IN VITRO CONSERVATION OF THE UNIQUE *DILLENIA INDICA* GROWN IN EGYPT UNDER MINIMAL-GROWTH CONDITIONS

ABD EL-KADER, SSAM M.[1], HWIDA, M. FATHY[1]and DALIA G. ASEEL[2]

1. *Timber Trees Dept., Hort. Res. Inst., A.R.C., Egypt.*
2. *Plant Protection and Biomolecular Diagnosis Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, New Borg El-Arab City, 21934, Egypt.*

(Manuscript received 18 September 2018)

Abstract

Dillnia indica is a very important medicinal unique tree grown in Egypt (a good source of betulenic acid). An experiment has been conducted to establish a safe protocol for minimal growth conservation using osmosis and growth retardants. Data revealed that abscisic acid (as retardant) (ABA) at 1, 2 or 3 mg/L can be able to save plant life at percentages of 66.67, 83.33 and 50, respectively up to nine months without subculturing. On the other hand, sorbitol (an osmotic substance) has a bad effect especially at high concentrations (6 and 9%) which is lead to 100% death after six months. The results show that the survived shootlets exhibited regeneration ability significantly differed after subculturing into fresh medium. At the end of conservation period (9 months), the regeneration ability of explants derived from shootlets conserved on medium augmented with 1.0 or 2.0 mg/L ABA recorded 100%. When the dendogram was constructed based on the obtained band patterns, results revealed that both the negative (mother plant) and positive controls (*in vitro* propagated plants) were grouped in separate cluster. Whenever, samples treated with abscisic acid at 1.0 and 3.0 mg/l are grouped in one cluster, which indicated that they are much closed to each other and ABA is genetically safe.

Key words: *Dillenia indica*, minimal growth, *in vitro* culture, abscisic acid, Ancymidol.

INTRODUCTION

The genus *Dillenia* belong to Family: Dilleniaceae includes 60 species, of which *Dillenia indica* (Linnaeus) is the most favorable edible species. It is an evergreen tropical tree originally found in Indonesia and cultivated from India to China. Its common names varied from country to another which includes Chulta (Bengali, Hindi); Bhavya (Sanskrit) and Elephant apple (English). It is a wide spreading tree that has beautiful white aromatic flowers, toothed leaves, and globose fruits with small brown seeds (Janick and Poull, 2008). *D. indica* is a good source of betulinic acid (Abd El-Kader and Abd EL Shakour, 2015) which is a natural pentacyclic triterpenoid known for its antiretroviral, antimalarial, and anti- inflammatory activities. Recently, it has been discovered that betulinic acid is a potential anticancer agent through inhibition of topoisomerase (Damle *et al*., 2013).

Using of *in vitro* culture techniques has a great importance for collection, preservation and multiplication of recalcitrant and vegetatively propagated

germplasms (Engelmann, 1991). Conservation techniques are differed according to the storage period requested. At short and medium term storage, the goal is reducing the growth and increasing subculturing times. This could be accomplished by altering the culture medium composition through addition of osmotic substances or growth retardants. Adding osmotic substances with or without incubation at low temperature has also confirmed to be an important technique to prolong subculture intervals. Interesting results in some species could be achieved by use of either increased or decreased doses of yielded sorbitol. Therefore, adding of ABA to the last subculture positively affected the survival rate of tobacco plantlets transplanted to *ex vitro* environmental conditions (Pospisilova *et al*., 2009). Also, Monoj *et al*. (2011) mentioned that there are several indicators suggesting that ABA may not only be involved in *in vitro* conservation but, it has a significant role in the *in vitro* stress tolerance and *ex vitro* acclimatization. Ancymidol (ANC), a-cyclopropyl-a-(4- methoxyphenyl)-5-pyrimidine methanol (EL-53 1) is synthetic pyrimidine analogues with growth retardant activity (Sherald *et al.,* 1973). Ancymidol is an N-containing heterocycle, e retardants block cytochrome P450-dependent monooxygenases, which inhibits oxidation of ent-kaurene into ent-kaurenoic acid (Rademacher, 2000).

Therefore, the current experiments had been achieved with view to find out the suitable protocol for the *in vitro* minimal growth conservation of *Dilleni indica* germplasm. Besides, the genetic stability of such germplasm will be determinate.

MATERIALS AND METHODS

This study was conducted in Tissue Culture & Germplasm Conservation Laboratory, Horiculture Research Institute, Agricultural Research Center, Giza, Egypt during the period 2015 to 2017.

Plant material

In vitro proliferated shoot grown on MS medium (Murashige and Skoog, 1962), enriched with 1.0 mg/L BAP, 30 g /L sucrose and 7 g/L agar (according to the protocol established by Abd El-Kader and Hammad, 2012) and incubated at 24±2 ◦C, with a 16-h photoperiod and 1500 Lux light intensity using 120 cm white florescent lambs.

Minimal growth treatments

Osmotic treatments

- Sorbitol was used at 0, 3, 6 or 9% (w/v).

Growth retardants

Two different additives were used at three different concentrations as follows:

- Abscisic acid (ABA) at 0, 1, 2 or 3 mg/L.
- Ancymidol (ANC) at 0, 1, 2 or 3 mg/L.

Each treatment was applied to 10 Jars as replicates. After the appropriate concentration was added to the media mentioned above, pH was adjusted to 5.7. Forty ml of medium was dispensed in screw-capped 400 ml. jars and autoclaved at 121 ◦C for 20 min. Four microcuttings (approx. 5 mm) were cultured in each jar. Survival, defined by the presence of intact, green shoot plants, was monitored, and subsequently regeneration was undertaken after 3, 6 and 9 months respectively. The measured regeneration capacity closely resembled the results of the visual survival observations (directly after each of all preservation period and recovery stage).

Genetic stability of *D. indica.*

DNA Extraction from plant leaves.

The leaves were grounded to a fine powder using nitrogen and plant DNA were isolated from seven *D. indica* (Treatments: negative control (original mother plant); positive control (After the propagation at the laboratory under normal growth media); ABA at 1.0, 2.0 and 3.0 mg/l; ANC at 1.0 mg/L and sorbitol 3%). Leaves were used I-Genomic Plant DNA Extraction Mini Kit (INRTON) according to manufacturer's instructions.

Random Amplification Polymorphism DNA-PCR (RAPD) and electrophoresis.

Six random primers were used to differentiate the genetic stability of the isolated DNA from seven *D. indica* samples (treatments gave the highest survival %) which were under our study. Sequences of primers were illustrated in (Table 1). The PCR reaction mixture was accomplished in a final volume of 25µL, consisting of (1 µL of DNA (20 ng); 6.5 µL of Sterile Milli Qwater; 5µL (50 pmol/µL) of each arbitrary primers; 12.5 µL of Master Mix PCR (Applied Biotechnology, Egypt). The applied PCR program was performed as follows: initial denaturation at 95°C for 5 min.; 40 cycles at 94°C for 1 min.; annealing ranged at 28-30°C for 1 min and elongation at 72°C for 1min. A final elongation step at 72°C for 5 min. PCR products were separated on agarose gel 2% (w/v) electrophoresis in 0.5x TBE buffer. The band size was estimated by using DNA molecular weight marker using gel decomentation system.

Table 1. Primers used for RAPD and ISSR analysis

	RAPD		ISSR	
No.	**Primers**	**Sequences 5` --- 3`**	**Primers**	**Sequences 5` --- 3`**
1	RAPD1	AGCCACCGAA	ISSR3	CACCACCACGC
2	RAPD3	ACCGCCGAAG	ISSR4	GTGTGTGTGTGTCG
3	RAPD5	TTCGACCCAG	ISSR5	GTGTGTGTGTGTCC
4	RAPD7	ACCTGAACGG	ISSR6	GAGAGAGAGAGACC
5	RAPD9	TGCCGAGCTG		
6	RAPD10	GAGAGCCAAC		

Inter Sequence Short Repeat (ISSR)

Four primers (obtained from Sigma) of eighteen to twenty nucleotides containing of six to eight fold iterates of short sequence of two nucleotides, and zero to three additional nucleotides, often deteriorated and usually set to the 3' primer. ISSR primers sequences are represented in Table (1) (EL-Bakatoushi and Aseel 2018). PCR programming for ISSR markers was: pre-heat (95°C) for 5 min, followed by 35 cycles of: (94°C; 38°C; 72°C) for one minute each., followed by final extension (72°C 10 min) then, cooling for one hour at 10°C. The amplified products were electrophoresis performed as mentioned above.

Analysis of RAPD and ISSR profiles:

The DNA bands from both RAPD and ISSR markers in the seven samples of *D. indica* were observed and scored as one for present or zero for absent. Cluster analysis was carried out on similarity matrix using the Unweighted Pairgroup Method Arithmetic Average (UPGMA) using NTSYS-PC, version 2.02 (Rohlf, 1998). Polymorphism Information Content (PIC) values were calculated for each RAPD and ISSR primer according to the formula: PIC = PICi = 2 fi (1-fi) given by Roldan-Ruiz *et al*. (2000) where PICi is the polymorphic information content of the marker i, fi is the frequency of the marker bands present and (1-fi) is the frequency of absent marker bands.

Statistical analysis

The collected data were statistically analyzed using analysis of variance method. LSD was used to compare means at 5% probability according to (Steel and Torrei,1980) using MSTAT-C statistical computer package.

RESULTS

Growth behaviors of *Dilleni indica* under minimal growth conditions

Longevity percentage

The results indicated that longevity of *D. indica* was significantly affected after three months without subculturing in all treatments except those cultured on medium containing ABA (at different concentrations used 1,2 and 3 mg/l) and ANC (1 mg/L) (fig. 1and plate 1). While, as time passed on up to six months, survival percentage declined to zero when sorbitol was used at 6 or 9% and ANC at 2.0 or 3.0 mg/L. whereas survival of cultures under control treatment decreased up to 33.33%. With the passage of time up to nine months, cultures of control couldn't survive. Generally, it could be concluded that osmotic treatments using sorbitol have bad effect on *D. indica* while ABA is more favorable for preservation of *D. indica.*

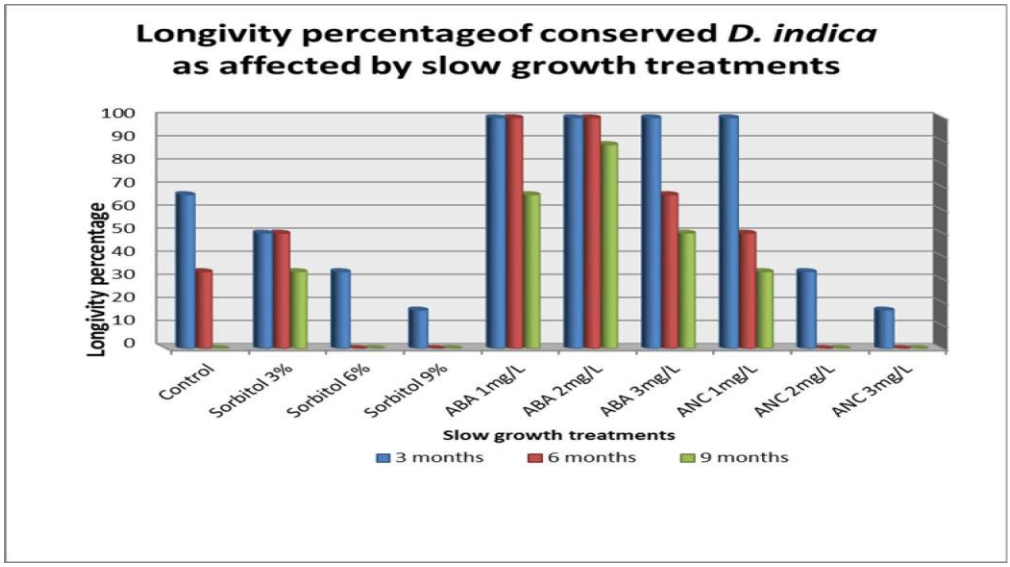

Fig. 1. Longevity percentage of *D. indica* as affected by minimal growth treatments under three conservation preiods .

Shootlets number per explant.

Data in Table (2) showed that shootlets number significantly decreased from 1.8 to 0.73 and insignificantly from 0.73 to 0.52 as the coservation time passed from 3 to 6 and 9 months, respectively. As the effect of conservation treatment played an important role, the highest significant shootlets number (2.28) was recorded for 3% sorbitol, while the lowest significant number of shootlets (0.17) was recorded for 3.0mg/L ANC. Data of interaction effect indicated that, after three months of conservation, medium enriched with 3% sorbitol allowed buds to form shoots at rate of 4.33 per explant (the highest significant shoot number). Whereas with duplicating and triplicating sorbitol added to medium, number of shoots formed per explants significantly decreased to 1 and 0.67, respectively with insignificant differences between them. Using ABA at 2.0 mg/L (which allowed explants to survive at 100% after 6 months and 88.33% after 9 months) minimized the development of buds to form one shoot per explant.

Shootlet length (mm)

As shown in Table (2) shootlet length is significantly affected by minimal growth treatment as well as conservation period. The maximum significant shootlet length (22.56 mm) was recorded for shootlets grown on MS with 2.0 mg/L ABA, while the lowest significant length (1.0mm) was measured for ANC at 3.0 mg/L. Shootlet length was insignificantly increased from 3 to 6 and from 6 to 9 months, while it was insignificantly increased from 3 to 9 months. Medium enriched with ABA at 2.0 mg/L allowed the shootlets to elongate up to 18.33 mm (the highest shootlet length) after three months of conservation. While using sorbitol at 9% reduced the shootlet length

to the lowest value (1.0 mm). It is also noticed that the length of shootlets was reversely related with concentrations of ANC and sorbitol added to the medium. As the time of conservation go on from 6 to 9 months, survived shootlets conserved on medium augmented with ABA at 2.0 or 3.0 mg/L stilled grow up from 15.0mm to 34.33mm and from 14.67 mm to 32.67mm, respectively.

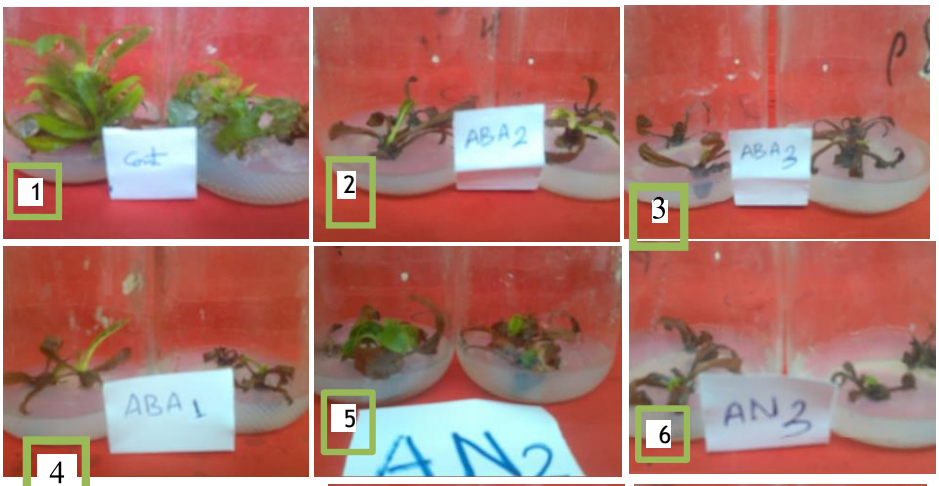

Plate (1) illustrate *D. indica* under minimal growth treatments 1). Regenerated shootlets aGer 3 months under control; 2, 3, 4) under ABA at 1,2,3 mg/L aGer 9 months; 5,6) under ANC at 2,3 mg/L; 7,8) under sorbitol at 6, 9 % aGer 6

Leaves number per shootlet

Data tabulated in Table (2) demonstrated that number of leaves formed per shootlet under minimal growth trail was significantly varied according to the treatments as well as conservation period. The greatest significant number of leaves per shootlet (8.67) was counted for shootlets grown on medium enriched with ANC at 1.0 mg/L after 6 months of conservation. On the other hand, the minute number of leaves per survived shootlet (0.67) was recorded with shootlets were conserved on medium with sorbitol 9% for 3 months.

Table 2. Growth behaviors of *D. indica* explants under minimal growth treatments

Treat. (B)	Growth parameters of viable plants											
	Shootlets number				Shootlet length (mm)				Leaves no./shootet			
	Conservation period (month) (A)											
	3	6	9	**Mean**	3	6	9	**mean**	3	6	9	**mean**
Control	2.67	1.33	0.0	**1.33**	13.9	15.0	0.0	**9.63**	6.5	4.3	0.0	**3.61**
Sorbitol 3%	4.33	1.67	0.83	**2.28**	6.95	15.33	17.67	**13.62**	7.93	7.0	3.9	**6.27**
Sorbitol 6%	1.0	0.0	0.0	**0.33**	4.0	0.0	0.0	**1.33**	2.17	0.0	0.0	**0.72**
Sorbitol 9%	0.67	0.0	0.0	**0.22**	1.0	0.0	0.0	**0.33**	0.67	0.0	0.0	**0.22**
ABA 1mg/L	2.67	1.0	1.17	**1.61**	9.5	20.0	15.0	**14.83**	7.67	8.33	3.67	**6.56**
ABA 2 mg/L	1.5	1.0	1.0	**1.17**	18.33	15.0	34.33	**22.56**	8.0	7.33	5.17	**6.83**
ABA 3 mg/L	2.17	1.33	1.0	**1.5**	15.0	14.67	32.67	**20.78**	7.33	6.67	7.0	**7.0**
ANC 1 mg/L	1.33	1.0	1.17	**1.17**	8.5	16.67	6.33	**10.50**	7.33	8.67	5.33	**7.11**
ANC 2 mg/L	1.17	0.0	0.0	**0.39**	5.5	0.0	0.0	**1.83**	4.67	0.0	0.0	**1.56**
ANC 3 mg/L	0.5	0.0	0.0	**0.17**	3.0	0.0	0.0	**1.0**	2.0	0.0	0.0	**0.67**
Mean(B)	**1.8**	**0.73**	**0.52**		**8.57**	**9.67**	**10.6**		**5.43**	**4.27**	**2.47**	
LSD	A= 0.59 B= 0.32 A×B= 1.02				A= 1.96 B= 1.07 A×B= 3.4				A=1.77, B=0.97 A×B= 3.06			

Root formation

At the end of conservation period (9 months) it is noticed that some of the survived plants formed roots at different ratios according to treatments. The highest significant rooting percentage (66.67%) was recorded for shootlets survived on medium supplemented with ANC at 1.0 mg/L while the lowest significant one (0.0 %) was recorded for ABA at 3.0 mg/L,control,sorbitol(6and9%) and ANC(2 and3) mg/L(Fig. 2).

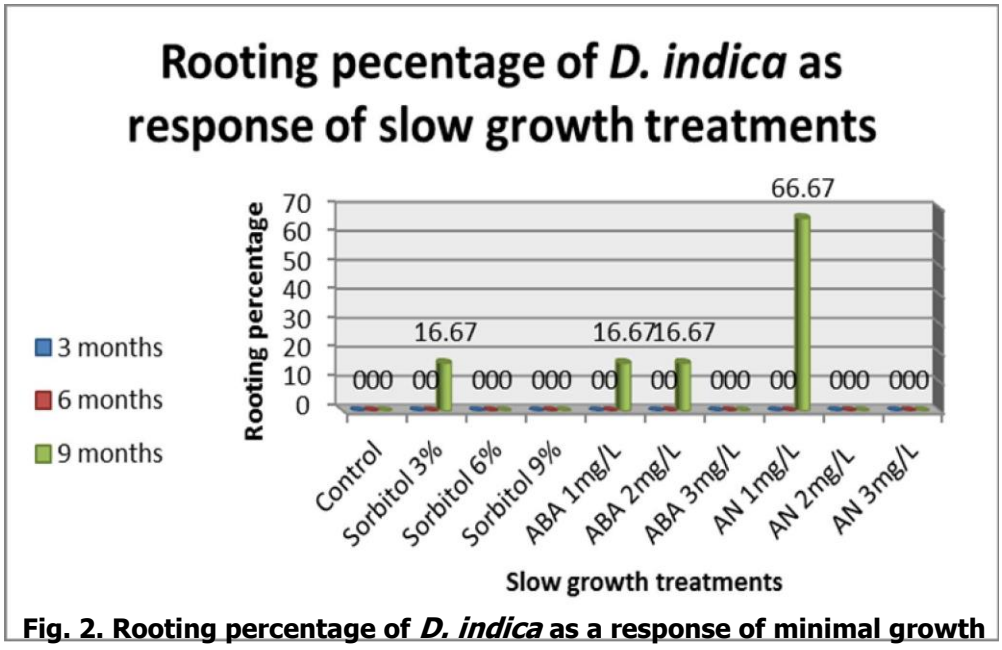

Fig. 2. Rooting percentage of *D. indica* as a response of minimal growth treatments under three conservation period.

Growth behaviors of recovered *D. indica* after minimal growth conditions

Regeneration ability

The results indicated that the survived shootlets differed significantly for the regeneration ability after conservation when transferred into the fresh medium (Fig 3). After three months of conservation, explants derived from control, 3% sorbitol and ABA at 2.0 mg/L treatments exhibited the highest significant shooting ability (100%). While those comes from shootlets conserved on medium supplemented with 6 or 9% sorbitol or 3.0 mg/L ANC couldn't form any shootlets and also couldn't survive (0.0% shootlets formation). With increasing ANC concentration from 1.0 to 2.0 mg/L, the sthe regeneration ability decreased from 100% to 83.33% for control whereas it was increased from 50 to 100% and 66.67 to 100% for

shootlets conserved on medium with 1.0 and 3.0 mg/L ABA, respectively. At the end of conservation period (9 months), the regeneration ability of explants derived from shootlets conserved on medium augmented with 1.0 or 2.0 mg/L ABA is not affected. It also noticed that the regeneration ability of explants derived from medium supplemented with ANC at 1.0 mg/L was not affected by conservation period (stable at 66.67%).

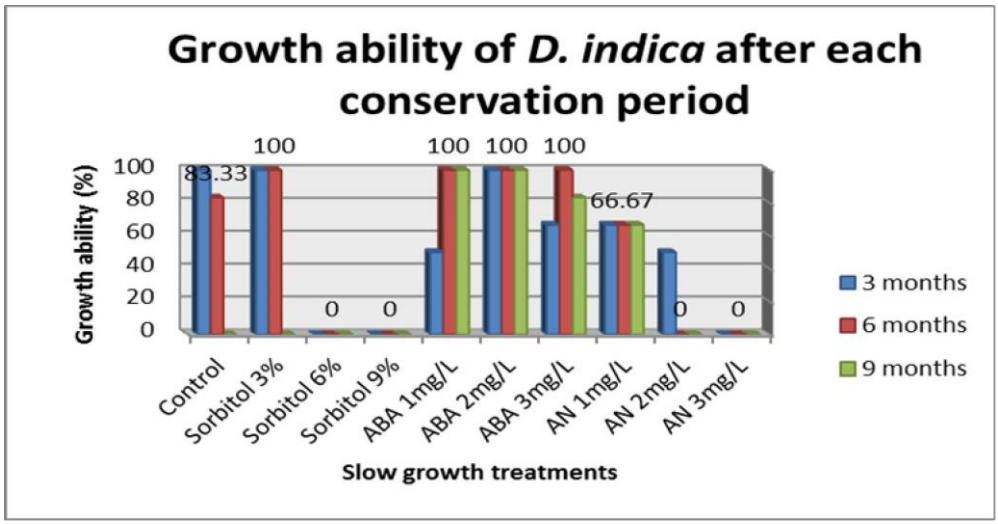

Fig. 3. Regeneration ability of *D. indica* after each conservation period

Shootlets number per explant

As shown in Table (3), there are significant differences among the average shootlet numbers formed per explant after exposing to different minimal growth storage for variable periods. After three months of storage, the highest significant shootlet number (5.17) was counted for shootlets recovered after storage on medium with 3% sorbitol while the lowest significant one (1.17) was recorded for explants derived from medium supplemented with 2.0 mg/L ABA. This means that ABA at 2.0 mg/L has the

physiological power to prevent buds to form shootlets. After six months of storage, the greatest shootlet numbers formed per explat (4.82) was detected for shootlets exposed to 3.0 mg/L ABA. While the lowest number (1.0) was significantly recorded for ANC at 1.0 mg/L. At the end of conservation period (9 months), the maximum number of shootlets formed per explant (4.83) was recorded for the recovered shootlets after conservation on medium with 3.0 mg/L ABA. On the other hand, the lowest one (0.0) was recorded for explants exposed to control, sorbitol (3, 6 and 9 %) and ANC (2 and 3 mg/L).

Shootlet length (mm)

Data in Table (3) revealed that conservation period had no significant effect on length of the formed shootlets, while the conservation treatments significantly affected it. The highest significant shootlet length (9.66 mm) was recorded due to ABA at 2.0 mg/L whereas the lowest significant one (5.11 mm) because of sorbitol at 3.0%. A significant difference was detected between the length of formed shootlets as affected by the interaction between treatments and conservation periods. The highest significant shootlet length (14.0 mm) was measured for regenerated shootlets after conservation on medium with 1.0 mg/L ABA.

Leaves number per shootlet

As shown in Table (3), the highest significant number of leaves (6.67) formed per shootlet was detected for those regenerated shootlets after six months of conservation on medium supplemented with 3.0 mg/L ABA. While the lowest significant number of the formed leaves (3.83) was counted for shootlets conserved for the same period on medium with 1.0 mg/L ABA.

able 3. Growth behaviors of recovered *D. indica* explants after minimal growth treatments.

Treat. (B)	Growth parameters of viable plants											
	Shootlets number				Shootlet length (mm)				Leaves no./shootlet			
	Conservation period (month) (A)											
	3	6	9	**Mean**	3	6	9	**Mean**	3	6	9	**mean**
Control	3.33	1.5	0.0	**1.61**	8.5	7.5	0.0	**5.33**	5.0	4.33	0.0	**3.11**
Sorbitol 3%	5.17	3.83	0.0	**3.0**	6.33	9.0	0.0	**5.11**	5.33	4.67	0.0	**3.33**
Sorbitol 6%	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**
Sorbitol 9%	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**
ABA 1mg/L	4.33	3.83	3.17	**3.78**	5.44	9.0	14.0	**9.48**	4.13	3.83	5.0	**4.33**
ABA 2 mg/L	1.17	3.5	2.83	**2.50**	8.33	8.33	12.33	**9.66**	6.0	4.00	5.83	**5.28**
ABA 3 mg/L	2.0	4.83	4.83	**3.33**	6.17	7.33	10.67	**8.06**	5.33	6.67	6.0	**6.00**
ANC 1 mg/L	3.0	1.0	2.0	**2.0**	6.03	7.17	10.67	**7.96**	6.0	5.50	6.0	**5.67**
ANC 2 mg/L	3.0	0.0	0.0	**1.0**	5.83	0.0	0.0	**7.94**	5.17	0.0	0.0	**1.72**
ANC 3 mg/L	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**
Mean(B)	**2.2**	**1.85**	**1.11**		**4.66**	**4.83**	**4.67**		**3.70**	**2.90**	**2.28**	
LSD	A= 0.49 B= 0.27 A×B= 0.85				A= 1.61 B= 0.88 A×B= 2.79				A= 0.80 B= 0.44 A×B= 1.39			

Genetic stability of *D. indica*

RAPD-PCR

RAPD–PCR was performed using six different arbitrary primers; RAPD1, 3, 5, 7, 9 and 10. All the used primers were succeeded to amplify different DNA patterns (Figure 4 and Table 4). The obtained band patterns have different molecular sizes which ranged from 3k to 100bp. Most of the observed variation was demonstrated with primers RAPD1 and RAPD7. On the other hand, most of the observed bands amplified by the other four primers are monomorphic. It was observed that the numbers of bands amplified by each primer ranged from 25 to 60 bands.

When the dendogram was constructed (Figure 5) based on the obtained banding patterns, results revealed that both negative and positive controls were grouped in separate cluster. Whenever, samples of *D. indica* regenerated from those treated with ABA at 1.0 mg/L and 3.0 mg/L are grouped in one cluster, which indicated that they are much closed to each other. But samples of regenerated shootlets from those conserved using ABA at 2.0 mg/L, ANC at 1.0 mg/L and sorbitol 3% are separated in one other cluster, while, regenerated shootlets produced from explants treated with ANC at 1.0 mg/L and sorbitol 3% are mostly identical but those derived from explants treated with abscisic acid at 2.0 mg/L considered the outer group of the two samples. These results indicated that *D. indica* treated with abscisic acid at 1.0 mg/L identical to that treated with abscisic acid at 3.0 mg/L but *D. indica* treated with ANC at 1.0 mg/L is identical for that treated with sorbitol 3%.

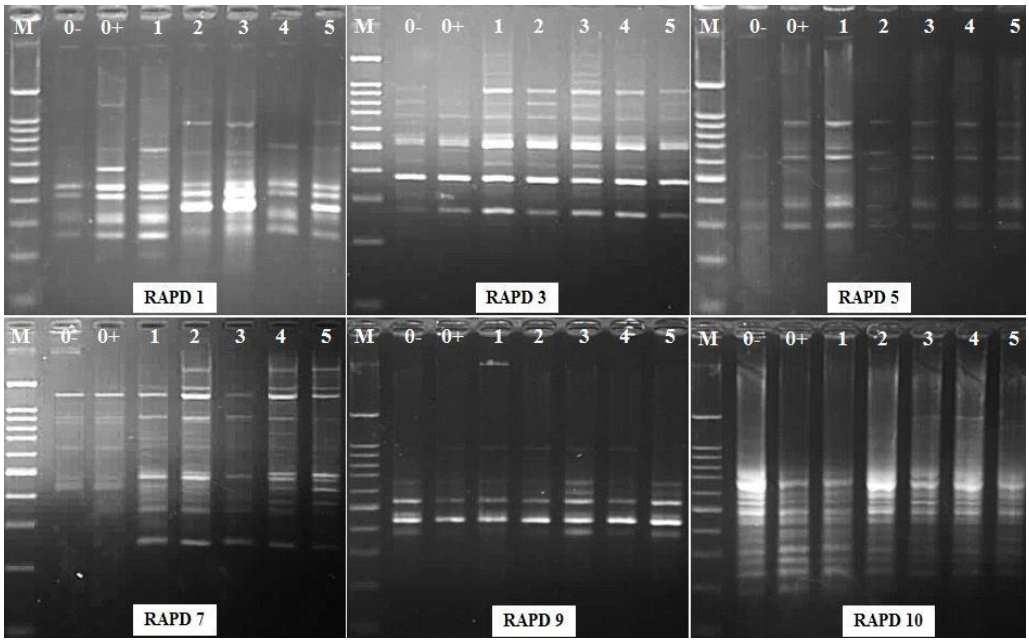

Fig. 4. DNA banding pattern generated by the 6 primers for different treatment of *D. indica*. [Lane, 0- (mother tree); Lane, 0+ (control of micropropagated shootlets); Lane, 1 (ABA at 1.0 mg/L); Lane, 2 (ABA at 2.0 mg/L); Lane, 3 (ABA at 3.0 mg/L); Lane, 4 (AN at 1.0 mg/L) and Lane, 5 (sorbitol 3%), respectively.]

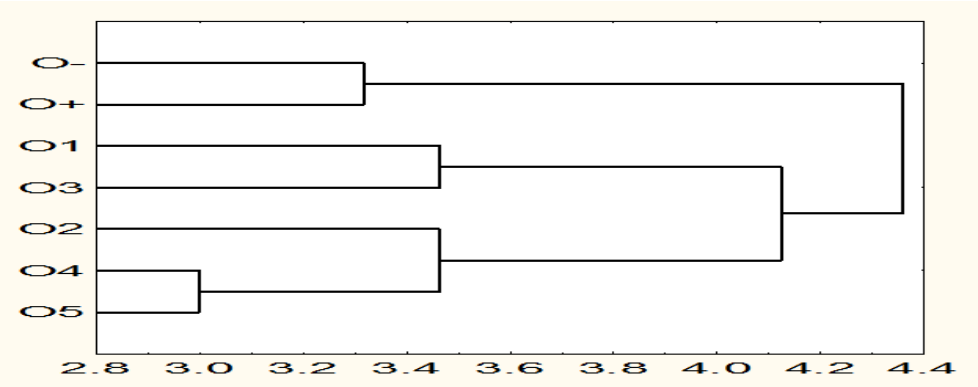

Fig. 5. Dendrogram showing genetic diversity for RAPD markers in *D. indica*.

ISSR-PCR

ISSR-PCR was performed using four different primers and the results presented in figure (6) and Table (4). Data in figure 6 revealed that the four ISSR primers succeeded to amplify different bands with different molecular size. The band size ranged from 1 k to 100 bp. The highest band variation was observed with primers ISSR 4 and 5. The number of polymorphic band is higher than the monomorphic ones. The total number of the obtained bands ranged from 32 to 59 bands.

The constructed dendogram based on the ISSR banding patterns as presented in figure (7) revealed that the examined samples are grouped into two main clusters. Cluster 1 contains the control samples (negative (mother plant) and positive (control micropropagated shootlets). Whenever, all the treated samples are grouped in one cluster and this cluster was divided into three different groups. Groups 1 included samples treated with ABA at 1.0, 3.0 mg/l and ANC at 1.0 mg/L but the other two groups each contain only one sample (either treated with ABA at 2.0 mg/l or sorbitol 3%. It was observed that samples ABA at 1.0 and 3.0 mg/L are very closed to each other.

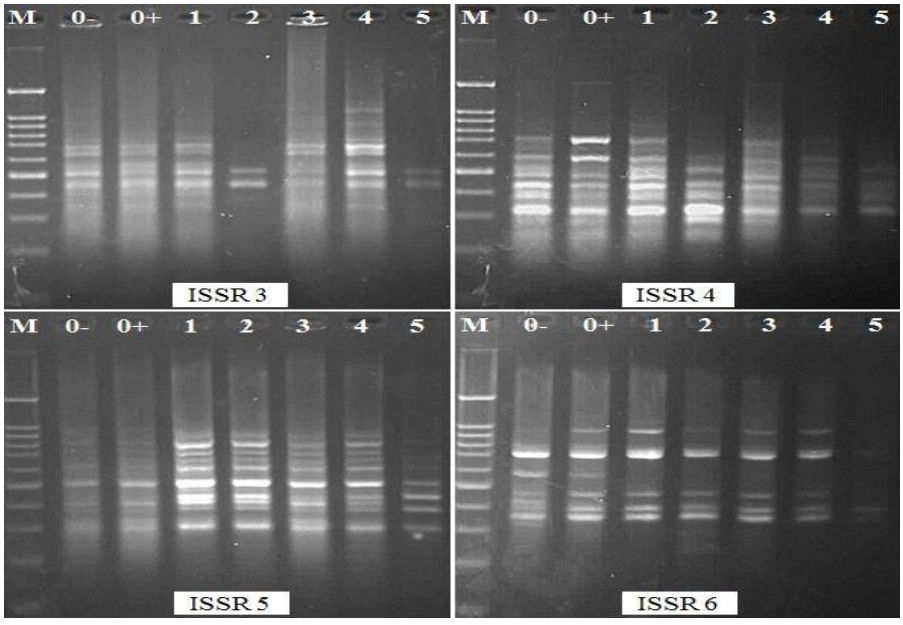

Fig. 6. 2% agarose gel electrophoresis for ISSR-PCR generated by the 4 primers.

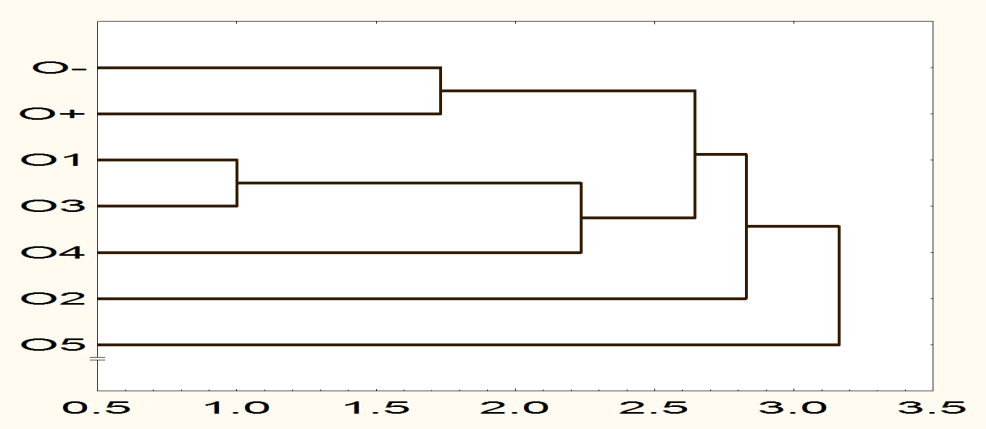

Fig. 7. Dendrogram showing genetic diversity for ISSR markers in *D. indica*

Table 4. Percentage of polymorphism of RAPD and ISSR of DNA fingerprint of *D. indica* under minimal conservation treatment

	No	Primers	Sequences 5` ---- 3`	Total number of bands	Number of monomorphic bands	Number of polymorphic bands	% of polymorphism
RAPD	1	RAPD1	AGCCACCGAA	15	4	11	73.3
	2	RAPD3	ACCGCCGAAG	13	5	8	6.15
	3	RAPD5	AAAGCTGCGG	7	3	4	57
	4	RAPD7	ACCTGAACGG	16	3	13	81.2
	5	RAPD9	TGCCGAGCTG	8	3	5	62.5
	6	RAPD10	GAGAGCCAAC	14	8	6	42.8
	Total		--------	--------	73	26	47
ISSR	1	ISSR3	CACCACCACGC	10	2	8	80
	2	ISSR4	GTGTGTGTGTGTCG	11	3	8	72.7
	3	ISSR5	GTGTGTGTGTGTCC	12	4	8	66.6
	4	ISSR6	GAGAGAGAGAGACC	8	4	4	50
	Total		--------	--------	41	13	28

DISCUSSIONS

The resumed growth of *D. indica* shootlets conserved *in vitro* and the avoidance of genetic variation after long periods in a minimum growth regime are fundamental to the success of *in vitro* conservation. Growth rate of *in vitro* cultures can be minimized and time of subculture expanded by altering the growth enhancers in culture medium. Minimum growth is the most direct way of restricting the growth and development of plantlets *in vitro.* Usually two main strategies were developed for minimal growth, there are removing the growth promoters or adding either an osmotic substance or growth retardant (Lata, *et al.*, 2010). ABA is one of the most important growth retardant substances used for minimal growth conservation. In the present study, using ABA at 2.0 m/L is the most effective treatment which kept the plant life for 9 months at survival rate of 88.33%, and the conserved shootlets had 100% regeneration ability. The important role of ABA is to inhibit auxins, cytokinins, and gibberelins. These hormones enhance growth, cell division and cell elongation in

plants (Swamy and Smith, 1999). ABA had different effects according to the plant under investigation; in this concern Wilkins *et al*. (1988) mentioned that the growth inhibitors were ineffective to inhibit growth for *in vitro* germplasm conservation of woody species. On the other hand, Westcott *et al*. (1977) showed that subculturing interval of microproagated potato could be expanded by using ABA enriched medium. Survival was improved at all concentration levels but the most successful results were observed when 5 and 10 mg /L ABA was added to the medium. These results demonstrated that types and concentrations of growth inhibitors which used to inhibit the growth of cultures depend upon the nature of plant species as well as endogenous levels of growth regulators in explants**.**

Genetic stability is an important requirement for genetic resource conservation. There are several techniques to ensure genetic stability. DNA can be defined by RAPD and ISSR techniques. In this regard, RAPD was used to confirm genetic stability of minimal growth conservation of cassava (Angel *et al.,* 1996). Also Negri *et al*. (2000) stated that using low concentrations of BAP and ABA resulted in the poorest storage of two apple genotypes while moderate concentration of BAP with or without ABA was successful for longer storage. The survival percentage and shoot regeneration ability of cultures stilled less than 30 % in both cases. Using ANC could not save plant life and it was less effective than ABA but it leads to root formation. Our results is in agreement with those found by Mix-Wagner and Eneva (1998) who reported that Ancymidol is a substituted pyrimidine with potent plant growth regulatory property, specifically suppression a series of oxidations in plant tissue. As a growth retardant inhibiting gibberellic acid biosynthesis, it has been shown to promote rooting formation in tissue culture.

It was observed that both RAPD and ISSR–PCR techniques succeeded to differentiate between the treated and non-treated samples in good manner. Not all the used primers gave high different banding patterns, but RAPD1 and RAPD 7 are the best. In case of the ISSR-PCR, ISSR 4 and 5 are the best primers which gave high differentiation between the examined samples. Samples treated with abscisic acid at 1.0 mg/L and abscisic acid at 3.0 mg/L are much closed to each other's when tested by RAPD and ISSR. These results confirm that the effect of the 3 mg/L of absaicic acid equal to the 1 mg/L. The highest effect of the absaicic acid was obtained with concentration 2 mg. On the other hand, the effect of Ancymidol at conc. 5 mg/L and Sorbitol 3% is equal. The main advantage associated with slow growth conservation is that slow growth did not require frequent subculture. After prolonged period of slow growth conservation, the culture can easily be retrieved for production of new plants with full genetic integrity (Ahmed *et. al*., 2010).

Generally, it can be recommended that conservation of *D. indica* shootlets up to 9 months was achieved when adding of ABA at 1 or 3 mg/L to conservation medium with maintaining the genetic stability of shootlets with optimum survival (100%) and enhancement in recovery characters.

REFERENCES

1. Abd El-Kadder, E. M. and H.H. Hammad, H. H. 2012. *In Vitro* Propagation of *Dillenia indica*. Australian Journal of Basic and Applied Sciences, 6(7): 452-457.
2. Abd El–Kader, E. M. and Zeinab T. Abd EL Shakour. 2015. Phytochemical and cytotoxicity investigation of *Dillenia idica* grown in Egypt. World Journal of Pharmaceutical Research, 4 (10): 334 – 348.
3. Ahmed, M. M.; Anjum, A.; Shah, A.H. and Hamid, A. 2010. In vitro preservation of Pyrus germplasm with minimal growth using different temperature regimes. Pak. J. Bot. 42(3): 1639-1650.
4. Angel, F.; Barng, V.F.; Tohm, J. and Roca, W.M. 1996. Stability of Cassava plants at the DNA level after retrial from 10 years of *in vitro* storage. Euphytica, 90: 307-313.
5. Damle, A.A.; Pawar, Y.P. and Narkar, A.A. 2013. Anticancer activity of betulinic acid on MCF-7 tumors in nude mice. Indian J. Exp. Biol., 51(7):485-91.
6. EL-Bakatoushi, R. and Aseel, D. G. 2018. Evaluation of genetic diversity in wild populations of *Peganum harmala* L.,a medicinal plant. Journal of Genetic Engineering and Biotechnology, 16 (1):143 – 151.
7. Engelmann, F. 1991. *In vitro* conservation of tropical plant germplasm - a review. *Euphytica* 57: 227-243.
8. Janick, J. and Paull, R. E. 2008. The Encyclopedia of Fruit and Nuts. 1st ed. London: CABI.
9. Lata, H.; Moraes, R.M.; Bertoni, B. and Pereira, A.M.S. 2010. *In vitro* germplasm conservation of Podophyllum peltatum L. under slow growth conditions. In Vitro Cellular and Developmental Biology-Plant, 46: 22-27.
10. Mix-Wagner, G. and Eneva, T. 1998. Plant regeneration from *Cichorium intybus* L. var. sativum leaf midrib explants induced by ancymidol supplemented culture medium. Landbauforschung Völkenrode 48: 53–55.
11. Manoj, K. Rai; Shekhowat N.S.; Harich;Amit Gupta K.; Phulworia M. ; Kheta R. and Jaiswal U. 2011. The role of abscisic acid in plant tissue culture: a review of recent progress. Plant Cell Tiss. Organ Cult. 106: 179-190.
12. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

13. Negri, V.; Tosti, N. and Standardi, A. 2000. Slow growth storage of single node shoots of apple genotypes. Plant Cell, Tissue and Organ Culture, 62: 159-162.
14. Pospisilova, J.; Haisel D; Synkova H.; Batkova Spoustova P. 2009. Improvement of ex vitro tranfer of tobacco plentlets by addition of abscisic acid to the last subculture. Biol. Plant, 53: 617-624.
15. Rademacher, W. 2000. Growth retardants: Effects on Gibberellin Biosynthesis and Other Metabolic Pathways. Annu. Rev. Plant Physiol. Plant Mol. Biol., 51:501–31.
16. Rohlf, F. J. 1998. NTSYS-PC. Numerical taxonomy and multivariate analysis system, version 2.00. Exeter Software, Setauket, NY.
17. Roldan-Ruize, I. E.; Calsyn, T.J.; Gilliand, R.; Coll, M.J.T.; Vaneijk, De and Loose, M. 2000. Estimating genetic conformity between related ryegrass (Lolium) varieties. 2. AFLP characterization. Mol. Breed., 6: 593-602.
18. Sherald, J. L.; Ragsdale, N. N. and Sisler, H. D. 1973. Similarities between the systemic fungicides triforine and triarimol. Pestic. Sci. 4: 719-727.
19. Steel,R.G.D. and Torrie S.H. 1980. Principle of Statistics. A biometrical Approach Second Ed.,McGraw-Hill,U.S.A.
20. Swamy, P.M. and Smith, B.N. 1999. Role of abscisic acid in plant stress tolerance. Current science 76(9):1220-1227.
21. Westcott, R.J.; Henshaw, G.G. and Roca, W.M. 1977. Tissue culture storage of potato germplasm: Culture initiation and plant regeneration. Plant Sci. Lett. 9: 309-315.
22. Wilkins, C.P.; Dodds, J.H. and Newbury, H.J. 1988. Tissue culture conservation of fruit trees. FAO/Intl. Board Plant Genetic Resour. Newslett. 73/74: 9-20.

الحفظ المعملي للدلينيا انديكا الوحيدة النامية في مصر تحت ظروف الحد الأدنى من النمو

عصام مصطفى عبد القادر[1]، هويدا محمد فتحي[1]، داليا جميل أصيل[2]

[1] قسم بحوث الأشجار الخشبية- معهد بحوث البساتين – مركز البحوث الزراعية – جمهورية مصر العربية
[2] قسم وقاية النبات و التشخيص البيوجزيئي – معهد بحوث زراعة الأراضي القاحلة – مدينة الأبحاث العلمية و التطبيقات التكنولوجية – برج العرب -*21934*- مصر

دلينيا انديكا شجرة فريدة في مصر لها أهمية طبية كبيرة (مصدر جيد لحمض البتيولنك). أجريت تجربة بهدف وضع بروتوكول آمن للحفظ المعملي تحت ظروف النمو الدنيا باستخدام الأسموزية و مثبطات النمو. أوضحت النتائج أن استخدام حمض الأبسيسك بتركيز 1، 2 أو 3 ملجم/لتر أمكن حفظ حياة النبات بنسبة 66.67 و 83.33 و 50% على التوالي لمدة تسعة أشهر بدون اجراء نقل للنباتات على بيئات جديدة. على الجانب الآخر، كان للسوربيتول (مادة أسموزية) تأثير سيئ خاصة عند استخدام تركيزات مرتفعة (6 و 9%) حيث أدى الى 100% موت بعد مرور ستة أشهر. أشارت النتائج الى اختلاف النباتات المحفوظة في قدرتها على استعادة انقسامها بعد نقلها على بيئة جديدة. بعد انتهاء مدة الحفظ (9 أشهر) سجلت المنفصلات النباتية التي مصدرها أفرع حفظت على بيئة مضافا اليها 1 أو 2 مليجرام/لتر حمض أبسيسك 100% قدرة على استعادة الانقسام. باجراء الدندوجرام المبني على نتائج البصمة الوراثية أوضحت النتائج ارتباط نبات الأم و النبات المكثر معمليا في مجموعة واحدة و ارتباط عينات النباتات المحفوظة على بيئة مضافا اليها 1 و 3 مليجرام/لتر حمض أبسيسك مما يدل على ارتفاع درجة الارتباط بينهما كما يدل على أن استعمال حمض الابسيسك آمن على التركيب الوراثي.