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In vitro Multiple Shoot Induction under Micropropagation Condition of *Cocculus laurifolius* (L.)

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ABSTRACT

In vitro trials were intended to find out a well protocol for micropropagation of *Cocculus laurifolius* plant. Shoots tips were effectively surface sterilized with 1.5 % NaOCl for 20 min which gave the best percentage of survival explant and free contamination. Shoot tip explants of the plants cultured on MS medium at full strength with activated charcoal to give the highest shoot length and number of leaves. For shoot formation, BA was better than Kin to produce the highest number of shoots/shoot. For rooting stage, 2.0 mg/l NAA was better than IBA to establish rooted shoots on full MS medium. Plantlets grew with a healthy appearance and the best percentage of acclimatization (81.67%) was observed when plantlets cultured in a peatmoss under plastic tunnel under plastic house conditions. RAPD-PCR (Randomly Amplified Polymorphic DNA) results confirmed the role of fragments polymorphism of micropropagated plants as compared to mother plants (*in vivo*) when using different growth regulators micropropagated Cocculus plants during tissue culture technique.

Keywords: Micropropagation, In vitro, Cocculus, Shoots tips, RAPD-PCR

1. Introduction

Cocculus laurifolius belongs to family Menispermaceae (Siddiqi, 1974). Plants are evergreen tree. It is native of China and Taiwan (Jain, *et al*., 2009). The plants contain many phytochemical compounds (Ajaib, *et al.*, 2017).

Cocculus hirsutus is antipyretic, lessens thirst, good for fractures, and useful in tubercular glands. It has a detoxifier. It is an aphrodisiac (Chatterjee, 1996). Roots were used in the treatment of some diseases (Chopra, *et al*., 1996).

Aqueous extracts for *Cocculus hirsutus* having new anti-TB agents (Jethva, *et al*., 2020). The studies confirm the presence of alkaloids as a inhibitors for the treatment of Alzheimer's disease (Thavamani, *et al*., 2013). The leaves contained alkaloids, flavonoids and glycosides (Rasheed *et al*., 1991). Isolated cohirsinine, hirsutine, jamtinine, jamitine- N - oxide, cohirsine, cohirsitine, haiderine, bis-benzyl isoquinoline and alkaloids were from stem and roots (Viquaruddin *et al*., 1991). *Cocculus hirsutus* has extremely in delaying the ejaculation, diuretic and laxative (Ganapathy, *et al*., 2002). Juice of the ripe fruits has hypotensive, cardiotonic and spasmolytic (Marya and Bothara, 2011).

Shoot tips 5 cm long of Gardenia plants were removed and sterilised by soaking in 20% bleach solution and then washed in sterile water (Minas, 2007). Multiplication of *Cocculus hirsutus* was achieved with axillary buds. Proliferation shoots were induced on media containing 0.5 mg/l BA. Shoots were rooted on quarter medium strength containing 0.5 mg/l IBA. The rooted plantlets were acclimatized in growth chamber (Meena, *et al*., 2012). Shoots were planted on media containing 2 mg/l BAP plus 0.5 mg/l NAA gave the highest shoot number of Gardenia (Mir, *et al*., 2009).

Cytokinins are used to induce cell division and induce of axillary buds. Naturally cytokinins which included zeatin or 2iP and the synthetic cytokinins that included benzyl adenine and kinetin (El-Shamy *et al*., 2009).

This study was executed to obtain the best protocol for micropropagation of *Cocculus laurifolius* and genetic fidelity. No research related to laboratory propagation of the plant under study was realized and exist so far and the research will be used as a guide.

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2. Materials and Methods

The experiments were carried out in the laboratory of Tissue Culture and RAPD-PCR in the Biotechnology Laboratory at Horticulture Research Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. Experiments were carried out during the period from 2022 to 2023.

Cocculus laurifolius plant is a rare and recently plant in Egypt. The mother plant was growing in Orman Botanical Garden originally from exchanging seeds between botanical gardens of different countries. Shoot tips and axilliary buds were used (0.5 cm) as explants. The culture media were MS at different strengths.

2.1. Surface Sterilization of Explants

Shoot tips of *Cocculus laurifolius* were excised then washed by soapy water for thirty minutes after that washed by tap water for 1h. Shoot tips were sterilized by sodium hypochlorite (NaOCl) solution (Clorox) at 0.50, 1.00 and 1.50 % NaOCl plus 6 drops of Tween 80 for 10, 20 and 30 minutes. Then, Shoot tips were washed 6 times with sterile water. The collected data were survival (decontamination) percentage, contamination percentage and mortality percentage.

2.2. Culture Media:

MS medium was solidified with 6.0 g/l agar. Sucrose was added at 30.0 g/l as a source of carbohydrate. The pH was set at 5.7. Media were poured fifty ml in 350 ml jars and sterilized by autoclave at 121°C for 20 min.

2.3. At the establishment stage

Explants were cultured under sterile conditions in 350 ml jars containing salt strength media at full, three quarter, half and quarter contained activated charcoal (AC) at 0.0 or 1.0 g/l and their combination were used for investigation the explants. After four weeks, the shoot length (cm) and number of leaves were recorded.

2.4. At the multiplication stage

For the multiplication stage, treatments were used with BA at 0.0, 0.5, 1.0, 2.0 or 4.0 mg/l and NAA at 0.0, o.25, 0.5, 1.0 or 2.0 mg/l. Also, Kin at 0.0, 0.5, 1.0, 2.0 or 4.0 mg/l and NAA at 0.0, o.25, 0.5, 1.0 or 2.0 mg/l were used for the same stage. These treatments were repeated three times by reculturing on the same media with the same clusters. After three subcultures mean shoot number/shoot, shoot length (cm), leaf number, and callus formation (as scores) were calculated.

2.5. At the rooting stage

For rooting stage, NAA or IBA were used at 0.0, 0.5, 1.0, 2.0 and 4.0 mg/l. After one month, recorded data were mean root number/shoot, root length (cm), shoot length (cm) and leaf number.

2.6. At the acclimatization stage

Singly plantlets were cultured into 6 cm plastic pots filled with culturing mixtures at 1:0:0, 0:1:0, 0:0:1, 1:1:0, 1:0:1 and 0:1:1 (v/v) peatmoss, sand and perlite under plastic tunnel at plastic house condition. The plastic tunnel was removed gradually to adapt the plantlets.

2.7. Experimental design and statistical analysis

A complete randomized design was carried with analysis of variance to show statistical differences between treatments using the L.S.D. at probability level (5%) (Snedecor and Cochran, 1989).

2.8. Randomly amplified polymorphic DNA (RAPD)

Plant tissues were ground using liquid nitrogen to a fine powder, followed by DNA extraction was performed using DNeasy plant Mini Kit (QIAGEN). Polymerase Chain Reaction (PCR) amplification was performed using six random 10 mer arbitrary primers (synthesized by Operon biotechnologies, Inc. Germany) with the following sequences: A2 = (5'-TGCCGAGCTG-3'), A5 = (5'-AGGGGTCTTG-3'), A10 = (5'-GTGATCGCAG-3'), B18 = (5'-CCACAGCAGT-3'), C19 = (5'-GTTGCCAGCC-3'), D18 = (5'-GAGAGCCAAC-3').

Amplification reaction has been done in volume of 25 µL containing 2.5 µL of dNTPs (2.5 mM), 2.5 µL $MgCl_2$ (25 mM), 2.5 µL of 10x buffer, 2.5 µL of primer (10 pmol), 2.5 µL of template DNA (25 ng µL-1), 1.0 µL of Taq polymerase (1U µL-1) and 11.5 µL of sterile dd H_2O. The DNA amplifications has been done in an automated thermal cycle (T100 Thermal Cycler, Bio-Rad) programmed for one cycle at 94°C for 4 min for initial denaturizing, followed by 45 cycles for 1 min at 94°C (denaturation), 1 min at 36°C (annealing) and 2 min at 72°C (extension), then 10 min at 72°C for final extension (Rajapakse *et al*., 1995). The reaction was finally stored at 4°C. Amplified products were size-fractioned (using 50 bp ladder marker) by electrophoresis in 1.5% agarose gel.

3. Results and Discussion

3.1. Effect of sodium hypochlorite and soaking periods on surface sterilization of shoot tips

Data in Table (1) demonstrated that, increasing sodium hypochlorite concentrations led to increased survival percentage and mortality percentage of explant and decreased percentage of contaminated explant. The highest percentage of survival (76.67 %) was observed using 1.5 % NaOCl. 1.5 % NaOCl which gave 16.67 mortality percentage and 6.67 contamination percentage.

For soaking period, increasing the soaking period of immersed explant increased the survival percentage. The highest survival percentage (73.33 %) was calculated when the explants were immersed for 30 min. Soaking period for 30 min gave 13.33 mortality and contamination percentage.

For the interaction between NaOCl and soaking period, the best percentage of explant survival and free contamination of explants (90.0 and 00.0%, respectively) were recorded when explants were immersed in 1.5 % NaOCl for 20 min. 1.5 % NaOCl for 20 min gave 10 % mortality percentage.
Results obtained here are in harmony with those obtained elsewhere when 30% Clorox for 25 min for explant sterilization which gave the best result with *Gardenia jasminoides* (Arafa *et al.,* 2013).

Table 1: Effect of NaOCl percentage and soaking periods (min) on survival (%), contamination (%) and mortality (%) of *Cocculus laurifolius* sterilization.

NaOCl (%)	Soaking Periods (min)											
	10	20	30	Mean (A)	10	20	30	Mean (A)	10	20	30	Mean (A)
	Survival (%)				Contamination (%)				Mortality (%)			
0.50	40.00	50.00	60.00	50.00	60.00	50.00	30.00	46.67	0.00	0.00	10.00	3.33
1.00	60.00	70.00	80.00	70.00	30.00	20.00	10.00	20.00	10.00	10.00	10.00	10.00
1.50	60.00	90.00	80.67	76.67	20.00	0.00	0.00	6.67	20.00	10.00	20.00	16.67
Mean (B)	53.33	70.00	73.33		36.67	23.33	13.33		10.00	6.67	13.33	
LSD $_{0.05}$ for												
NaOCl , % (A)				14.27				15.29				9.99
Periods, min (B)				14.27				15.29				9.99
(AxB)				24.71				26.49				17.30

3.2. Effect of MS medium strength and activated charcoal on establishment stage

Data recorded in Table (2) and Figure (1a) show that the tallest shoots and highest number of leaves were recorded at MS medium at full strength with activated charcoal (2.67 cm and 4.67 leaves, respectively). There were significant differences in shoot length and number of leaves between the different treatments.

All salt strengths of MS medium when activated charcoal was used resulted in significantly highest shoot length and number of leaves when compared to medium without activated charcoal.
Results obtained here are in harmony with those obtained elsewhere when medium at full strength was used on its own with *Agapanthus africanus* (Hassan, 2012).

Table 2: Effect of MS medium strength and activated charcoal on mean of shoot length (cm) and number of leaves at *Cocculus laurifolius* establishment.

MS medium	Shoot length (cm)	No. of leaves
Full	1.00	3.00
3/4	1.00	2.67
1/2	1.00	2.33
1/4	1.00	2.00
Full + AC	2.67	4.67
3/4 + AC	1.83	3.67
1/2 + AC	1.67	2.66
1/4 + AC	1.00	2.33
LSD $_{0.05}$	0.46	0.93

3.3. Effect of BA and NAA on multiplication stage

Results in Table (3) and Figure (1b) recorded that BA at different concentrations increased shoot number/shoot, shoot length (cm) and leaf number.

Table 3: Effect of BA and NAA on mean shoot number/shoot, shoot length (cm), leaf number and callus formation of *Cocculus laurifolius* multiplication.

BA (mg/l)	NAA (mg/l)											
	0.00	**0.25**	**0.50**	**1.00**	**2.00**	**Mean (A)**	**0.00**	**0.25**	**0.50**	**1.00**	**2.00**	**Mean (A)**
	Shoot number/shoot						**Shoot length (cm)**					
0.00	1.00	1.00	1.33	1.00	1.00	**1.07**	2.17	2.17	2.00	1.83	1.67	**1.97**
0.50	5.00	4.33	4.33	4.00	1.00	**3.73**	3.33	3.17	2.33	2.17	1.67	**2.53**
1.00	7.67	5.67	4.67	4.33	1.33	**4.73**	4.67	4.33	3.00	2.33	1.50	**3.17**
2.00	6.33	5.33	5.00	2.67	2.33	**4.33**	4.50	3.33	2.33	2.17	1.33	**2.73**
4.00	6.00	5.67	3.00	3.33	3.00	**4.53**	2.83	2.33	2.00	1.50	1.67	**1.97**
Mean (B)	**5.20**	**4.40**	**4.00**	**3.07**	**1.73**		**3.50**	**3.07**	**2.33**	**2.00**	**1.47**	
LSD$_{0.05}$ for												
BA (A)	0.26						0.18					
NAA (B)	0.26						0.18					
(A×B)	0.59						0.39					

Table 3: Continued

BA (mg/l)	NAA (mg/l)											
	0.00	**0.25**	**0.50**	**1.00**	**2.00**	**Mean (A)**	**0.00**	**0.25**	**0.50**	**1.00**	**2.00**	**Mean (A)**
	Leaf number						**Callus formation (as scores)**					
0.00	8.00	7.67	7.33	7.00	7.00	**7.40**	0.00	0.50	1.50	2.00	3.0	**1.40**
0.50	8.67	8.33	7.67	7.00	6.33	**7.60**	0.00	0.00	1.00	1.75	2.5	**1.05**
1.00	10.67	8.67	7.00	6.67	5.00	**7.60**	0.00	0.00	1.25	2.25	2.00	**1.10**
2.00	9.67	7.67	6.67	6.33	4.67	**7.00**	0.00	1.25	1.50	2.00	2.25	**1.40**
4.00	9.33	8.00	6.00	5.67	4.33	**6.67**	0.00	0.75	1.50	1.25	1.00	**0.90**
Mean (B)	**9.27**	**8.07**	**6.93**	**6.53**	**5.47**		**0.00**	**0.50**	**1.35**	**1.85**	**2.63**	
LSD$_{0.05}$ for												
BA (A)	0.31						0.24					
NAA (B)	0.31						0.24					
(A×B)	0.70						0.53					

Footnote: Results for callus formation were calculated visually as scores (according to Pottino, 1981) Negative (-) =1, Below average (+) =2, average (++) =3, Good (+++)= 4

The highest mean shoot number/shoot, shoot length (cm) and leaf number were 4.73 shoots, 3.17 cm and 7.60 leaves, respectively at 1.0 mg/l BA. While, the best callus formation (1.40) was observed on a medium contained 0.0 mg/l BA.

For the different concentrations of NAA, mean shoot number/shoot, shoot length (cm) and leaf number were decreased by increasing the level of NAA. The highest shoot number/shoot, shoot length (cm) and leaf number were recorded (5.20 shoots, 3.50 cm and 9.27 leaves, respectively) at 0.0 mg/l NAA. The best callus formation (2.63) was recorded on medium contained NAA at 2.0 mg/l.

Regarding the interaction between BA and NAA, the shoots which were cultured on medium contained 1.0 mg/l BA gave the highest mean shoot number /shoot, shoot length (cm) and leaf number (7.67 shoots, 4.67 cm and 10.67 leaves, respectively). While, the best callus formation (3.0) was observed on medium contained 2.0 mg/l NAA.

On the other plant, elsewhere shoot length of *Liquidambar* plant was decreased by increasing of BA concentration (El-Shamy, *et al*., 2009).

3.4. Effect of Kin and TDZ on multiplication stage

For Kin concentrations, data calculated in Table (4) show that Kin concentrations induced the mean shoot number/shoot, shoot length (cm) and leaf number. The highest mean shoot number/shoot, shoot length (cm) and leaf number (5.60 shoots, 2.67 cm and 8.40 leaves, respectively) were calculated with Kin at 2.0 mg/l.

Also, results indicated that mean shoot number/shoot, shoot length (cm) and leaf number were decreased due to TDZ. Treatments showed that the shoots were tallest and highest leaf number (3.01 cm, 8.33 leaves, respectively) at 0.25 mg/l TDZ. The highest mean shoot number/shoot was calculated on a control treatment without TDZ.

Similarly, 2.0 mg/l Kin and 0.25 mg/l TDZ recorded the highest mean shoot number/shoot, shoot length (cm) and leaf number (7.67 shoots, 4.50 cm and 10.00 leaves, respectively).

So in general, for the sake of shoot formation, BA at concentrations utilized was better than Kin concentrations.

Table 4: Effect of Kin and TDZ on mean shoot number/shoot, shoot length (cm) and leaf number of *Cocculus laurifolius* multiplication.

Kin (mg/l)	TDZ (mg/l)																	
	0.00	**0.25**	**0.50**	**1.00**	**2.00**	**Mean (A)**	**0.00**	**0.25**	**0.50**	**1.00**	**2.00**	**Mean (A)**	**0.00**	**0.25**	**0. 50**	**1.00**	**2.00**	**Mean (A)**
	Shoot number/shoot						**Shoot length (cm)**						**Leaf number**					
0.00	1.00	2.33	2.67	3.00	3.33	**2.47**	2.17	2.17	2.00	1.83	1.67	**1.97**	8.00	8.33	8.33	8.00	5.67	**7.67**
0.50	1.67	2.00	3.33	4.00	3.67	**2.93**	2.50	2.33	1.83	1.67	1.50	**1.97**	8.67	7.67	8.00	6.33	5.33	**7.20**
1.00	2.33	2.33	2.67	3.00	3.67	**2.80**	2.50	2.37	1.67	1.50	1.33	**1.87**	9.00	7.33	6.67	5.67	5.00	**6.73**
2.00	3.00	7.67	7.33	5.33	4.67	**5.60**	2.67	4.50	3.33	1.70	1.17	**2.67**	9.33	10.00	9.67	6.67	6.33	**8.40**
4.00	3.33	6.33	5.33	4.33	4.67	**4.80**	3.00	3.67	3.17	1.60	1.00	**2.48**	6.00	8.33	7.00	6.67	6.67	**6.67**
Mean (B)	**5.20**	**4.40**	**4.00**	**3.07**	**1.73**		**2.57**	**3.01**	**2.40**	**1.66**	**1.33**		**8.20**	**8.33**	**7.93**	**6.60**	**5.80**	
$LSD_{0.05}$ for																		
BA (A)				0.26					0.21			0.32						
NAA (B)				0.26					0.21			0.32						
(A×B)				0.57					0.48			0.70						

3.5. Effect of different concentrations of IBA on rooting stage

For IBA concentrations, results represented in Table (5) and Figure (1c) show that there were increases in mean root number/shoot, root length (cm), shoot length (cm) and leaf number from increasing IBA concentrations from zero to 2.0 mg/l. Results showed that the highest mean root number/shoot, root length (cm), shoot length (cm) and leaf number (3.33 roots, 7.67 cm, 10.00 cm and 8.00 leaves, respectively) were obtained at 2 mg/l IBA when compared to most IBA concentrations.

Table 5: Effect of IBA at different concentrations on mean number of roots/shoot, root length (cm), shoot length (cm) and number of leaves of *Cocculus laurifolius* rooting.

IBA (mg/l)	Root number /shoot	Root length (cm)	Shoot length (cm)	Leaf number
0.00	2.33	4.33	2.67	5.33
0.50	2.67	4.33	3.00	6.33
1.00	3.00	5.33	5.67	7.33
2.00	3.33	7.67	10.00	8.00
4.00	3.00	6.33	6.67	7.67
LSD $_{0.05}$	0.91	0.36	1.11	1.06

3.6. Effect of NAA concentrations on rooting

For NAA concentrations, data in Table (6), showed that the best NAA concentration for rooting was 2.0 mg/l. The best mean root number/shoot, root length (cm), shoot length (cm) and leaf number was 3.67 roots, 6.67 cm, 6.33 cm and 7.67 leaves, respectively.

NAA was still preferable on because it gave higher root number/shoot with desirable features which was suitable for the rooting stage.

Earlier elsewhere, IBA gave the best mean number of root/shoot and root length of Gerbera when compared to NAA (El-Shamy, *et al*., 2009).

Table 6: Effect of NAA at different concentrations on mean number of roots/shoot, root length (cm), shoot length (cm) and number of leaves of *Cocculus laurifolius* rooting.

NAA (mg/l)	Root number /shoot	Root length (cm)	Shoot length (cm)	Leaf number
0.00	2.33	4.33	2.67	5.33
0.50	2.33	5.00	5.33	6.67
1.00	3.00	5.67	5.67	7.00
2.00	3.67	6.67	6.33	7.67
4.00	1.33	3.33	5.00	5.33
LSD $_{0.05}$	0.97	0.92	1.09	1.44

3.7. Effect of different mixtures of peatmoss, sand and perlite on acclimatization stage

Data recorded in Table (7) and Figure (1d) demonstrate that the plantlets grew with a healthy appearance. A high percentage of acclimatization (%), plantlet length (cm) and number of leaves was recorded by transplanting of plantlets in pots containing peatmoss alone. The best percentage of acclimatization (%), plantlet length (cm) and number of leaves (81.67%, 7.00 cm and 8.33 leaves) was observed when plantlets were cultured in a peatmoss medium. After four weeks, no abnormalities in physical appearance and growth habits were observed on the transplanted plantlets.

Table 7: Effect of peatmoss, sand and perlite on acclimatization percentage (%), plantlet length (cm) and number of leaves during acclimatization stage of *Cocculus laurifolius.*

Peatmoss	Sand	Perlite	Acclimatization (%)	Plantlet length (cm)	Number of leaves
1	0	0	81.67	7.00	8.33
0	1	0	0.00	0.00	0.00
0	0	1	0.00	0.00	0.00
1	1	0	56.67	5.33	5.33
1	0	1	75.00	5.83	7.67
0	1	1	0.00	0.00	0.00
LSD $_{0.05}$			5.16	0.61	0.79

A B C D

Fig. 1: *In vitro* micropropagation of *Cocculus laurifolius* plant
a. Establishment (with and without activated charcoal).
b. Multiplication.
c. *In vitro* rooting (with activated charcoal).
d. Acclimatization.

3.8. Effect of growth regulators on shoots RAPD analysis of *in vitro Cocculus laurifolius* plants.

True-to-type clonal fidelity is one of the most important required in micropropagation. A major problem in vitro culture is the presence of somaclonal variation amongst of one parental line, arising for micropropagation culture. RAPD fingerprints (Randomly Amplified Polymorphic DNA) was adopted for evaluating the variation between the mother plant and that obtained from plant treated with free growth regulators, 4 mg/l BA and 2 mg/l NAA or 2 mg/l TDZ and 4 mg/l Kin. Six RAPD primers (A2, A5, A10, B18, C19 and D18) were used in this study, the primers produced good reproducible and scorable patterns and the amplification profiles were screened among the mother plants and micropropagated plants of *Cocculus laurifolius* (Figure 2).

All primers showed polymorphic bands obtained between the primers except for primers A10 and D18 showed monomorphic bands of all fragments.

Primer A2 showed a number of total bands are eleven ranging between 292.5 to 1932.3 bp, only two polymorphic bands were detected, were one was unique band for 4 mg/l BA and 2 mg/l NAA representing polymorphic percentage of 18.18 %, whereas the other nine fragments were monomorphic, since they were seen in all plants.

The results of primer A5 showed a number of total bands are thirteen ranging between 245.6 to 1774.2 bp, only two polymorphic bands representing polymorphic percentage of 15.38%, whereas the other eleven fragments were monomorphic, since they were seen in all plants.

Primer B18 exhibited nine DNA fragments with molecular sizes from 268.1 to 1387.4 bp, only one band was polymorphic representing that percentage of 11.11%, whereas the other eight fragments were monomorphic, since they were seen in all plants.

The pattern produced by primer C19 showed a total number of bands are eleven ranging between 456.4 to 1294.1 bp, there are five polymorphic bands were detected, were one was unique band for mother plant representing polymorphic percentage of 45.45%, whereas the other six fragments were monomorphic, since they were seen in all plants.

Obtained results revealed that, four of six were used primers for RAPD–based DNA fingerprints gave evidence of somaclonal variation for used of micropropagation protocol. The RAPD profiles

revealed high similarity between mother plant and the micropropagated plants at free growth regulators and 4 mg/l BA and 2 mg/l NAA. However, between mother plant and micropropagated plants at 2 mg/l TDZ and 4 mg/l Kin enabled us to explore the DNA polymorphism.

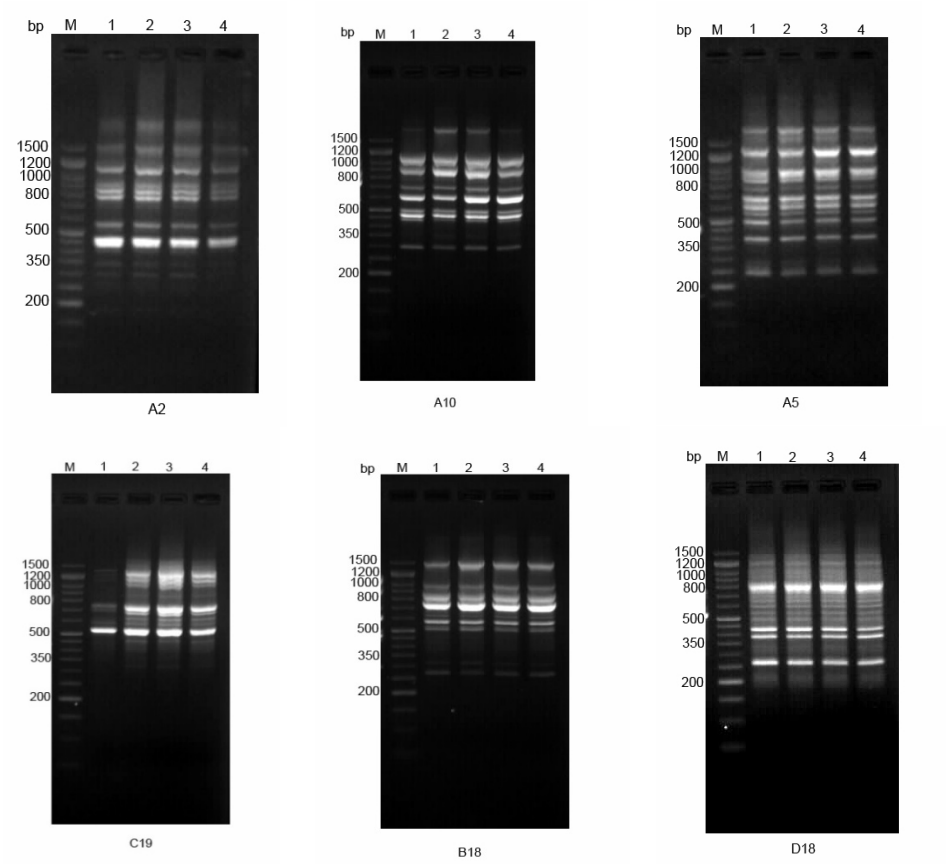
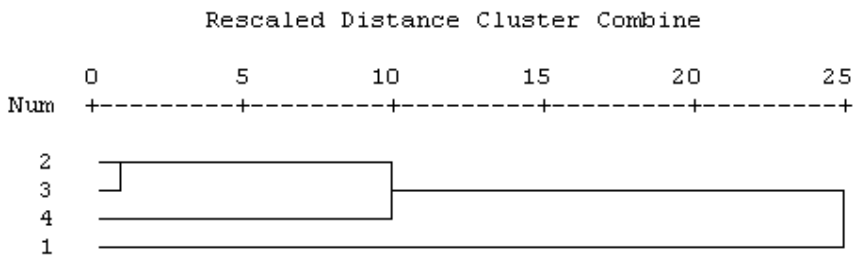


Fig. 2: DNA amplification pattern obtained for RAPD primers:
M: DNA ladder bp**, 1:** mother plant, **2:** free growth regulators, **3:** 4 mg/l BA and 2 mg/l NAA and **4:** 2 mg/l TDZ and 4 mg/l Kin.

According to similarity index (Table 8) and genetic distance dendrogram (Figure 3), none of the micropropagated plants was typically as compared to each other's or with *Cocculus* mother plant using different growth regulators of tissue culture technique.

Table 8: Similarity index (Pairwise comparison) for micropropagated plants with *Cocculus* mother plant when using different growth regulators during tissue culture technique on RAPD-PCR.

	1= Mother plant	**2=** Free hormone	**3=** 4 mg/l BA and 2 mg/l NAA	**4=** 2 mg/l TDZ and 4 mg/l Kin
Plants	**1**	**2**	**3**	**4**
1	1.000	0.955	0.947	0.930
2	0.955	1.000	0.993	0.977
3	0.947	0.993	1.000	0.970
4	0.930	0.977	0.970	1.000



1= Mother plant **2=** Free growth regulators **3=** 4 BA and 2 NAA **4=**2 TDZ and 4 Kin

Fig. 3: A dendrogram showing the genetic distance to micropropagated Cocculus plants using different growth regulators (mg L-1) as compared to Cocculus mother plant during tissue culture technique using RAPD-PCR data.

The most similar plants to the Cocculus mother plant when using different growth regulators during tissue culture technique were those obtained using free hormone and BA at mg/l 4 and 2 mg/l NAA (plants No. 2 and 3) recorded 0.955 and 0.947 similarity, respectively. While, the most dissimilar plant was that multiplicated using growth regulators TDZ at 2 mg/l and 4 mg/l Kin (plants No. 4) represents 0.930 similarities.

Abbreviations:

Kin= Kinetin,
BA = BAP = 6- benzylaminopurine,
TDZ = Thidiazuron, IBA = Indolbutric acid,
NAA = Naphtaleneacetic acid,
RAPD= Randomly Amplified Polymorphic DNA.

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