recorded with using 20% Clorox for 5 min., followed by 0.2% mercuric chloride for 5 min.

Table 1: Effect of different types and concentrations of disinfectants on decontamination and survival percentages of *Dillenia indica* explants *in vitro*.

Disinfectant	Decontamination%	Survival%
Clorox 10%	40	100
Clorox 15%	85	95
Clorox 20%	100	50
Mercuric chloride 0.1%	20	70
Mercuric chloride 0.2%	40	70
Mercuric chloride 0.3%	65	30
L.S.D.	31.78*	22.93*

2. Shoot Multiplication Stage:

Effect of Different Types and Concentrations of Cytokinens on Shoot Multiplication:

Shoot proliferation and multiple shoot production is the aim of this stage. At this stage, each explant has expanded into a cluster of small shoots Fig. (1B). Data in Table 2 explain the effect of different types and concentrations of cytokinens on the average shoot number per explant , shoot length (cm) and leaves number per shootlet. As for the average shoot number formed per explant, data indicated that the maximum significant shoot number (7.1) formed per explant was recorded when explants of *Dillenia indica* were cultured on MS medium supplemented with BAP at 2.0 whereas the lowest shoot number was obtained (1 shootlet per explant) when MS medium was used free (without growth regulators). It also noticed that average shoot formation was increased with increasing BAP increasing from 0.5 to 1.0 , 1,5 and 2.0 mgl^{-1} and the the number of shootlets was decreased when the BAP was increased from 2.0 to 2.5 mgl^{-1}. As for the other two cytokinens used (kinetin and 2-iP) the average shoot number per explant was increased as the increasing cytokinen concentration till it reached 1.5 mgl^{-1} and then the shootlets number were decreased with increasing growth regulators concentration.

As for the effect of type and concentration of cytokinen on shootlet length, the highest significant shootlet length (5.9 cm) whereas the lowest significant one (1.2 and 1.3cm) has been achieved when explants were cultured on MS enriched with 1.5 2-iP and 2.0, 2.5 mgl^{-1} and MS free medium.

Leaves number per shootlet as a shoot multiplication parameter was also significantly affected by cytokinen type and concentration. Maximum leaves number per shootlet (7.2) was recorded when *Dillenia indica* was cultured on MS medium augmented with 1.5 mgl^{-1} BAP. While the lowest number was achieved with 2-iP at 2.5 mgl^{-1}. The effect of cytokinens on shoot multiplication was studied by many authors. Ružić and Vujović (2008) studied the effect of BAP, 2iP, kinetin (Kin) and thidiazuron (TDZ) at different concentrations on shoot multiplication parameters of *Prunus avium* L. and they found that the highest multiplication index was found with BAP.

Table 2: Effect of different types and concentrations of cytokinins on shoot multiplication of *Dillenia indica*.

MS basal medium + 3% sucrose (w/v)	Concentration of cytokinin (mgl^{-1})	Average shoot number per explant	Shoot length (cm)	Leaves number per shootlet
Control	0.0	1.0	1.3	4.1
BAP	0.5	2.4	4.1	6.3
	1.0	2.7	4.2	5.6
	1.5	5.2	2.6	7.2
	2.0	7.1	1.9	6.4
	2.5	4.4	2.0	5.7
Kin	0.5	1.2	5.9	5.3
	1.0	2.4	4.1	6.8
	1.5	2.7	3.9	6.5
	2.0	1.9	3.5	6.4
	2.5	1.4	3.8	5.9
2-iP	0.5	1.2	2.4	4.3
	1.0	1.7	2.3	4.1
	1.5	2.1	1.2	4.1
	2.0	1.5	1.3	4.0
	2.5	1.3	1.3	3.9
L.S.D		0.37*	0.33*	0.87*

3- Rooting Stage:

Effect of Different Concentrations of IBA with or Without Silver Nitrate on in vitro Rhizogenasis of Dillenia indica:

Rooting stage is an essential stage to complete micropropagation process (fig. 1C). Data in Table 3 shows the effect of IBA with or without $AgNO_3$ on rooting capacity of shootlets of *Dillenia indica in vitro*. Data revealed that the treatments under investigation had a significant influence on rooting stage parameters. The highest rooting rate (100%) was recorded for more than treatment; MS half strength enriched with 10 mgl^{-1} silver nitrate in combination with all IBA concentration used (0.5, 0.75 and 1.0 mgl^{-1}) and silver nitrate at 5mgl^{-1} plus IBA at 0.75 and 1.0 mgl^{-1}. Whereas the lowest significant rooting percentage was recorded when shootlets were cultured on medium supplemented with 0.25 or 0.5mgl^{-1} IBA. It also noticed that using silver nitrate in combination with IBA enhanced rooting response. Other rooting parameters; number of roots formed per shootlet and length of roots take the same trend. These findings are in agreement with those reported by Chithra *et al*. (2004). They showed that silver nitrate enhanced rooting *in vitro* on *Rotula aquatic* L. shoots. Also, Steephen *et al.* (2010) tested different concentrations of IBA for rooting of *Vitex negundo* and found that 0.5 mgl^{-1} was more effective for inducing roots *in vitro*.

Table 3: Effect of different concentrations of IBA with or without silver nitrate on *in vitro* rooting of *Dillenia indica.*

Half strength MS medium with		Rooted	No of roots/	Root length
IBA concentration mgl^{-1}	$AgNO_3$ concentration mgl^{-1}	plants, %	Plant	(cm)
0.25	0	25	2.3	1.8
0.50	0	28	2.4	1.7
0.75	0	34	3.1	2.4
1.0	0	52	3.5	3.2
0.25	5	68	4.1	3.1
0.25	10	73	5.1	3.7
0.50	5	81	5.8	3.6
0.50	10	100	7.3	4.4
0.75	5	100	6.1	4.1
0.75	10	100	6.5	3.7
1.0	5	100	6.2	3.9
1.0	10	100	6.6	3.6
L.S.D.		19.77*	1.38*	1.80*

4- Acclimatization Stage:

All rooted plantlets were successfully acclimatized in peat moss under green house with hundred percent of success (fig 1E).

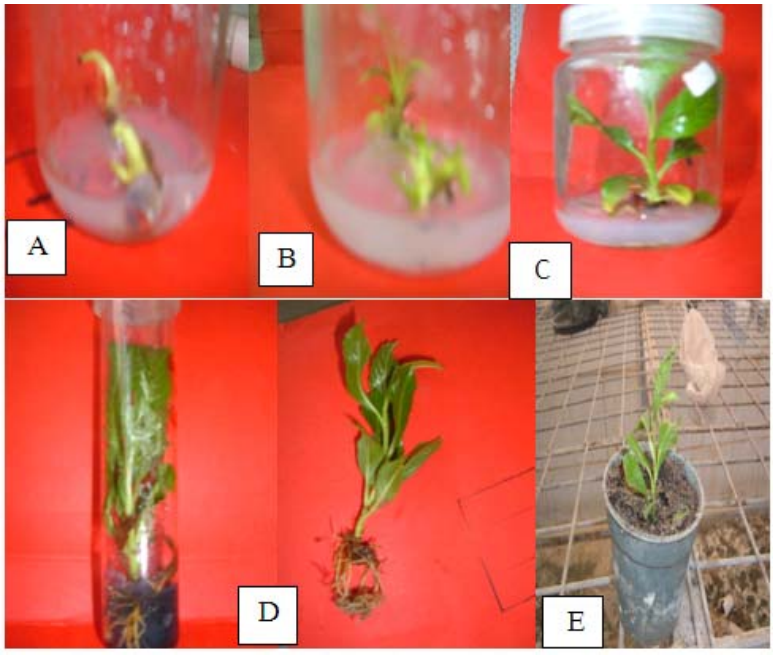

Fig. 1: *In vitro* clonal propagation of *Dillenia indica* L. A, Nodal explants after the first 6 weeks of culture. B, Multiplication of shoots. C, prerooting stage. D, Complete rooted plantlet. E, Acclimatized plants.

REFERENCES

Abdille, M.H., R.P. Singh, G.K. Jayaprakasha and B.S. Jena, 2005. Antioxidant activity of the extracts from *Dillenia indica* fruits. Food Chem., 90: 891-6.

Abou Dahab, A.M., A.M. Habib; M.K. El-Bahr and A.M.M. Gabr, 2010. Establishment of an *in vitro* propagation protocol for *Taxodium distichum* and *Taxodium distichum* var. 'distichum'. Nature and Scince, 8(9): 216-227.

Anis, M., M. Faisal and S.K. Singh, 2003. Micropropagation of Mulberry (*Morus alba* L.) through *in vitro* culture of shoot tip and nodal explants. Plant Tissue Cult., 13(1): 47-51.

Bais, H.P., G. Sudha and G.A. Ravishankar, 2001. Influence of putrescence, silver nitrate and polyamine on morphogenetic response in untransformed and transformed tissue of *Cichorium intybus* and their regenerates. Plant Cell Report, 20: 547-555.

Bais, H.P., G. Ssdha, B. Suresh and G.A. Ravishankar, 2000. AgNO3 influences *in vitro* root formation in *Decalepis hamiltonii* Wight, Arn. Current Science, 79: 894-898.

Bhakuni, D.S., M.L. Dhar, M.N. Dhar, B.N. Dhawan and B.N. Mehrotra, 1969. Screening of Indian plants for biological activity, II. Indian J. Exp. Biol., 7: 250.

Chithra, M., K.P. Martin, C. Sunandakumari and P.V. Madhusoodanan, 2004. Silver nitrate induced rooting and flowering in vitro on rare rhoeophytic woody medicinal plant, Rotula aquatic Lour. Indian Biotech, 3: 418-421.

Deepak, K., M. Sumana, V. Joseph and C.P. Bikas, 2010. Anti-leukemic activity of *Dillenia indica* L. fruit extract and quantification of betulinic acid by HPLC Phytomedicine: International J. Phytotherapy and Phytopharmacology, 3(1): 241-247.

Hartmann, H.T., D.E. Kester, F.T. Davies and R.L. Geneve, 2002. Plant Propagation: Principles and Practices, 7th edn. Prentice-Hall, Inc., Englewood Cliffs, NJ, USA.

Jaakola, L., A. Tolvanen, K. Laine and A. Hohtola, 2001. Effect of N6-isopentenyladenine concentration on growth initiation *in vitro* and rooting of bilberry and lingonberry microshoots. Plant Cell, Tissue and Organ Culture, 66: 73-77.

Janick, J. and R.E. Paull, 2008. The Encyclopedia of Fruit and Nuts. 1st ed. London: CABI.

Khalifa, S.F. and M.H. Louttfy, 2006. Ornamental Cultivated Plant Collection: The First Occasion of the First International Conference on Strategy of Botanic Gardens, pp: 61.

Kuotsu, K. and A.K. Bandyopadhyay, 2007. Development of oxytocin nasal gel using natural mucoadhesive agent obtained from the fruits of *Dellinia indica*. L Science Asia, 33: 57-60.

Mohan Jain, S. and H. Haggman, 2007. Protocols for Micropropagation of Woody Trees and Fruits, Publisher: Springer-Verlag, the Netherland, pp: 559.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Osterc, G., M. Zavrl Fras, T. Vonedik and Z. Luthar, 2005. The propagation of chestnut (*Castanea sativa* Mill.) nodal Explants. Acta Agric. Slov., 85: 2.

Perez-Tornero, O., J.M. Lo pez, J. Egea and L. Burgos, 2000. Effect of basal media and growth regulators on the *in vitro* propagation of apricot (*Prunus armenica* L.) cv. Canino. J. Hort. Sci. Biotechnol., 75: 283-286.

Rahman, M.A. and J. Blake, 1988. Factors affecting *in vitro* proliferation and rooting of shoots of jackfruit (*Artocarpus heterophyllus* Lam.), Plant Cell, Tissue and Organ Culture, 13(3): 179-187.

Reddy, B.O., P. Giridhar and G.A. Ravishankar, 2001. *In vitro* rooting of *Decalepis hamiltonii* Wight and Arn., an endangered shrub by auxins and root-promoting agents. Current Science, 81(11): 1479-1481.

Ružić, D.J., M. Sarić, R. Cerović and L.J. Ćulafić, 2001. Changes in macroelement content of the media and in sweet cherry Inmil GM 9 shoots during in vitro culture. J. of Hort. Science & Biotech., 76: 295-299.

Ružić, D.J. and T.I. Vujović, 2008. The effects of cytokinin types and their concentrationon *in vitro* multiplication of sweet cherry cv. Lapins (*Prunus avium* L.). Hort. Science, 35(1): 12-21.

Sharma, H.K., L. Chhangte and A.K. Dolui, 2001. Traditional medicinal plants in Mizoram, India. Fitoterapia, 72: 146-61.

Shome, U., R.K. Khanna and H.P. Sharma, 1980. Pharmacognostic studies on *Dillenia indica* Linn: II- Fruit and Seed. Proc Indian Acad Sci (Plant Sci), 89: 91-104.

Steel, R.G.D. and J.H. Torrie, 1980. Principle of Statistics. A Biometrical Approach 2nd, MC Graw-Hillkogakusha, Ltd.

Steephen, M., S. Nagarajan and D. Ganesh, 2010. Ploroglucinol and silver nitrate enhances axillary shoot proliferation in nodal explants of *Vitex negundo* L.- an aromatic medicinal plant. Iranian J. of Biotech., 8(2): 82-89.