

EFFECT OF CYTOKININ TYPE AND CONCENTRATION ON THE MULTIPLICATION RATE OF SOME ORNAMENTAL PLANTS

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ABSTRACT

This study was carried out in the Tissue Culture Laboratory of Zohriya Botanical Garden, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt during the period from 2007-2009 to establish an applicable protocol for the rapid micropropagation of *Clerodendrum glabrum* and *Mussaenda luteola*. The tabulated data showed that, in the establishment stage, Clorox concentration had a significant effect on survival percentage of both applying Clorox at 15%, value of survival percentage of both plants explants rose to the significantly highest level. On the other hand, in the multiplication stage, cytokinin type and concentration had a significant effect on the number of shoots and shoot length. In both plants, the significantly highest number of shoots was found on medium supplemented with BAP at 1 ppm, while all media free of cytokinins of any type (the control treatment) resulted in the significantly lowest number of shoots. On the contrary, the untreated control shoots were significantly the longest, while the shortest ones were those treated with BAP. The interaction between cytokinin type and concentration had a significant effect on the number of leaves of *Clerodendrom glabrum* only. However, the response of the two plants was somewhat contradictory to each other. The highest number of leaves was produced by the untreated control *Mussaenda* and *Clerodendron* shoots treated with BAP at 3 ppm, while the lowest number belonged to *Mussaenda* shoots treated with TDZ at 2 ppm and the shoots grown on the untreated control media.

INTRODUCTION

Clerodendrum glabrum E. Mey. Family Lamiaceae ,is a small to medium deciduous tree, widespread from Tropical to Southern Africa. In common with others of this family, its bark is covered in prominent white lenticels. *C. glabrum* has delightful aromatic flowers that can be used as pot-pourri, while the leaves when crushed emit a strong odor with insect repellent qualities that has been used by indigenous populations for generations as a wash or lotion to prevent insect infestation of the skin. So soothing is the leaf considered by some tribes, that it is pounded and put under the neck or armpit of a fretful child. Many tribes, including the Zulus, use it as a snake bite remedy.

Mussaenda luteola Delile; Family, Rubiaceae, is a very interesting frost-tender tropical evergreen erect twiggy slender small shrub or sub shrub,

about 2m high. Leaves petiolate 4-5cm long. The unusual flowers of dwarf *Mussaenda* feature golden star shaped 1.8 cm across flowers, surrounded by creamy yellow bracts. Sepals are large and yellow corollas are small. The ornamental value of this plant is due to one or more petaloid sepals. The color of the petaloid calyx lobes pale yellow-white, varieties with white, yellow, pink to red petaloid sepals are available. The different parts of this plant viz. leaves, roots, flowers as well as the whole plant are used in curing coughs, ulcers and leprosy possibly due to the presence of saponins. *Mussaenda luteola* can be used to remove heavy metals from the polluted soils (Sharma and Sindhu, 1999). These trees are rare in Egypt, its are only found in Zohriya Botanical Garden. Plants propagation by tissue culture is usually aimed at the possibly highest multiplications rate. In order to obtain high multiplication rates, relatively large amounts of cytokinins are used in multiplications media. It was affirmed that the mass micropropagation performances of many plants are widely controlled by a numerous factors from which the ingredient of culture media is the most important factors one. For sterilization of the explants Clorox can be used as reported by Sawsan & Secham (2007) and Hadake et al., (2008). However, in the micropropagation of some plants the multiplication rate decreases with increasing the concentration of cytokinins in the medium (Corchete et al., 1997).

The effect of cytokinins is most noticeable in tissue cultures where they are used to stimulate cell division and control morphogenesis. When added to shoot culture media, they overcome apical dominance and release lateral buds (George, 1993). Synthetic cytokinins comprise the N⁶-substituted aminopurines such as benzyladenine (BA or BAP), 6-furfurylaminopurine (kinetin or Kin). There are also Synthetic cytokinin compounds most notable of which are the diphenylurea-type compounds, such as thidiazuron, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea or *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (Taiz and Zeiger, 2002). GenLin et al. (2003) remarked that BA was found to be suitable for the multiplication (production) of adventitious shoots of *Ilex latifolia* from nodal segments. On the other hand George et al. (2008) mentioned that among the cytokinins, BA was superior to kinetin in terms of the number of shoots produced per explant of *Baliospermum montanum* (Fam. Euphorbiaceae).

Hosoki et al. (2003) and Lucchesini et al. (2003) on chocolate cosmos (*Cosmos atrosanguineus*) and *Passiflora incarnata*, respectively claimed that the optimal multiplication rate was achieved on medium containing 0.20-0.25 ppm BA. Kulkarni et al (2004) worked on banana cultivars belonging to triploid (AAA, AAB) and diploid (BB) genomic background. They declared that BAP was essential for proliferation in shoot tip cultures. The number of shoots showed proportionate increase to BAP concentration. Riordain et al., (1983) studied the effect of BA levels on growth, number of shoots and propagates of *cordyline* produced *in vitro*. They found that the highest rates of shoots were obtained with the medium supplemented with 0.2 mg/L BA. Therefore, the current experiments are achieved on *Mussaenda luteola* and *Clerodendron glabrum* to investigate the effect of types of cytokinins and concentrations on shoot proliferation response of explants *in vitro*.

MATERIALS AND METHODS

This study was carried out in the Tissue Culture Laboratory of Zohriya Botanical Garden, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt during the period from 2007-2009 to establish an applicable protocol for the rapid micropropagation of *Clerodendrum glabrum* and *Mussaenda luteola*.

1-Explant source and preparation: Healthy shoots (20-30 cm long) were collected from plants growing in Zohriya Garden. The collected shoots were brought to the tissue culture lab where they were cleaned by removing soil particles and dead parts, cut into nodal segments, thoroughly washed up by liquid soap and rinsed several times by tap water to rid them of surface contaminants as much as possible. They were then put in beakers of distilled water for sterilization treatments.

2-Glassware: Glass jars of 11.5 cm height x 6.5 cm diameter with their polypropylene caps were used during establishment, multiplication and rooting stages. In order to avoid contamination problems, jars were washed then soaked in a detergent solution for 24 hours, sterilized with 10 % solution of sodium hypochlorite (NaOCl) for 2 hours, rinsed with distilled water and autoclaved at 1.05 kg/cm² and 121°C for 25 minutes before being filled with 40 ml of the MS medium/jar.

3-Culture medium: Murashige and Skoog medium (1962) [MS] was used. This medium contained, in addition to the prescribed salts and vitamins, 150 mg/liter citric acid, 100 mg/liter ascorbic acid, 100 mg/liter myoinositol, 20 g/l sucrose and 6 g/l agar. It was adjusted to 5.8 pH, poured in the jars and autoclaved at 121°C for 20 minutes under 1.05 kg/cm² pressure, left to cool and stored at 25±2°C for one week before being used.

4-Plant growth regulators: In the multiplication stages, three types of cytokinins, incorporated in the media individually at different concentrations (0, 1, 2 and 3 ppm), were used. These were benzyladenine (6-benzylaminopurine), referred to for short as "BA or BAP", kinetin "Kin" and thidiazuron "TDZ".

This investigation comprised 2 experiments for each plant as follows:

Experiment 1: Effect of Clorox concentration:

Determination of the best concentration of Clorox Sodium hypochlorite (NaOCl) solution (about 0.75% active ingredient "Cl"), was the aim of this experiment. Surface sterilization was carried out by either one of the following concentrations: 0, 15, 20 and 25%. Sodium hypochlorite (NaOCl) solution. Explants were inoculated on ½MS medium under aseptic conditions using a laminar airflow cabinet. Jars were incubated at 25/20°C (day/night) ±2°C, 70% relative humidity. Two fluorescent tubes/shelf were installed at 30 cm above explants to provide light intensity of 2200-2400 lux at explants level. Total number of treatments was four. Each treatment comprised 3 replicates. Each replicate contained 3 jars with 1 explant/jar. Data obtained in this experiment were percentage of survived. This percentage represents the explants that were freed of contamination, stood surface sterilization and responded positively to the medium by growth and shoot proliferation.

Experiment 2: Effect of cytokinins (BA, Kin and TDZ) on multiplication:

A factorial experiment was carried out to study the effect of two factors on the multiplication process, the cytokinin type (BA, Kin and TDZ) and the cytokinin concentration (0, 1, 2 and 3 ppm) and the interaction between these two factors. The outcome of these two factors represented 9 treatments, each included 3 replicates, with 3 jars of one explant, in each replicate. Data obtained in this experiment were: number of shoots, shoot length and number of leaves. Shoots obtained from this experiment were used as a source of explants as lateral buds were excised and used for the next experiment. Design of all experiments was a completely randomized one. Data obtained were statistically analyzed using SAS 1995 computer program, and means were compared by L.S.D. method according to Snedecor and Cochran (1980) at 5% probability level.

RESULTS AND DISCUSSION

1 – Establishment stage:

Data recorded on the effect of Clorox concentration on survival percentage of *Clerodendron glabrum* and *Mussaenda luteola* explants are shown in table (1).

Table (1): Effect of Clorox concentration on survival percentage of *Mussaenda luteola* and *Clerodendron glabrum* explants

Clorox conc. %	<i>Mussaenda luteola</i>	<i>Clerodendron glabrum</i>
0	00.00 C	0 0.00 C
15	80.95 A	85.71 A
20	33.33 B	42.86 B
25	00.00 C	0 0.00 C
LSD at 5%	10.64	10.64

It is found that Clorox concentration had a significant effect on survival percentage. When no Clorox was used for sterilization, all explants suffered contamination and died eventually and the survival percentage was nilled. On applying Clorox at 15%, value of this character rose to the significantly highest level (80.95% and 85.71%, respectively). As Clorox concentration was increased to 20%, survival percentage decreased. Further increase in the concentration of the sterilizing agent to 25% had a detrimental effect, as no explant survived this relatively high concentration. These results were in an agreement of those obtained by Sawsan and Seham (2007) mentioned that for sterilization of *Cotoneaster horizontalis*, Decne explants, using (15% Clorox for 10 min) gave the best result for free contamination explants and the highest survival percentage.

2 – Multiplication stage:

2.1-Effect of cytokinin type and concentration on number of shoots/ explant of *Clerodendron glabrum* and *Mussaenda luteola*.

Data in table (2) showed that the cytokinin type affected significantly on number of shoots of *Clerodendron glabrum* and *Mussaenda luteola*. The

significantly highest numbers of shoots (3.31 and 3.19 shoots/ explants, respectively) were obtained by applying BAP, followed with a significant difference by those resulted when kinetin was used (2.28 and 2.06 shoots/ explants, respectively). The significantly lowest number of shoots (1 and 1.20 shoots, respectively) was observed in media supplemented with TDZ.

The cytokinin concentration had a significant effect on the number of shoots. In the absence of cytokinins from the media, the significantly lowest number of shoots was produced. Media augmented with cytokinins at 1 ppm resulted in the significantly highest number of shoots. As cytokinin concentration was raised to 2 ppm, value of this character decreased significantly. Further increase in cytokinin level to 3 ppm significantly decreased number of shoots. Media supplemented with TDZ produced the least value in this concern, i.e. 1.53 and 1.2 shoots, respectively.

Table (2): Effect of cytokinin type and concentration on number of shoots / explants of *Clerodendron glabrum* and *Mussaenda luteola*

Cytokinin conc. ppm	Cytokinin type			Mean
	BAP	Kin	TDZ	
<i>Mussaenda luteola</i>				
0	1.00 e	1.00 e	1.00 e	1.00 D
1	4.73 a	2.73 c	1.40 e	2.96 A
2	4.07 b	2.20 d	1.40 e	2.56 B
3	2.97 c	2.30 d	1.00 e	2.09 C
Mean	3.19 A	2.06 B	1.20 C	
LSD at 5%	Type 0.21	Conc. 0.24	Interaction 0.42	
<i>Clerodendron glabrum</i>				
0	1.00 f	1.00 f	1.00 f	1.00 D
1	4.97 a	3.20 c	2.40 d	3.52 A
2	4.07 b	2.50 d	1.50 e	2.69 B
3	3.20 c	2.40 d	1.20 ef	2.27 C
Mean	3.31 A	2.28 B	1.53 C	
LSD at 5%	Type 0.19	Conc. 0.22	Interaction 0.37	

The interaction between cytokinin type and concentration was found to have a significant effect on the number of shoots of /explant, as trends of the three curves representing values of this character as affected by each cytokinin at the levels used, were not the same according to the regression analysis. The significantly highest number of shoots/explant belonged to shoots grown on media augmented with BAP at 1 ppm, while the lowest record in the same concern was that of the untreated control shoots, in addition to those treated with TDZ at 3 ppm. As each of the three curves representing the effect of the three cytokinins at different levels had a different trend, the interaction between cytokinin type and concentration had a significant effect on the number of shoots. The significantly highest number of shoots (4.97 and 4.73, respectively) was found on medium supplemented with BAP at 1 ppm. All media free of cytokinin of any type (the control treatment) and those having TDZ at any concentration resulted in the significantly lowest number of shoots (either 1.00 or 1.40 shoots, with no

significant difference between the two records). These results were in harmony with Dahab (2007) who stated that MS medium plus BA at 2 mg/liter resulted in the highest number of shoots/explant of *Hydrangea macrophylla*. BA at 4 mg/liter produced the highest number of leaves/explant. Also, George (1993) stated that it was necessary to use BA to obtain multiple shoots of *Castanea* explants. However,

2.2 - Effect of cytokinin type and concentration on shoot length

The effect of cytokinin type on shoot length of two plants were found to be significant. The significantly longest shoots were a result of using TDZ, while the significantly shortest shoots were found on media supplemented with BAP. Media augmented with Kin resulted in an intermediate shoot length, significantly different from the two previously mentioned categories.

The cytokinin concentration effect on shoot length was significant. Apart from the increase in shoot length of *Mussaenda luteola* from 5.16 to 5.57 cm, and *Clerodendron glabrum* from 5.05 to 5.32 cm as cytokinin concentration rose from 2 to 3 ppm, the general effect of concentration was a negative one. This could be clearly discerned when a regression equation is deduced and a regression line is drawn. Accordingly, the longest shoots were the untreated control ones, the regression lines of these effects showed a progressive decline in shoot length as concentrations increased from 0 to 3 ppm. Regression line representing BAP effect declines at a sharper angle compared to that of the TDZ effect, while the Kin angle of decline was intermediate.

Table (2.2) Effect of cytokinin type and concentration on shoot length (cm) of *Mussaenda luteola* and *Clerodendron glabrum*

Cytokinin conc. Ppm	Cytokinin type			Mean
	BAP	Kin	TDZ	
<i>Mussaenda luteola</i>				
0	7.40 a	7.40 a	7.40 a	7.40 A
1	4.80 d	5.20 cd	7.20 a	5.73 B
2	3.95 e	6.07 b	5.47 bc	5.16 C
3	3.97 e	5.90 b	6.83 a	5.57 B
Mean	5.03 C	6.14 B	6.73 A	
LSD at 5%	Type 0.30	Conc. 0.35	Interaction 0.61	
<i>Clerodendron glabrum</i>				
0	7.73 a	7.73 a	7.73 a	7.73 A
1	3.97 e	5.47 cd	5.37 cd	4.93 B
2	3.68 e	6.03 cb	5.43 cd	5.05 B
3	4.23 e	5.10 d	6.63 b	5.32 B
Mean	4.90 B	6.08 A	6.29 A	
LSD at 5%	Type 0.36	Conc 0.41.	Interaction 0.71	

The effect of interaction was significant on shoot length as the curves representing shoot length at different concentrations of every cytokinin inclined downwards at different angles according to the regression analysis. The significantly longest shoots were those untreated with any cytokinin, while the corresponding shortest ones were those treated with BAP at 1, 2 or 3 ppm, respectively. Dahab (2007) stated that MS medium plus BA at 2

mg/liter resulted in the highest shoot length of *Hydrangea macrophylla*. BA at 4 mg/liter produced the highest number of leaves/explant. Kale *et al* (2004) remarked that BAP (0.1-2.0 mg/liter) had significant effects on plantlet height of sugarcane (Co.740). Sawsan and Seham (2007): on *Cotoneaster horizontalis* Decne found that, shoot let length increased by adding (2 mg/l BA) to MS-medium. On *Philodendron scandens*, Koriesh and Al-Manie (2000) found that the media Supplemented with 2 mg/L BA significantly increased the number and length, of shoots. Economou and Spanoudaki (1984) studied the influence of growth regulators on *in vitro* *Gardenia jamioides*. BA produced most shoots but length and quality were less than with KI. Evaldsson (1985) cultured shoots of *Cordyline terminalis* with various cytokinins. Shoot length decreased with increasing concentration of BA. For kinetin the opposite was true. Mujib and Pal (1995) obtained the highest number of *carnation* shoots in the presence of low concentration of BA (0.5mg /L) but KI increased shoot length. Schwengber *et al.*, (1999): on apple rootstock cv. Mark TDZ showed better results than BAP for shoot length. Concentrations between 6 and 7 micro M of both TDZ and BAP showed the best results.

2.3 - Effect of cytokinin type and concentration on number of leaves of *Mussaenda luteola*.

The effect of cytokinin type on the number of leaves of was found to be insignificant. However, the highest number of leaves belonged to shoots treated with BAP, and the lowest one to shoots of *Mussaenda luteola* treated with Kin. TDZ treatment resulted in an intermediate value in this concern (9.29).

While, the number of leaves of *Clerodendron glabrum* was significantly affected by cytokinin type. The greatest number of leaves (8.73) was produced by shoots grown on media augmented with BAP. The lowest value in this regard belonged to shoots grown on media supplemented with TDZ (7.41 leaves). However, this later value was not significantly different from the corresponding value of shoots grown on media supplemented with Kin (7.83 leaves).

The cytokinin concentration affected the number of *Mussaenda luteola* leaves significantly and positively. As concentration rose from 0 to 1 ppm, this record increased from 6.77 to 7.97 leaves, respectively. When cytokinin level increased to 2 ppm, the number of leaves increased, though insignificantly. Further increase in cytokinin concentration to 3 ppm resulted in a significant increase in the number of leaves to the highest value.

The interaction between cytokinin type and concentration proved to have a significant effect on the number of *Clerodendron glabrum* leaves as the curves representing shoot length at different concentrations of every cytokinin inclined downwards at different angles according to the regression analysis. The significantly highest number of leaves (10.10) was a result of using BAP at 3 ppm, while the significantly lowest values were observed on media of the untreated control media (6.77 leaves) and on media supplemented with TDZ at 2 or 3 ppm (7.17 and 7.53 leaves, respectively).

The interaction between cytokinin type and concentration had an insignificant effect on the number of *Mussaenda luteola* leaves. Despite this

fact, the highest number of leaves was produced by the untreated control shoots (12.17 leaves), while the lowest number (7.30 leaves) belonged to shoots treated with TDZ at 2 ppm

Table (2.3) Effect of cytokinin type and concentration on number of leaves of *Mussaenda luteola* and *Clerodendron glabrum*

Cytokinin conc. Ppm	Cytokinin type			Mean
	BAP	Kin	TDZ	
<i>Mussaenda luteola</i>				
0	12.17 a	12.17 a	12.17 a	12.17 A
1	7.87 a	7.73 a	9.30 a	8.30 B
2	8.97 a	7.77 a	7.30 a	8.01 B
3	9.87 a	9.33 a	8.40 a	9.20 B
Mean	9.72 A	9.25 A	9.29 A	
LSD at 5%	Type	N.S.	conc 1.31	Interaction N.S
<i>Clerodendron glabrum</i>				
0	6.77 f	6.77 f	6.77 f	6.77 C
1	8.67 b-d	7.07 f	8.17 de	7.97 B
2	9.40 ba	8.30 c-e	7.17 f	8.29 B
3	10.10 a	9.17 a-c	7.53 ef	8.93 A
Mean	8.73 A	7.83 B	7.41 B	
LSD at 5%	Type 0.50	Conc 0.58	Interaction 1.00	

Evaldsson (1985) on *Cordyline* and Araújo *et al.*, (2004): on *Sinningia speciosa*. On the other hand Khattak *et al.*, (2004) reported that, the number of formed leaves from apple seed increased with 60 (8.6) and 40 micro M BA (8.0) but decreased with thidiazuron at all concentrations.

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تأثير نوع السيتوكينين وتركيزه علي معدل التضاعف لبعض نباتات الزينة
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تم اجراء هذه التجربة في معمل زراعة الأنسجة بحديقة الزهرية التابع لمعهد بحوث البساتين بالجيزة بغرض تأسيس بروتوكول اكثار سريع لنباتي الموزيندا و الكليرودندرون من خلال تقنية زراعة الأنسجة. ولقد اظهرت النتائج المتحصل عليها ان لتركيز الكلوروكس تأثير معنوي على النسبة المئوية لبقاء منفصلات الموسيندا والكليرودندرون. وعند استعمال الكلوروكس بتركيز ١٥% فإن هذه النسبة للنباتين وصلت لأعلى مستوى لها بدرجة معنوية . من ناحية أخرى كان لنوع السيتوكينين تأثيرا معنويا على عدد الأفرع وطول الأفرع لكل من الموسيندا والكليرودندرون . وقد كان عدد الأفرع الأعلى معنويا عند استعمال BAP . أما العدد الأقل معنويا فقد لوحظ في البيئات المزودة بمادة TDZ . وعلى العكس من ذلك ، كان تأثير السيتوكينينات الثلاثة على صفة طول الأفرع. وكان للتفاعل بين نوع السيتوكينينات وتركيزها أثرا معنويا على عدد الأفرع وطول الأفرع لكل من الموسيندا والكليرودندرون. ففي كلا النباتين نتج العدد الأكبر معنويا من الأفرع على البيئة المزودة بمادة BAP بتركيز ١ جزء في المليون ، بينما تسببت البيئات الخالية من أى نوع من السيتوكينينات (معاملة الكنترول) في إنتاج العدد الأقل معنويا من الأفرع. وعلى العكس من ذلك ، فإن الأفرع الغير معاملة كانت الأطول معنويا ، بينما كانت الأقصر معنويا هي تلك التي عوملت بمادة BAP . إقتصرت الأثر المعنوي للتفاعل بين نوع السيتوكينينات وتركيزها على عدد الأوراق على نبات الكليرودندرون فقط. ولقد اوضحت النتائج إن إستجابة النباتين متناقضة نوعا ما. فقد نتج العدد الأكبر معنويا من الأوراق على أفرع الموسيندا الغير معاملة (الكنترول) وعلى أفرع الكليرودندرون المعاملة بمادة BAP بتركيز ٣ جزء في المليون. بينما نتج العدد الأقل من أفرع الموسيندا المعاملة بمادة TDZ بتركيز ٢ جزء في المليون ، وعلى أفرع الكليرودندرون النامية على بيئات الكنترول.