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NEW ALTERNATIVE METHOD OF DRUMSTICK TREE (*Moringa oleifera* Lam.) MICROPROPAGATION.

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ABSTRACT

Tissue culture protocol reported in this study is an alternative method suggested to produce plantlets of drumstick tree (Moringa oleifera Lam.) with uniform genotypes for breeding, in vitro and in vivo production. The highest rate of micropropagtion will often depend not only on the selection of the most suitable explants, but also on sterilization agent and the best basal medium for that tissue. Sterilizing shoot tip explants of drumstick, using 15 % of clorex for 15 minutes showed the highest survival percentage and lowest contamination as well as sufficient browning percentage.

For multiple shoot induction, the shoot tip explants of drumstick were cultured on Murashige and Skoog (MS) agar medium supplemented with different concentrations of 2-isopentinyl adenine (2iP) at (0.0,0.5,1.0 and 2.0 mg/L) alone or in combination with indol butyric acid (IBA) at (0.0,0.1,0.5and 1.0 mg/ L). The highest shoot multiplication rate was found in the 3rd subculture. Moreover, the highest shoot number/explant (8.8) was obtained with the medium contained 0.5mg/l 2iP with 0.1mg/ L IBA and maintained at 25±2C compared to all other treatments. The control treatment alone significantly showed the highest shoot length compared to all other treatments Low concentrations (0.5mg/L) with (0.1ml/L IBA) were more effective in increasing the number of leaves. Plantlets grown on the control medium produced the maximum number of healthy roots. The rooted plantlets were hardened and successfully established in the soil.

Key words: *Moringa oleifera* Lam., drumstick tree, shoot tip, sterilization, shoot multiplication.

INTRODUCTION

Moringa oleifera Lam. Family: *Moringaceae* is adapted to a wide range of soil types but it grows best in well drained loam to clay loam soil. Moringa is not a nitrogen fixing tree, but its fruits, flowers and leaves

contain 5 to 10% protein in average (F/FRDP, 1992). All of these parts are eaten widely as vegetables, providing excellent food for human. The pods are cooked and eaten like green beans and its reported to contain 2.5 g protein/100g and the same mass of drumstick leaves also contain 440 mg Calcium, 70 mg P, 7mg Fe, 110 µg Cu, 5.1, µg I, 11300 IU Vit A, 10µg Vit B, o.8 mg nicotinic acid and 220 mg ascorbic acid/100 g. According to Burkill (1966) the seeds yield a clear inodorous oil to the extent of 22 to 38.5%,this oil is sweet and non-sticking, It often extracted for lubricating watches other delicant machinery. Pterygospermin is a bactericidal and fungicidal compound isolated from drumstick. Root bark yields two alkaloids, moringine and moringinine. Moringinine acts as cardiac stimulant, produces rise of blood pressure, and acts on sympathetic nerve endings as well as smooth muscles all over the body. The flowers, leaves, roots, barks are used in folk remedies for tumers, abdominal discomfort, boils, cold, high blood pressure, hysteria, relapsing fever, skin diseases, rheumatism etc. Hartwell (1967-1971). The main objective of the research is how could be brought moringa plants to human throughout the year. The shoot multiplication rate is most significant in terms of shoot production. Cytokinins are usually added to culture media to stimulate axillary or adventitious shoot development. The type and concentration of cytokinin used have profound effects on shoot multiplication Therefore, we aimed to establish an efficient *in vitro* method (new source of plant) of propagation of *Moringa oleifera* Lam., that was conducted by studying the following:

1. Effect of different concentrations of the more cheaper aseptic agent chlorex (sodium hypochlorite solution 5.25 mg/L) on explant sterilization of *Moringa oleifera* Lam. *in vitro.*
2. Effect of different concentrations of (2iP), IBA and there combinations on number of shoots, shoot length and number of leaves of *Moringa oleifera* Lam. *In vitro.*

MATERIALS AND METHODS

All experiments of this part were carried out in Tissue Culture Laboratory, Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City University, Sadat City, Egypt during the year 2013.

1-Plant materials, explant preparation and sterilization:

Mother plants of *Moringa oleifera* Lam. were obtained from Agriculture Research Center, Egypt, the plants characterized by growth

vigor. Shoot tips of 1 cm length and 0.5 thicknesses were used as explants. Explants were washed under running tap water for one hour. To sterilize those explants, the experiment was conducted to study the effect of different concentrations and sterilization periods of chlorex (sodium hypochlorite solution 5.25mg/L) on explants surface sterilization of *Moringa oleifera* Lam. *in vitro*. According to the following treatments.

The explants (shoot tips) were immersed in different sodium hypochlorite levels (5, 10, 15 and 20 %) at different immersing periods (5, 10,15 and 20 min). Two drops of tween 20 emulsifier were added to each treatment. Then, they were rinsed three times in sterile distilled water to remove the residuals of disinfectants. After surface sterilization the external parts of explants were removed and the explants of 0.5cm were placed on the nutrient free hormone MS agar medium. After 4 weeks, data were recorded as survival, contamination and browning percentages of drumstick tissue cultures.

Media preparation:-

Murashige and Skoog (MS) basal medium (1962) supplemented with 30 gram sucrose and 8 gram agar per liter was used in all stages of this study. After preparing the medium and prior to agar addition, the pH was adjusted to 5.8 by using a few drops of either potassium hydroxide (1 N KOH) or hydrochloric acid (1 N HCL). The nutrient media were heated to dissolve the agar. The medium was poured into culture jars of size 150 ml and polypropylene closure caps, where each jar contained 20 ml of medium. The medium was autoclaved at 121^{o}cand 1.2 kg/cm^2 for 20 min, then, it was cooled and kept in an appropriate position for one week in order to examine any infection or contamination in jars before the culturing process.

Culture conditions:

The cultured jars were kept at 25 ± 2^{o}c with light intensity 1500 lux for 16 hr photoperiod using cool white fluorescent lamps.

2- In vitro shoot induction and multiplication: -

Survived plantlets, obtained from the previous stage were cultured on the experimental media that contained MS medium supplemented with 2-isopentinyl adenine (2ip) at different concentrations (0.0, 0.5, 1.0, 2.0 and 3.0 mg/L) in combination with indol butyric acid (IBA) at different concentrations (0.0, 0.1, 0.5 and 1.0 mg/L). The medium contained 30 g /L sucrose and 8 g /L agar. This experiment was conducted to study shoot formation of drumstick *in vitro*. Each treatment was represented by 12 replicates and each jar contained one explant. The regenerated numbers of shoots and the highest proportional shooting response were recorded after

three weeks of culture. Regenerated shoots were subcultured every three weeks onto the freshly prepared medium that was subjected to produce the highest proportional response. Numbers of shoots, shoots length and numbers of leaves per cluster were recorded at the end of each three weeks culture period for a total of five generations. The experiments were carried out as a factorial in completely randomized block design (CRBD) and repeated twice and analyzed by analysis of variance (ANOVA) according to Gomez and Gomez (1984). Explants were cultured in 350 ml jars each contained 50 ml medium. Each treatment was replicated three times.

3- In vitro Rooting and Acclimatization:

This stage aimed to produce hardened plantlets of *Moringa oleifera* Lam. Therefore, the obtained shoots transferred onto the rooting media, consists of MS medium alone to initiate the maximum percentage of rooting Shahina Islam *et al*. (2005). After 4 weeks of transfer to rooting media, plantlets were removed from the culture medium with well-developed shoots and roots washed gently under running tab water then transferred in plastic pots (7.5×10.5 cm), containing peat moss: sand (1:1, v: v) every pot contained one plantlet covered with plastic bag to maintain high relative humidity around the plantlet in the greenhouse. The plastic bags were bored every day in order to get rid of excess humidity. Then bags were removed after one month of transplanting. The obtained plants were transferred into 30 cm pots and kept to grow *in vivo* for another month before transplanting to the open field.

RESULTS AND DISCUSSION

1. Chemical sterilization of explants:

- Effect of different concentrations of chlorex (sodium hypochlorite solution 5.25mg/l) on explant sterilization of *Moringa oleifera* Lam. ***in vitro.***

Sporadic records were obtained in survival, contamination and brownig of explants when the sterilization was carried out using different concentrations of NaOCl. Data in Table (1) indicate that the highest percentage of the shoot tip survival (75.2%) was recorded when the explants were treated with 15 % chlorex for 15 minutes. Lowest survival percentage (17 %) was observed at the concentration of 5 % chlorex for 5 minutes. As for the concentration, the minimum contamination percentage (35.0 %) was observed when the explants were treated with 15 % chlorex for 15 minutes. Concerning the browning, the lowest browning percentage (18.6 %) was recorded when the explants were treated with 5 % chlorex for 5 minutes.

Table 1: Effect of chlorex concentrations (%) on explant sterilization of *Moringa oleifera* Lam. *in vitro*(%).

Chlorex	Time (min)	Survival	Contamination	Browning
5.0	5	17.0	90.0	18.6
	10	14.3	87.5	20.6
	15	14.2	80.9	35.2
	20	14.6	80.0	66.3
10.0	5	18.4	65.2	33.0
	10	21.1	59.8	32.8
	15	24.5	52.6	40.5
	20	22.0	49.0	60.1
15.0	5	53.3	60.0	23.3
	10	60.5	60.9	23.3
	15	75.2	35.0	45.0
	20	30.0	70.0	51.6
20.0	5	20.0	70.5	56.6
	10	16.6	66.4	73.0
	15	13.3	66.0	76.6
	20	13.3	63.3	100 .0

The previously mentioned results concluded that, using 15 % of clorex for 15 minutes showed the highest survival percentage and lowest contamination as well as sufficient browning percentage compared to the other treatments. These results are in agreement with those obtained by Mazrou (2008) on lemongrass, recommended to use shoot tips in micropropagation of lemongrass. Also, Maksoud (2007) concluded that explants were washed thoroughly with running tap water then; they were immersed in 1.5% NaOCl to obtain clean tissue culture of rosemary plant.

2- Multiplication stage:

- Effect of cytokinin (2iP) and auxin (IBA) on multiplication stage of *Moringa oleifera* Lam. ***in vitro.***

Number of shoots:

In Table 2 and Figure 1, data on the main effect of 2iP concentrations indicate that the cultured explants of *Moringa oleifera* Lam on MS nutrient medium with 0.5mg/l 2iP recorded the best result for number of shoots (6.92/explant) compared to the other treatments. Concerning the main effect of IBA using MS medium with 0.1mg/L IBA significantly presented higher number of shoots (4.65 /explant). Data of the interaction clear that, the greatest shoot number/explant (8.8) was obtained with the medium contained 0.5mg/L 2iP with 0.1mg/L IBA compared to other treatments (Table 2).

Table 2: Effect of different concentrations of (2iP), IBA and their combinations on number of shoots of *Moringa oleifera* Lam ***in vitro.***

Cytokinin (2iP) mg/L	Number of shoot				Mean(A)
	IBA (mg/L)				
	0.0	**0.1**	**0.5**	**1.0**	
Control	1.20	1.30	1.20	1.40	**1.27**
0.5	5.50	8.80	5.80	5.60	**6.92**
1.0	5.20	5.30	4.70	4.90	**5.27**
2.0	4.40	4.50	3.22	3.80	**3.98**
Mean (B)	**4.07**	**4.65**	**3.73**	**3.92**	
LSD 5%					
A	**0.76**				
B	**0.52**				
A*B	**1.52**				

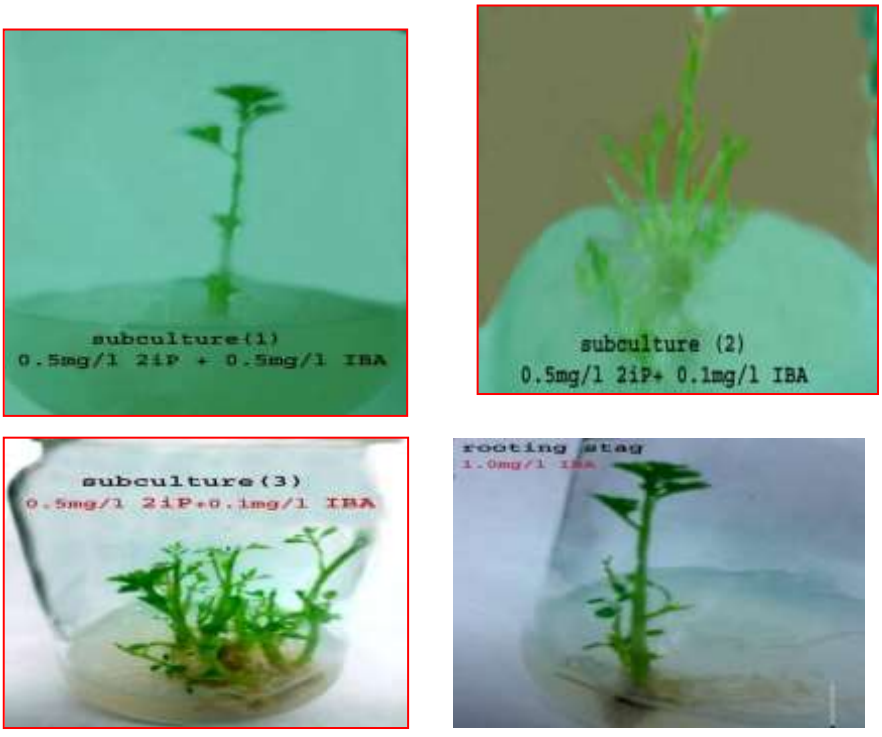

Figure 1: Effect of different concentrations of (2iP), IBA and their combinations on number of shoots of *Moringa oleifera* **Lam *in vitro.***

Shoot length:

Data on the main effect of cytokinin (2iP) concentrations on shoot length (Table 3) observe that the addition of 2ip to the medium did not stimulate the shoot length, and highest shoot length was significantly obtained with hormone-free medium (control). Regarding the main effect of IBA on shoot length, the same trend as that of different cytokinins was observed, and control surpassed all IBA concentrations in that concern. Concerning the interaction, interestingly, the control treatment alone significantly showed the highest shoot length compared to all other treatments. Such results were in harmony with Thidarat *et al.* (2012) who stated that explants grown on MS medium without growth regulator were elongated and grown to be new drumstick plantlets with healthy roots.

Table (3): Effect of cytokinin (2iP) and IBA on shoot length (cm) of *Moringa oleifera* Lam ***in vitro.***

Cytokinin (2iP) mg/L	**Shoot length**				**Mean(A)**
	IBA (mg/L)				
	0.0	**0.1**	**0.5**	**1.0**	
Control	4.50	3.00	2.800	2.80	**3.150**
0.5	2.40	2.60	2.40	2.40	**2.450**
1.0	2.80	3.60	2.80	2.40	**2.900**
2.0	2.40	1.60	1.60	1.40	**1.750**
Mean(B)	**3.02**	**2.70**	**2.40**	**2.25**	
LSD at Level 5% A	**0.34**				
B	**0.26**				
AxB	**0.69**				

Number of leaves:

In Table (4) data on the main effect of cytokinins (2iP) on number of leaves/explant indicate that the treatment contained 0.5mg/L 2iP resulted in the highest value of number of leaves/explant (20.35) compared to other treatments including control. However, data on the main effect of IBA on number of leaves/explant show different response, as the control and the medium contained 0.1mg/L IBA (15.95) /explant) significantly showed higher response and surpassed all treatments in increasing the number of leaves. The original data show that the higher and significantly similar increase in number of leaves /explant was observed with the treatments contained 0.5mg/L BA with 0.1mg/L IBA (25.40 /explant).

Interestingly, combinations contained 2iP especially at low concentration (0.5mg/L) with (0.1ml/L IBA) were more effective in increasing the number of leaves compared to other treatments including

Table (4): Effect of different concentrations of (2iP), IBA and their combinations on number of leaves of *Moringa oleifera* Lam**.** ***in vitro*.**

Cytokinin 2ip (mg/L)	Number of leaves				Mean (A)
	IBA (mg/L)				
	0.0	**0.1**	**0.5**	**1.0**	
(Control)	6.6	7.6	6.8	8.0	**5.12**
0.5	16.0	25.4	20.8	19.2	**20.35**
1.0	15.6	16.4	13.0	12.9	**14.47**
2.0	13.0	14.4	12.6	12.0	**13.00**
Mean(B)	**12.**	**15.9**	**13.3**	**13.0**	
LSD at Level 5% A	**0.24**				
B	**0.12**				
AxB	**0.49**				

control. Such stimulating effects of combinations between auxin and cytokinin were well supported by Husain and Anis (2009) on *Melia azedarach* L.

Acclimatization

At acclimatization, the survival percentage of plantlets was 95% when calculated in the greenhouse after 30 days of transplanting to a soil mixture contained sand, peatmoss in equal volumes Figure 1. In that concern, Aboshama and Emara (2004) on *Gerbera jamesonii* H. BOLS demonstrated that the plantlets were successfully acclimatized when transferred to pots contained soil mixer of peat moss: sand in equal volumes (1:1).

Conclusively, it was recommended to use shoot tips in micropropagation of *Moringa oleifera* Lam**.** and to sterilize these explants using 1.5% NaOCl. In addition, the highest shoot number/explant (8.8) was obtained with MS medium contained 0.5m g/L 2iP and 0.1 mg/L IBA. Plantlets grown on free hormone MS medium with healthy roots could be successfully acclimatized. The survival percentage of plantlets was 95% in the greenhouse.

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طريقة بديلة لإكثار شجرة المورنجا معمليا

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أجريت هذه الدراسة باستخدام تقنية زراعة الأنسجة النباتية لإنتاج شتلات المورنجا المتماثلة وراثيا لاستخدامها في برامج التربية والإنتاج معمليا و حقليا. هذا ولا يتوقف الحصول على أعلى معدل للإكثار الدقيق على اختيار المنفصل النباتي فقط ولكن على طريقة تعقيمه وعلى مكونات بيئة زراعة الأنسجة أيضا.

أدى استخدام القمم النامية لأفرع المورنجا وتعقيمها باستخدام الكلوركس (٢,٥% صوديوم هيبوكلورايت) بتركيز ١٥% لمدة ١٥ دقيقة للحصول على أفضل النتائج من حيث أعلى حيوية المنفصل النباتي وأقل نسبة تلوث و تلون بني للأنسجة. في مرحلة التضاعف ولإنتاج العديد من الافرع تم زراعة القمم النامية على بيئة موراشيج وسكوج المدعمة بالأجار وبتركيزات مختلفة من الايزوبنتينيل ادينين 2iP بتركيزات (صفر، ٥و٠ ، ١، ٢ ملليجرام/لتر) وحمض الاندول بيوتيريك IBA بتركيزات (صفر، ١و ،٥و ، ١ ملليجرام/لتر) .

أوضحت النتائج أن أعلى معدل للتضاعف حصل عليه بعد النقلة الثالثة وأعلى عدد للأفرع معنويا (٨ و٨) تم الحصول عليه من البيئة المحتوية على ٥و ملليجرام/لتر2iP مع ١و ملليجرام/لتر IBA مقارنة بالمعاملات الأخرى واطول الأفرع معنويا تم الحصول عليه من بيئة الكنترول.

التوصية: أدى استخدام التركيزات المنخفضة ٥و٠ ملليجرام/لتر 2iP - ١و٠ ملليجرام/لتر IBA إلى زيادة عدد الأوراق. أعطت الأفرع النامية على بيئة الكنترول أكبر عدد من الجذور السليمة و قد تم أقلمة الشتلات وزراعتها فى اصص محتوية على خليط من البيتموس والرمل بنسبة ١:١ لمدة شهر ثم تم نقلها الى التربة بنجاح.