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IN VITRO PLANT REGENERATION FROM SHOOT TIP EXPLANTS OF *LOROPETALUM CHINENSE*

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Abstract

This study was carried out in the Plant Tissue Culture Laboratory, Horticulture Research Institute, Agriculture Research Center, Ministry of Agriculture and land reclamation, Giza, Egypt, during the period from 2016 to 2019. An efficient direct shoot regeneration micropropagation protocol has been established successfully for commercial *in vitro* propagation of *Loropetalum chinense*, an ornamental plant with medicinal values. The optimized sterilization conditions for the terminal node stem cuttings with its two axillary buds explants were exposure to clorox (sodium hypochlorite 5.25%) at 0.25 % for 20 min and 0.5 % for 15 or 20 min gave the highest value of survived explants (100 %). In establishment stage, MS medium supplemented with 0.5 mg/l IBA gave the highest number of shoots (3.0 shoots). The multiplication stage should be cultured on MS medium supplemented with 2.0 mg/l BA which recorded 7.67 shoots at the end of the third subculture. For rooting the regenerated shoots, using NAA at 1.0 mg/l resulted in significantly highest number of roots than that recorded with other treatments. Growing medium consists of peatmoss and vermiculite at the ratio of 1:1 (v/v) was the best for acclimatization. Inter simple sequence repeats (ISSR) analysis detected similarity to the mother plant in the in *vitro* propagated plants, that the protocol will be useful for *Loropetalum chinense* production. This plant protocol could be used for large scale regeneration of *Loropetalum chinense.*

Key words: *Loropetalum chinense*, Tissue culture, IBA, NAA, BA, ISSR analysis

Introduction

Fring flower plant (*Loropetalum chinense var.* rubrum), belongs to hazel family (Hamamelidaceae) and is defined as a variety of *Loropetalum chinense*. It is a diploid (2n=24) beautiful shrub, fast growing and tolerant to diseases and insects. *Loropetalum chinense* var. rubrum is versatile and can be grown in mild coastal climates in light shade to full sun, and can tolerate the more extreme winter and summer temperatures associated with other regions **(**Lian and Xiao, 2001). It is a desirable plant for the cool greenhouse and grown with good quality in pots containing peaty and sandy soil (Gawel *et al*., 1996). The name derives from the Greek and loron, athong or strap, referring to the masses or flowers with twisted, strap – like, creamy white petals that hang on the shrub in spring. It does best in warm – temp rate climate in sun or dappled shade and prefers will drained slightly acid soil (Burnie *et al*., 2004). It has various medical uses as promoting wound healing, anti-bacteria, anti-inflammatory, antioxidant, adjusting fat metabolism and antitumor but there is no available review of literature on *in vitro* micropropagation of *Loropetalum chinense* (Jing *et al*., 2014).

Selection of the explant and developing a genetically stable regeneration protocol are important in the starting of an *in vitro* study. Direct regenerated shoots from explants without passing callus has fewer somaclonal variation and more genotype-dependency (Ebrahimie *et al*., 2007) and are true to mother plant type while callus derived plants are variable. Shoot apex has high endogenous auxin level. Shoot tips are suitable in inducing roots because of the presence of procambial-like tissue in the structure surrounding the vascular tissue. Sterilization aims at using explants contamination free before establishment of micropropagation. Kind of sterilization substances or concentration and duration of exposing to the sterilant are important to keep the explant sterilized. Effectiveness of sodium hypochlorite and silver nitrate as an antibacterial is great. For the establishment of a new protocol in tissue culture, a universal basal media

like Murashige and Skoog's (MS) medium, Gamborg (B5) medium and Woody Plant (WPM) medium are suitable to supply different growth regulators for growth, besides to its high content of macronutrients, compared to other media strength . Growth regulators are the most effective variables in plant tissue culture media, especially auxins and cytokinins. The auxins commonly used in plant tissue culture media are indole-3-butyric acid (IBA), Indoleacetic acid (IAA), 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 1-napthaleneacetic acid (NAA). Auxins stimulate growth from shoot tips culture in establishment stage and to induce root formation. Auxins stimulate growth from shoot tips culture in establishment stage and to induce root formation. These growth regulators generally cause cell elongation and swelling of tissues, cell division, callus formation and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation (Pierik, 1997). Cytokinins used in the culture media in multiplication stage include 6- benzyladenine (BA) and N-(2-furanylmethyl)-1H-puring-6- amine (kin). These growth regulators in tissue culture media are incorporated mainly to initiate cell division and differentiation of adventitious shoots from callus and organs. These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance (Bhojwani and Razdan 1996). A balance between auxin and cytokinin required for the formation of adventitious shoots and roots. High levels of auxin relative to cytokinin stimulated the formation of roots, whereas high levels of cytokinin relative to auxin led to the formation of shoots (Taiz & Zeiger, 1991).

Tissue culture technique produces homogenized and genetically stabilized plants, having similar banding patterns to that of mother plant. Somaclonal variation occurred at DNA level, clonal stability can be assessed by studying chromosome numbers, isozyme profile and PCR-based molecular markers like random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and most recently start codon targeted (ScoT) (Devi *et al*., 2013 andRathore *et al*., 2014). So, this study was carried out as a first attempt to investigate the most suitable treatments for micropropagation of *Loropetalum chinense* plants.

Materials and Methods

This study was carried out in the Tissue Culture Laboratory, Horticulture Research Institute, Agriculture Research Center, Ministry of Agriculture and Land Reclamation, Giza, Egypt, during the period from 2016 to 2019 to determine the suitable protocol for propagating of *Loropetalum chinense* plant using tissue culture technique.

Culture Media

In the first four experiments, all culture media contained 30 g/l sucrose and were solidified with 6 g/l agar (pH was adjusted at 5.7 ± 0.1 prior to addition of agar). Media were autoclaved for 20 min at 121°C and 1.5 kg/cm^2, then they were cooled and kept for 7 days before use, and contaminated media were discarded. After culturing, the culture jars were directly plugged with polypropylene closure caps.

Source of explants

Active growing shoots were excised from 2-years-old seedlings of *Loropetalum chinense* plant, which were imported from China. Terminal node stem cuttings with its two axillary buds were prepared by dividing the shoots into 1.0-1.5 cm segments. The explants were washed with tap water, followed by a soap solution for 10 min and rinsed under a running tap-water for one hour, then redistilled water for 3 min, before soaking in sterilizers.

This study included five experiments, as follows:

Experiment I: Surface sterilization of explants

The aim of this experiment was to evaluate the effect of some sterilization treatments, by using sodium hypochlorite solution (commercial clorox containing 5.25% sodium hypochlorite, NaOCl). Clorox was used at 0.25, 0.50, 1.00 or 2.00% for 15, 20, 25 or 30 min and Silver nitrate ($AgNO_3$) at 0.25, 0.50 or 1.0 g/l for 3, 6 or 12 min on the contamination, mortality and survival percentages of explants. A few drops (0.1 %) of Tween 20 (polyoxyethylene sorbitan monolaurate) were added to the sterilizing solution as a wetting agent per 100 ml of sterilizing solution for each treatment. After sterilization, the explants were rinsed in sterilized distilled water (three times) to remove all traces of the disinfectant. After that, the explants were cut further to 0.3 cm segments, and were placed in 150 ml culture jars containing 20 ml of MS, B5 and WPM media. This stage contained twenty five treatments with two substances and each treatment consisted of four replicates and each replicate consisted of three jars and each jar contained one explant. Jars were incubated for 28 days in a growth room at 25±2ºC and 16 hrs illumination of 2000 lux (white fluorescent lamps). Contamination, mortality and survival percentages were recorded. All steps of the sterilization experiment have been done under aseptic condition inside the culture cabinet (Laminar air flow).

Experiment II: Establishment stage

The aim of this experiment was to investigate the suitable media (MS, B5 and WPM) supplemented with

various concentrations of NAA or IBA for studying the effect of different treatments on establishment stage. Uniform sterilized explants that produced from the aseptic culture were transferred to the MS, B5 and WPM media supplemented with NAA and IBA at 0.0, 0.5, 1.0, 2.0 or 4.0 mg/l. This stage contained twenty seven treatments and each treatment consisted of three replicates and each replicate consisted of three jars and each jar contained one explant. Jars were incubated as described in experiment I. After four weeks, shoot length (cm), number of shoots and number of leaves were recorded.

Experiment III: Multiplication stage

In these experiments shoot tip explants were cultured in multiplication stage. The aim of this experiment was increasing shoot formation and determine the most suitable of cytokinin kinds and concentrations, including benzyl adenine (BA) and kinetin (Kin) for axillary buds proliferation of *Loropetalum chinense*. Uniform sterilized shoot explants (1.0 cm long shoot) were cultured on MS and WPM media. Cultures were incubated under the same conditions previously described in experiment I for four weeks. The resulted uniform 1.5 cm shoot length with 2 leaves were subcultured on MS and WPM media supplemented with BA and Kin each at 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l in addition to MS and WPM media hormone free as the control. Subculturing the shoots was done onto the same medium every four weeks, and three subcultures were done. This experiment consisted of twenty two treatments, each treatment consisted of three replicates and each replicate consisted of three jars and each jar contained two explants. After the three subcultures, number of shoots, shoot length (cm) and number of leaves were recorded.

Experiment IV: Rooting stage

The aim of this experiment was to study the effect of WPM medium supplemented with auxins concentrations including Indole butyric acid (IBA) or Naphthalene acetic acid (NAA) on root formation of *Loropetalum chinense*.

Uniform shoots (2.0 cm long with 2 leaves) were cultured on WPM medium supplemented with IBA or NAA each at 1.0, 2.0 or 4.0 mg/l, in addition to a hormone-free WPM medium as the control. Activated charcoal (AC) was added to the rooting medium at the rate of 3 g/l in order to improve root formation. This experiment consisted of seven treatments, each treatment consisted of nine jars and every jar contained two individual shoots. Cultures were incubated for one month under the conditions previously described in experiment I. After one month, at the end of this experiment, plantlet length, number of leaves, number of roots, root length (cm) and rooting percentage were recorded.

Experiment V: Acclimatization stage

This experiment was conducted *in vivo* to evaluate the effect of growing media on plantlet growth of *Loropetalum chinense* during acclimatization stage. The shoots (2.0 cm long, with 2 leaves, grown on WPM medium supplemented with 1.0 mg/l NAA + 3.0 g/l AC) were cultured for rooting (the best rooting medium, as proven in Experiment IV) for one month before being used in the acclimatization experiment.

Resulting plantlets of suitable size (3.0 cm long with 4.0 leaves and 3 roots) were selected, washed thoroughly with tap water to remove the remains of medium from the root system, and individually transplanted into 5 cm plastic pots filled with one of the following media: peatmoss, peatmoss + sand (1:1, 2:1 v/v), peatmoss + perlite (1:1, 2:1 v/v) and peatmoss + vermiculite (1:1, 2:1 v/v). Each treatment consisted of three replicates and each replicate consisted of three pots. Before planting, the roots of plantlets were rinsed in water containing a fungicide (Benlate, 0.1 %) for 5 min, after that the roots were washed thoroughly with tap water to remove the remains of the fungicide from the root system. The plantlets were held in the greenhouse. The relative humidity around the plantlets was kept high by surrounding each pot with a white polyethylene bags for two weeks. The polyethylene bags were perforated gradually starting from the third day, with the aim of gradually reducing the relative humidity of the air surrounding the plantlets. The pots were irrigated by diffusion (by placing the basal part of pots in water) twice during these two weeks, and then the bags were removed after three weeks. After 4 weeks, plantlet length (cm) number of leaves/plantlet, number of shoots/plantlet and survival percentage (%) were recorded.

Molecular genetic identification

DNA isolation

Young and fresh leave samples were collected from *Loropetalumchinense*. All the selected leaves were free from any physiological or pathogenic symptoms, saved in ice box and quickly transferred to laboratory. Plant tissues were ground to a fine powder in the presence of liquid nitrogen (-196 ^{o}C).

The DNA extraction was performed using DNeasy plant Mini Kit(QIAGEN).

Data analysis

ISSR-DNA fragments were scored as 1 or 0 for the presence or absence of bands, respectively. Only sharp

bands were scored ("ghost" bands were not scored). The obtained binary data were subjected to analysis with GelAnalyzer 3 (Egygene) software (Alzohairy, 2008).

Statical analysis: The first three experiments were factorial, while the fourth and fifth experiment includes one factor. All experiments were conducted using a completely randomized design. All data were averaged and differences among the means of the different treatments were compared using the "Least Significant Difference, L.S.D." test at the 5% level, as described by Little and Hills (1978). In case of percentages in the first, fourth and fifth experiments, the original data were arcsine-transformed prior to statistical analysis.

Results and Discussion

Experiment I: Surface sterilization of explants

a. Surface sterilization with Silver nitrate

Contamination percentage

Data presented in table 1 show that the percentage of contaminated Loropetalum chinense explants significantly decreased with increasing silver nitrate (AgNO3) concentration. The significantly lowest percentage of contaminated (0.0%) explants was recorded by using 0.5 and 1.0 g/l $AgNO_3$.

Increasing soaking period from 3 to 12 min significantly decreased percentage of contaminated explants.

The interactions between $AgNO_3$ and soaking period revealed that using $AgNO_3$ at 0.25 g/l for 3 min resulted in the highest contamination %, wheras other combination treatments resulted in no contamination.

Table 1: Effect of different concentrations of silver nitrate ($AgNO_3$) and soaking period on surface sterilization of *Loropetalum chinense.*

Silver nitrate (A) (g/l)	Soaking periods (B)			Mean (A)
	3 min	6 min	12 min	
Contamination %				
0.25	50.00 a	0.00 b	0.00 b	16.67 a
0.50	0.00 b	0.00 b	0.00 b	0.00 b
1.00	0.00 b	0.00 b	0.00 b	0.00 b
Mean (B)	16.67 a	0.00 b	0.00 b	
Survival %				
0.25	25.00 bc	75.00 a	75.00 a	58.33 a
0.50	50.00 ab	0.00 c	0.00 c	16.67 b
1.00	50.00 ab	0.00 c	0.00 c	16.67 b
Mean (B)	41.67 a	25.00 a	25.00 a	
Mortality %				
0.25	25.00 b	25.00 b	25.00 b	25.00 b
0.50	50.00 b	100 .00 a	100 .00 a	83.33 a
1.00	50.00 b	100. 00 a	100 .00 a	83.33 a
Mean (B)	41.67 b	75.00 a	75.00 a	

Survival percentage

Data presented in table 1 showed that the significantly highest survival percentage of *Loropetalum chinense* explants (58.33%) was recorded with using $AgNO_3$ at 0.25 g/l followed by that recorded with $AgNO_3$ at 0.50 and 1.00 g/l (16.67%).

Increasing soaking periods from 3 min to 6 or 12 min insignificantly decreased survival percentage, giving the highest value with 3 min soaking period.

Soaking explants with $AgNo_3$ at 0.25 g/l for 6 or 12 min gave the significantly highest value of survived explants (75%) of *Loropetalum chinense* explants.

Mortality percentage

Data presented in table 1 showed that the mortality percentage of *Loropetalum chinense* explants increased with increasing $AgNO_3$ concentration. The significantly highest mortality percentage of explants was recorded by using 0.5 and 1.0 g/l $AgNO_3$, while the lowest values was recorded with 0.25 g/l $AgNO_3$.

The highest mortality percentage of explants was recorded with soaking period for 6 and 12 min. The lowest percentage of mortality was recorded with explants soaked for 3 min.

Generally, the lowest percentage of mortality explants (25%) was resulted from soaking the explants in 0.25 $AgNO_3$ for 3 or 12 min.

From the above results the best sterilization treatments were soaking explants in 0.25 g/l $AgNO_3$ for 3, 6 or 12 min giving 75 % survival, 25 % mortality and 0.0 % contamination.

Results are in agreement with prior studies as treatment with $AgNO_3$, 1 % $AgNO_3$ for 20 minutes was the best for controlling the infection of 'Oblaèinska' sour cherry buds gave the 96.67% healthy explants with 3.3% contaminated explants (Ines *et al*., 2013). Meanwhile, 2 % $AgNO_3$ for 5 minutes was found to be optimum for the disinfection of shoot tip explants of *Ginkgo biloba* L. (Lilyana and Valeria, 2017).

Silver is known to attack a broad range of biological processes in microorganisms including the alteration of cell membrane structure and functions. Silver also inhibits the expression of proteins associated with ATP production (Jo *et al*., 2009).

b. Surface sterilization with Sodium Hypochlorite

Contamination percentage

Results recorded in table 2 showed that Clorox treatments had no significant effect on contamination percentages. Clorox treatment at 0.25 % resulted in the highest value of contaminated explants (6.25 %). No contamination was recorded with the explants immersed in 0.50, 1.00 and 2.00 % Clorox treatments.

Regarding the effect of soaking periods on contamination percentages, data clearly revealed that soaking periods had no significant effect on contamination percentages. Generally, increasing soaking period from 15 to 20, 25 and 30 min resulted in preventing contamination.

The data of the interaction between the concentration of Clorox and soaking periods indicated that only the explants soaked in Clorox at 0.25 % for 15min resulted in the significantly highest contamination while other treatments resulted in no contamination.

Survival percentage

Data presented in table 2 indicate that. Clorox treatments at 0.5 % resulted in the significantly highest survival percentage while 2.0 % Clorox resulted in the significantly lowest survival percentage.

Concerning the effect of soaking period on survival percentage, the significantly highest survival percentage was recorded with 15 min followed by that recorded after 20 min with no significant difference between them. On the other hand, increasing soaking period to 25 min resulted in significant lower survival percentages.

Table 2: Effect of different concentrations of Sodium hypochlorite (NaOCl) and soaking period on surface sterilization of *Loropetalum chinense.*

Clorox % (A)	Soaking periods (B)				Mean (A)
	15 min	20 min	25 min	30 min	
Contamination %					
0.25	25.00 a	0.00 b	0.00 b	0.00 b	6.25 a
0.50	0.00 b	0.00 b	0.00 b	0.00 b	0.00 a
1.00	0.00 b	0.00 b	0.00 b	0.00 b	0.00 a
2.00	0.00 b	0.00 b	0.00 b	0.00 b	0.00 a
Mean (B)	6.25 a	0.00 a	0.00 a	0.00 a	
Survival %					
0.25	75.00 ab	100 .00 a	50.00 b	50.00 b	68.75 ab
0.50	100 .00 a	100 .00 a	50.00 b	75.00 ab	81.25 a
1.00	50.00 b	50.00 b	50.00 b	75.00 ab	56.25 b
2.00	50.00 b	0.000 c	0.000 c	0.000 c	12.50 c
Mean (B)	68.75 a	62.50 a	37.50 b	50.00 ab	
Mortality %					
0.25	0.00 c	0.00 c	50.00 b	50.00 b	25.00 bc
0.50	0.00 c	0.00 c	50.00 b	25.00 bc	18.75 c
1.00	50.00 b	50.00 b	50.00 b	25.00 bc	43.75 b
2.00	50.00 b	100.00 a	100.00a	100.00 a	87.50 a
Mean (B)	25.00 b	37.50 bc	62.50 a	50.00 ab	

Results indicated that the use of different concentrations of Clorox for various soaking periods had significant effect on survival percentage. Using Clorox at 0.25 or 0.50 % for 20 min as well as 0.50 for 15 min gave the significantly highest value of survived explants (100 %). On the other hand, using Clorox at 2.00 % for 20, 25 or 30 min resulted in no survived explants (0.0 %).

Mortality percentage

The results represented in table 2 indicate that the use of sodium hypochlorite on mortality percentage of explants had positive significant effects when 2.00 % of Clorox was used as compared with the lower concentration. The highest percentage of moralized explants (87.50 %) was recorded when soaking the explant in 2.00 % Clorox.

Mortality percentage increased with the increase of soaking period to 25 min. The highest percentage of moralized explants (62.50 %) was recorded at 25 min. Soaking period for 15 min gave the lowest value of moralized explants (25.0 %).

The interactions between sodium hypochlorite and soaking period were significant with the highest value of moralized explants (100%) when the mixture contained 2.00 % Clorox for 20, 25 and 30 min soaking period. The significantly lowest value of moralized explants (0.0 %) was recorded with using 0.25 or 050 % Clorox for 15 and 20 min.

From the above results the best sterilization treatment was Clorox at 0. 25% for 20 min and 0.50 % for 15 or 20 min soaking period which gave 100% survival, 0.0% mortality and 0.0 % contamination.

Results are in agreement with prior studies as treatment with NaOCl, addition of 70.0 % Ethyl alcohol for 30 sec and 30.0% commercial Clorox (1.30%

NaOCl) for 5.0 minute were the best treatment for seeds sterilization for *Antirrhinum majus* plant (Hamza *et al.*, 2013) . Also, Kuldeep *et al.*, (2015) stated that the most effective sterilization for *Gloriosa superba* was achieved using 1.0 % NaOCl treatment for 8 minutes.

Antimicrobial action of sodium hypochlorite depends on causing enzymatic inhibition of the bacterial important enzymes. The high pH of sodium hypochlorite interferes in the cytoplasmic membrane integrity with biosynthetic alterations in cellular metabolism and phospholipid degradation (Estrela *et al*., 2002).

Experiment II: Establishment stage

Shoot length (cm)

In this experiment, shoot tip explants were cultured on three different types of media for 4 weeks during establishment stage.

Data presented in table 3 revealed that supplied media with IBA at 2 mg/l gave the significantly tallest shoots. Shoots established on media supplemented with 2.0 mg/l NAA gave the shortest shoots compared to that cultured on other treatments.

The significantly tallest shoot was recorded on MS medium followed by that recorded on WPM medium, while B5 medium resulted in significantly shorter shoot length.

The significantly tallest shoots were recorded on MS medium supplemented with 2.0 mg/l IBA, while the significantly shortest shoots were recorded on B5 medium supplemented with 4.0 mg/l NAA.

Number of leaves

Concerning the effect of different concentrations of NAA and IBA on number of leaves, regardless of the effect of different media, data presented in table 4 clearly revealed that IBA at 0.5 or 1.0 mg/l gave the significantly highest number of leaves (9.67 and 9.00 leaves). Using NAA at 1.0 mg/l resulted in the lowest number of leaves.

The significantly highest number of leaves was formed on explants cultured on MS medium, whereas that cultured on WPM and B5 media gave the lowest values.

The significantly highest number of leaves was recorded on MS supplemented with 0.5 mg/l IBA and that on WPM medium supplemented with 1.0 mg/l IBA, while the significantly lowest number of leaves was recorded on B5 medium supplemented with 4.0 mg/l NAA.

Table 3: Effect of different concentrations of NAA, IBA and different media on shoot length during establishment stage of *Loropetalum chinense*.

Shoot length (cm)				
	Media (B)			
Auxins (A) (mg/l)	MS	B5	WPM	Mean (A)
Control	2.13 bc	1.67 g-i	1.77 f-i	1.86 bc
0.5 mg/l NAA	2.03 c-e	1.97 c-f	1.97 c-f	1.99 b
1.0 mg/l NAA	2.13 bc	1.63 hi	2.00 c-f	1.92 b
2.0 mg/l NAA	1.70 g-i	1.63 hi	1.63 hi	1.65 d
4.0 mg/l NAA	1.67 g-i	1.60 i	1.77 f-i	1.68 d
0.5 mg/l IBA	1.80 e-i	1.80 e-i	1.70 g-i	1.77 cd
1.0 mg/l IBA	2.00 c-f	1.83 d-i	2.07 cd	1.97 b
2.0 mg/l IBA	2.40 a	1.83 d-i	2.33 ab	2.19 a
4.0 mg/l IBA	1.87 d-h	1.90 c-g	1.90 c-g	1.89 bc
Mean (B)	1.97 a	1.76 b	1.90 a	

Table 4: Effect of different concentrations of NAA, IBA and different media on number of leaves during establishment stage of *Loropetalum chinense*.

Number of leaves				
	Media (B)			
Auxins (A) (mg/l)	MS	B5	WPM	Mean (A)
Control	7.33b-f	6.33e-g	5.33f-h	6.33bc
0.5mg/lNAA	7.00c-f	7.33b-f	6.67d-g	7.00b
1.0mg/lNAA	5.67e-h	5.00f-h	5.00f-h	5.22d
2.0mg/lNAA	6.67d-g	4.33gh	6.33e-g	5.78b-d
4.0mg/lNAA	6.33e-g	3.67h	6.00e-h	5.33cd
0.5mg/lIBA	10.0a	9.67ab	9.33a-c	9.67a
1.0mg/lIBA	9.00a-d	8.00a-e	10.0a	9.00a
2.0mg/lIBA	6.67d-g	6.67d-g	6.67d-g	6.67bc
4.0mg/lIBA	6.33e-g	6.67d-g	6.67d-g	6.55b-d
Mean(B)	7.22a	6.41b	6.89ab	

Number of shoots

Data presented in table 5 showed that IBA treatments gave higher number of shoots than that recorded with NAA treatments. The significantly highest number of shoots was obtained on media supplemented with 0.5 and 1.0 mg/l IBA. On the other hand, the significantly lowest number of shoots was obtained on media supplemented with 1.0 mg/l NAA.

The results recorded in table 5 revealed that the explants were successfully cultured on MS, B5 and WPM media. Furthermore, all media (MS, B5 and WPM) gave positive response with number of shoot with no significant difference among them.

The interaction effect between the types of media and NAA or IBA concentrations showed that there were significant differences on number of shoots resulted with

Table 5: Effect of different concentrations of NAA, IBA and different media on number of shoots during establishment stage of *Loropetalum chinense*.

Number of shoots				
	Media (B)			
Auxin (A) (mg/l)	MS	B5	WPM	Mean (A)
control	1.67bc	1.33bc	1.33bc	1.44bc
0.5mg/lNAA	1.00c	1.67bc	1.67bc	1.45bc
1.0mg/lNAA	1.00c	1.33bc	1.33bc	1.22c
2.0mg/lNAA	1.33bc	1.33bc	1.67bc	1.44bc
4.0mg/lNAA	2.0a-c	1.33bc	1.33bc	1.55bc
0.5mg/lIBA	3.00a	1.67bc	2.33ab	2.33a
1.0mg/lIBA	2.33ab	2.33ab	2.33ab	2.33a
2.0mg/lIBA	2.33ab	2.00a-c	2.33ab	2.22a
4.0mg/lIBA	1.67bc	2.00a-c	2.33ab	2.00ab
Mean(B)	1.81a	1.67a	1.85a	

different treatments. The highest number of shoots was obtained on MS medium supplemented with 0.5 mg/l IBA, whereas the lowest number of shoots was obtained on MS medium supplemented with 05 or 1.0 mg/l NAA.

From the above results the best treatment for establishment stage was MS medium supplemented with 0.5 mg /l of IBA which gave 3.0 shoots and 10.0 leaves.

Similar results was reported by Abdallah *et al.,* (2017) they reported that MS medium was the best medium for establishment stage of *Origanum syriacum* which gave the best number of shoots, shoot length, number of leaves andnumber of leaves/shoot (1.83, 1.75 cm, 6.67 and 3.33, respectively).

Also, Uddin *et al.*, (2016) mentioned that the highest response (100%) for *Cestrum nocturnum* was gained at establishment stage from lateral bud explants on MS medium supplemented with 1.5 mg /l BA with most of NAA concentrations.

Experiment III: Multiplication Stage

Number of shoots

Concerning the effect of different concentrations of Kin and BA on number of leaves, regardless of the effect of different media, data presented in table 6 clearly reveal that BA at 2.0 mg/l gave the significantly highest number of shoots (6.33 shoots) . Using Kin at 5.0 mg/l resulted in the significantly lowest number of shoots (1.83 shoots).

Data demonstrated that number of shoots was significantly increased by using MS medium than using WPM medium after the third subculture.

The interaction effect between Kin, BA and types of media, after three subcultures, revealed that the shoots were cultured on MS medium supplemented with 2.0 mg/ l BA produced the significantly highest number of shoots. The lowest number of shoots was obtained on MS medium supplemented with 5.0 mg/l Kin.

Great shoot development of *Euphorbia Pulcherrima* was observed on MS medium supplemented with BA and NAA as compared with the use of BA or Kin separately (Layla and Rafail, 2012). Cytokinins stimulate cell division and axillary shoot proliferation. Cytokinins promote gene expression in tissue culture that stimulates the shoot formation (Howell *et al*., 2003).

Shoot length

Data presented in table 7 showed that using Kin and BA treatments resulted in significant effect on shoot length as compared to the control (hormone – free medium). The tallest shoots were recorded with 2.0 mg/ l BA. The significantly shortest shoot was obtained with 5.0 mg/ l Kin.

It was found that MS medium resulted in taller shoots compared to WPM medium with no significant difference between them.

The interaction effect between the cytokinins treatments and types of media showed significant effect on shoot length. It was found that the tallest shoots were recorded on MS medium supplemented with 2.0 mg/l BA, while, the shortest shoots were recorded on MS medium supplemented with 5.0 mg/l Kin.

Number of leaves

Regarding the effect of different cytokinins at various concentrations on number of leaves, Data in table 8 revealed that using different treatments resulted in significant effect on number of leaves as compared with control (hormone – free medium) in most cases. The highest number of leaves was recorded with medium

Table 6: Effect of MS or WPM media and different concentrations of Kin or BA on number of shoots during multiplication stage of *Loropetalum chinense*.

Cytokinins (A) (mg/l)	Media (B)		Mean (A)
	MS	WPM	
Control	3.67e-h	3.33f-i	3.50c-e
1.0mg/lKin	3.67e-h	4.33c-f	4.00cd
2.0mg/lKin	5.33bc	4.67c-e	5.00b
3.0mg/lKin	3.67e-h	3.00g-j	3.33d-f
4.0mg/lKin	2.67h-k	2.33i-k	2.50f-h
5.0mg/lKin	1.67k	2.00jk	1.83h
1.0mg/lBA	4.00d-g	6.33b	5.16b
2.0mg/lBA	7.67a	5.00cd	6.33a
3.0mg/lBA	5.33bc	3.33f-i	4.33bc
4.0mg/lBA	3.33f-i	2.67h-k	3.00e-g
5.0mg/lBA	2.67h-k	2.00jk	2.33gh
Mean(B)	3.97a	3.54b	3.50c-e

Table 7: Effect of MS or WPM media and different concentrations of Kin or BA on shoot length during multiplication stage of *Loropetalum chinense*.

Cytokinins (A) (mg/l)	Media (B)		Mean (A)
	MS	WPM	
Control	1.80i	2.03gh	1.92d
1.0mg/lKin	2.57cd	2.70bc	2.64b
2.0mg/lKin	2.87b	2.67bc	2.77ab
3.0mg/lKin	1.77i	1.53jk	1.65e
4.0mg/lKin	1.27lm	1.40kl	1.34gh
5.0mg/lKin	1.17m	1.37k-m	1.27h
1.0mg/lBA	2.13fg	2.40de	2.27c
2.0mg/lBA	3.40a	2.33ef	2.87a
3.0mg/lBA4.0mg/lBA	1.83hi1.63ij	1.83hi1.50jk	1.83d1.57ef
5.0mg/lBA	1.53jk	1.37k-m	1.45g
Mean(B)	2.0a	1.92b	

Table 8: Effect of MS or WPM media and different concentrations of Kin or BA on number of leaves during multiplication stage of *Loropetalum chinense*.

Cytokinins (A) (mg/l)	Media (B)		Mean (A)
	MS	WPM	
Control	5.67 e-g	6.33 d-f	6.00 e
1.0mg/l Kin	7.00 c-e	7.67 cd	7.33 d
2.0mg/l Kin	11.33 ab	8.33 c	9.83 b
3.0mg/l Kin	7.00 c-e	4.67 f-i	5.83 e
4.0mg/l Kin	3.67 h-j	4.33 g-j	4.00 gh
5.0mg/l Kin	2.67 j	3.33 ij	3.00 h
1.0mg/l BA	6.33 d-f	10.33 b	8.33 cd
2.0mg/l BA	12.33 a	10.33 b	11.33 a
3.0mg/l BA	11.67 ab	5.67 e-g	8.67 bc
4.0mg/l BA	5.67 e-g	5.33 e-h	5.50 ef
5.0mg/l BA	5.33 e-h	3.67 h-j	4.50 fg
Mean (B)	7.15 a	6.36 b	

supplemented with 2.0 mg/l BA.

It was found that using MS medium resulted in significant higher number of leaves compared with WPM medium.

Regarding the interaction effect between different cytokinins treatments and different types of media, data presented in table 8 clearly revealed that the significantly highest number of leaves was recorded with MS medium supplemented with 2.0 mg/l BA. On the other hand, the lowest number of leaves was recorded on MS medium supplemented with 5.0 mg/l Kin.

From the above results the best treatment for multiplication stage was MS medium supplemented with 2.0 mg /l BA which gave 7.67 shoots with length 3.40 cm and 12.33 leaves.

These results were in agreement with that obtained by Bekheet *et al.*, (2015) who found that MS medium + 1 mg/l BA gave the highest number of shoots of Jojoba plant, the greater value of shoot length and the maximum number of nodes were observed on the medium containing 1 mg/l BA + 1.5 mg/l Kin.

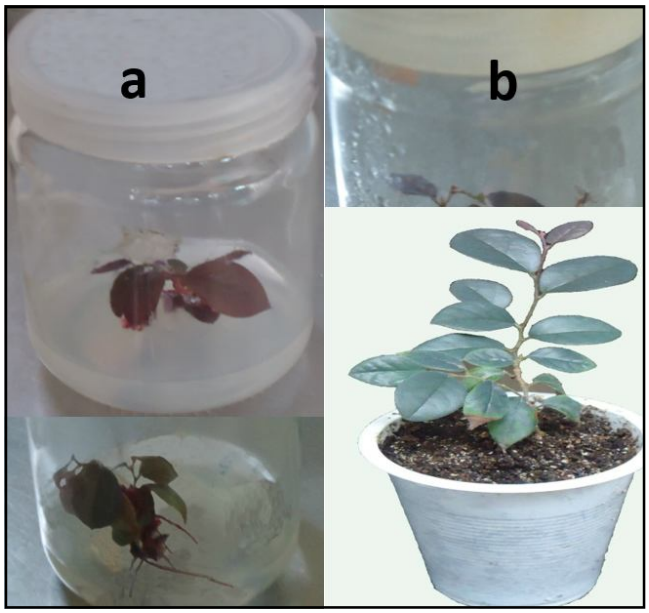

Fig. 1: *In vitro* micropropagation of *Loropetalum chinense.*
a. Shoot formation on MS medium supplemented with 0.5 mg/ l IBA during establishment stage.
b. Shoot multiplication on MS medium supplemented with 2.0 mg/l BA during multiplication stage.
c. Root formation on WPM medium supplemented with 1.0 mg/l NAA during rooting stage.
d. Shoot acclimatization on peatmoss and vermiculite at the ratio of 1:1 (v/v) during acclimatization stage.

Elenav *et al.*, (2016) showed that the highest multiplication rate of *Rosa canina* (7.5 shoots per explant) was achieved on MS medium supplemented with 1.0 mg /l BA. Also, Mayur *et al.,* (2018) mentioned that the maximum number of shoots of *Kalanchoe blossfeldiana* (7.0 shoots per explant) was observed when callus was grown on MS + BAP (0.2 mg/l) and NAA (0.1 mg/l).

Table 9: Effect of WPM medium supplemented with different concentrations of NAA and IBA on plantlet length , number of leaves, number of roots , root length and rooting percentage during rooting stage of *Loropetalum chinense*.

Auxins (m/g)	Plantlet length (cm)	Number of leaves	Number of roots	Root length (cm)	Rooting perce-ntage
Control	1.53 d	7.67 ab	0.00 c	0.00 b	0.00 b
1.0mg/l NAA	2.50 b	8.33 a	4.00 a	2.17 a	100.00 a
2.0mg/l NAA	1.73 d	7.00 bc	2.67 b	2.23 a	100.00 a
4.0mg/l NAA	1.50 d	6.33 c-e	0.00 c	0.00 b	0.00 b
1.0mg/l IBA	2.17 c	6.67 b-d	0.00 c	0.00 b	0.00 b
2.0mg/l IBA	2.83 a	5.67 de	0.00 c	0.00 b	0.00 b
4.0mg/l IBA	2.23 bc	5.33 e	0.00 c	0.00 b	0.00 b

Experiment IV: Rooting stage

Plantlet length, number of leaves, number of roots, root length, and rooting percentage

Data presented in table 9 and illustrated in Fig. 1 reveal that using WPM medium supplemented with IBA resulted in significantly plantlet length than that recorded with NAA. While, the lowest number of leaves was recorded with WPM medium supplemented with IBA than that recorded with NAA. Using NAA at 1.0 mg/l resulted in significantly highest number of roots than that recorded with other treatments. For root length (cm) and rooting percentage, using NAA at 1.0 or 2.0 mg/l resulted in significantly highest mean value than that recorded

with other treatments with no significant different between them. While, the other treatments gave negative response with number of roots, root length and rooting percentage which resulted no rooting.

Similar results was reported by Papafotiou and Skylourakis (2010), they observed that microshoots of *Callistemon citrinus* rooted when transferred in WPM Medium with 0.5 mg /l NAA which gave 100% rooting percentage, 6.2 of roots and 0.3 cm root length. Also, Sun *et al.*, (2009) found that the optimal rooting culture mode for Jasmine plant was two-step rooting method, i.e., pre-culturing shoots on the rooting medium of 1/2 WPM+ 1.0 mg/l NAA for 7 days, then transferring pre-cultured shoots to 1/2 WPM hormone free, the rooting rate was up to 98.41%.

Experiment V: Acclimatization stage

Plantlet length (cm), number of leaves/ plantlet, number of shoots/plantlet and survival percentage (%)

Data presented in table 10 and illustrated in Fig. 1 reveal that, the significantly tallest plantlets with the significantly highest number of leaves, number of shoots/ plantlet and the significantly survival percentage were recorded on the growing medium containing peatmoss + vermiculite (1:1, v/v). Peatmoss + sand (2:1, v/v) treatments gave no positive response with plantlet length, number of leaves, number of shoots/plantlet and survival percentage. The significantly lowest plantlet length, number of leaves, number of shoots/plantlet and survival percentage was recorded on growing peatmoss media.

In the same trend, peat moss + vermiculite + sand (1:1:1) was the best for the acclimatization of two cultivars of Strawberry plant (Marquez and Festival) which gave survival percentage 96 and 93%, respectively (Wafaa and Wahdan 2017). Also, Hamza *et al.*, (2013) mentioned that total of 90% survival of *Antirrhinum majus* was achieved and an increase in shoots length (12.71cm) when rooted explants were acclimatized *ex vitro* using 1:1 soil: vermiculite mixture.

The above mentioned result can be explicated by that peatmoss holds nutrients and water in the growing media (Hussein 2004). Vermiculite equally increases water-holding capacity, it is 3-4 times its weight of water and holds positively charged nutrients such as K, Mg and Ca (Handreck and Black 1994). Perlite improve drainage, aeration, nutrients and retain moisture due to the high porosity (Grillas *et al*., 2001).

Inter Simple Sequence Repeats (ISSRs)

In the present study, Five ISSR primers in table 11 were applied to investigate the level of polymorphism among the *Loropetalum chinense* (mother plant and micropropagated). All primers produced amplified amplicons, the five ISSR primers successfully amplified DNA bands of the *in vitro* plant under investigation with a total number of 33 bands where 5 polymorphic and 28 bands were monomorphic (84.9 % monomorphic). The range of DNA band size was between (95.65 to 1326.97 bp).

The primer 844-A produced a total number of 7 bands. Two bands were polymorphic (28.6% polymorphism). Meanwhile, 5 monomorphic DNA bands were recorded (71.4%). The range of molecular size was between 486.16 to 1326.97 bp.

The primer 844-B produced 4 DNA fragments with molecular size ranging from (319.56 to 769.27 bp), all bands were monomorphic bands (100% monomorphic).

The ISSR profile generated by the primer 17898-A gave a total of 8 DNA fragments with molecular size ranging from (234.31 to 919.68 bp), seven bands were monomorphic bands (87.5%), while

Table 10: Effect of different growing media on plantlet length, number of leaves, number of shoots/plantlet and survival percentage during acclimatization stage of *Loropetalum chinense.*

Growing media	Plantlet length (cm)	Number of leaves	Number of shoots /plantlet	Survival percentage (%)
Peatmoss	3.67 f	4.33 d	1.33 bc	22.22 de
Peatmoss + sand (1:1)	4.30 e	5.33 cd	1.67 b	33.33 cd
Peatmoss + sand (2:1)	0.00 g	0.00 e	0.00 c	0.000 e
Peatmoss+ perlite (1:1)	5.00 d	6.00 c	1.67 b	55.56 bc
Peatmoss + perlite (2:1)	5.60 c	6.67 bc	2.00 ab	55.56 bc
Peatmoss + vermiculite (1:1)	8.80 a	10.33 a	3.33 a	100.0 a
Peatmoss + vermiculite(2:1)	6.20 b	7.67 b	2.67 ab	77.78 ab

Table 11: Type and number of amplified DNA bands generated by five ISSR primers used for the identification of the *Loropetalum chinense.*

Primer code BandType	Primer (844-A)	Primer (844-B)	Primer (17898-A)	Primer (17898-B)	Primer HB-14	Total
Monomorphic	5	4	7	6	6	28
Polymorphic	2	0	1	1	1	5
Total bands	7	4	8	7	7	33
monomorphic (%)	71.4	100	87.5	85.7	85.7	Mean: 84.9%
Fragment size range (bp)	486.16-1326.97	319.56 -769.27	234.31 - 919.68	686.6 -1169.42	95.65 -390.65	

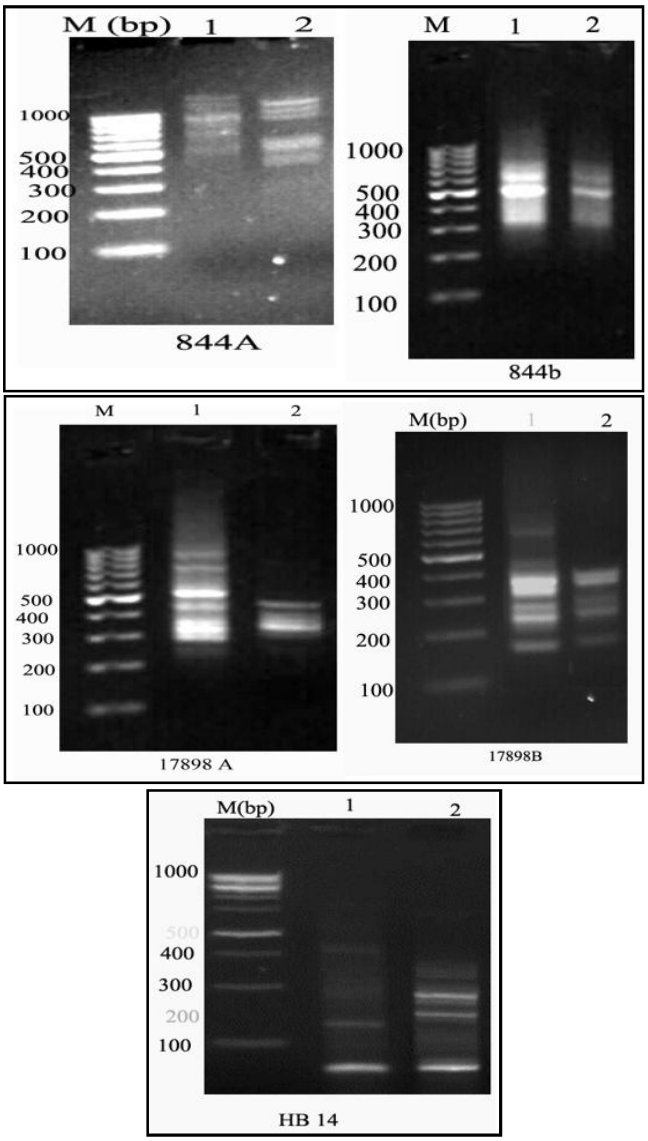

Fig. 2: Gel electrophoresis of ISSR fragments profiles generated by using 5 primers: 844-A 844-B, 17898 A, 17898-B and HB-14).

one polymorphic band (12.5 % polymorphism).

The ISSR profile generated by the primer 17898-B produced a total of 7 bands with molecular size ranging from (686.6 -1169.42 bp), with only one polymorphic band (14.3% polymorphism), while six bands were monomorphic bands (85.7%).

Primer HB-14 resulted in the amplification of 7 bands with molecular size ranging from (95.65 to 390.65 bp), six bands were monomorphic bands (85.7 %) and one polymorphic band (14.3% polymorphism), respectively as noticed in Tables 11 and Fig. 2.

Dong *et al.*, (2014) reported that ISSR analysis of *Loropetalum chinense* recorded ISSR fingerprinting amplified by 14 ISSR primers revealed a total number of 203 unambiguous bands, of which 148 ones were polymorphic and the polymorphism frequency was 72.9%.

Prakash *et al*., 2004 reported that RAPD analysis of *Curcuma amada* recorded 103 scorable bands from 10 primers, representing 8.7% polymorphism.

Obtained results explained by that *in vitro* results in breakdown of normal cellular controls causing genetic and epigenetic instabilities and therefore alterations in thegene expression and derivation of new phenotypes happens (Phillips *et al*., 1994 and Kaeppler *et al*., 2000).

Conclusion and Recommendation: An efficient direct shoot regeneration micropropagation protocol has been established successfully for commercial *in vitro* propagation of *Loropetalum chinense* as follow. The optimized sterilization conditions for the terminal node stem cuttings with its two axillary buds explants were exposure to clorox (sodium hypochlorite 5.25%) at 0.25 % for 20 min and 0.5 % for 15 or 20 min to obtain 100% survived explans. MS medium supplemented with 0.5 mg/l IBA was the best medium for establishment stage, whereas MS medium supplemented with 2.0 mg/l BA was the best medium for the multiplication stage. The best rooting was obtained by using NAA at 1.0 mg/l. Growing medium consists of peatmoss and vermiculite at the ratio of 1:1 (v/v) was the best for acclimatization.

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