J. Plant Production, Mansoura Univ., Vol. 9 (3): 281 - 288, 2018

In Vitro Callus Induction and Plant Regeneration of *Aloysia triphylla*, a High Value Aromatic and Medicinal Plant

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ABSTRACT

An efficient *in vitro* callus induction and plant regeneration Protocol has been established for a value aromatic and medicinal plant, *Aloysia triphylla* by using shoots tips and leaf disc as explants. The nutrient MS medium with different concentrations of auxins NAA, IBA and 2,4-D alone or in combination with different cytokinins BA, TDZ and Kin were used to induce callus formation. Shoots tips and leaf disc cultured on MS medium supplemented with 2,4-D at 1.5 mg/L in combination with BA at 1 mg/L induced Maximum callus formation percentage of 91.7 and 100 %, respectively coupled with the highest callus fresh weight of 9.71 and 10.66 g, respectively. For Callus differentiation, callus derived from shoot tip and culture on half strength MS media fortified with TDZ at 2 mg/L and GA_3 at 0.5 mg/L recorded 100 % callus differentiation percentage and significantly the highest value of shoots number (12.92 shoots). Healthy regenerated shoots were rooted *in vitro* on MS containing 0.5 mg/L IBA. Plantlets with well-developed root and shoot systems were successfully acclimated (97%) and established in pots containing mixture of soil, sand and peatmoss (1: 1: 1).

Abbreviations: Murashig and Skoog (MS), a-naphthalene acetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), 6-benzyladenine (BA), thidiazuron (TDZ) and Kinetin (Kin)

INTRODUCTION

Aloysia triphylla. L'her (lemon verbena) is an aromatic and medicinal semi-shrub which belongs to family Verbinaceae. It is native to South American countries (Vogel *et al.*, 1999; Botta, 1979). Lemon verbena is cultivated mainly due to the lemon aroma emitted from its leaves which are the most economical part of the plant that can be used to add a lemony taste in tea, salads, milk, jellies and ice creams (Hanna *et al.*, 2011; Beemnet *et al.*, 2013). Also, the aromatic oil obtained from the leaves is used in food flavoring industries, fragrance industries, soft drink industries and folk medicine. Traditionally, it is used for treatments of cold, spasms and fever as folk remedy (Carnat *et al.*, 1999), colic, flatulence, asthma, diarrhoea, insomnia, indigestion and anxiety (Newal et al., 1996; Cowan, 1999; Durat and Chritina, 2005). *Aloysia*'s essential oil has been shown anti-oxidant, antibacterial and anti-fungal properties due to its chemical composition (Hanna *et al.*, 2011).

Recently, Egypt gave a big concern to production and cultivation of new essential plants like lemon verbena to cover the increasing demands of the local industries as well as, for export. In order to expand cultivation of *A.triphylla*, the first step is the production of high quantities of genetically homogeneous plant material, since it is difficult to obtain seeds owing to Egyptian climate; usual multiplication procedure is vegetative propagation (cutting). However these methods are not efficient enough to produce mass production needed because it isn't easy through seeds, since it need to maintain a very high soil temperature and even cutting are tricky as mentioned by Keville (1999). So it appears that using micropropagation technique is very important to offers a potential deliver of large quantities of disease-free, true-to type healthy stock within a short span of time. There are few reports on direct micropropagation of *A.triphylla*, which reported by Zhang *et al*. (2005), Severin *et al.* (2006) and Sharaf El-Din *et al.* (2011). But tell this study, there was no mention of indirect micropropagatio of *A.triphylla*.

Hence, the present study aimed to establish a methodology for indirect micropropagatio of lemon verbena (*A.triphylla*) to achieve high-frequency shoot induction and plant regeneration, starting from a callus.

MATERIALS AND METHODS

The present research was performed in Mansoura University, Faculty of Agriculture, Vegetable and Floriculture Department at the experimental station and tissue culture laboratory from March 2012 to December 2013.

Plant material Preparation:

Healthy young shoots of *Aloysia triphylla* were collected from an adult plant growing in the Faculty of Agriculture, Mansoura University at the farm of Medicinal and Aromatic plant. The terminal shoots were about 10 cm in length and about one month old. The shoots were washed thoroughly under running tap water containing a few amount of Tween- 20 detergent for one hour. The surface sterilization was done with 3 % sodium hypochlorite (NaOCl) solution for 8 min and finally washed four times with sterile distilled water for 3 min each. In the laminar flow hood cabinet Shoots were cut into two explant parts (shoot tips and mature leaves) with length of 1-1.5 cm for the first explant and leaf disc about 1.0× 1.0 cm^2 for the second explant.

Culture conditions and media composition:

The nutrient medium used in all experiences was MS medium (Murashig and Skoog medium, 1962) which fortified with 3% (w/v) sucrose and before solidify the medium with 7g agar /l the pH of the medium was adjusted to 5.75 using 0.1 N NaOH or 0.1 N HCl. About 25 ml of the media were added in cultured jars (250 ml) and after sterilization the media by autoclaving at 121° C and under 1.2 bar pressure for 20 min they were preserved in plant growth room under constant fluorescent light of 2500 Lux for 16/8 h (light/ dark) photoperiod at 25 ± 2°C.

Induction of callus:

The experiment was conducted in order to study the effects of various concentrations of different auxins on callus induction from the two explants (shoot tips and mature leaves), the nutrient MS basal medium was supplemented with various auxins types NAA, IBA and 2,4-D at different concentrations of 0.0, 0.5, 1, 1.5 and 2 mg/L. Then the superior auxin type result obtained from the previous experiment (2,4-D at 1.5 mg/L) were further combined with three cytokinins; Kin, BA and TDZ at different concentrations of 0.25, 0.5, 1 and 1.5

mg/L in order to study the effect of combination between auxin and cytokinin on callus induction from the two explants. A factorial experiment was used with 4 replicates included 12 jars for each treatment. After 30 day of culture, data were recorded.

Callus differentiation:

For plant regeneration, different friable callus resources (callus from shoot tip and leaf disc) derived from MS medium supplemented with 2,4-D at 1.5 mg/L in combination with BA at 1 mg/L (the best result in the callus induction experiments) were cut into piece of 1 × 1 cm^2 and inoculated on MS medium supplemented with three types of cytokinins (BA, Kin and TDZ) each alone at different concentrations of (1.0, 1.5, 2.0 and 2.5 mg/L).

In order to improve plant regeneration another experiment was carried out by using MS medium at full, ¾ and ½ strength in combination with GA_3 at different concentrations (0.0, 0.5 and 1.0 mg/L), supplemented with TDZ at 2.0 mg/L and cultured with just friable callus obtained from shoot tip (the best result in the previous experiment). After 4 weeks of culture the experiments, data of callus percentage forming shoots, shoots number and shoot length were registered. A factorial experiment was used with 4 replicates included 12 jars for each treatment.

Induction of rooting and acclimatization:

For root induction, individual *in vitro* healthy shoots (3 – 4 cm long) were takeout from 4 weeks old shoot regeneration and transmitted to half strength MS basal medium supplemented with IBA at concentration of 0.5 mg/L for 4 weeks. The *in vitro* rooted plants were takeout from the medium, removing all traces of media by washing with warm water and then single plantlets were transmitted to pots filled with soil: sand: peatmoss (1: 1: 1) for acclimatization.

Statistical analysis:

A factorial experiments in a complete randomize design were used with the all experiments designed. Data of all experiment was subjected to analysis of variance (ANOVA) by using COSTAT v. 63 statistical software. Mean comparisons between groups were achieved using the least significant difference (LSD) at $P \leq 5$ % according to (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

I- Callus induction stage:

1- Effect of explant type and auxin type at different concentrations on callus induction.

Effect of two explant types and various auxins (NAA, IAA and 2,4-D) at different concentrations were studied to improve callus induction of lemon verbena (Table 1). The response of callus formation % and callus fresh weight varied with the type of explants, auxin type and their concentration. However, it was a matter of importance to mention that culturing the two explant types on the control medium (free auxin medium) failed entirely to give callus for both of them.

In general, callus induction was observed in both explant types with all auxins tested but the frequency of callus induction from leaf disc was higher rather than shoot tip. Also, it was observed that 2,4-D had a higher effect on increasing the callus formation % and callus fresh weight, when compared with NAA and IBA at the same concentration with the two explants types. The next positive effects were recorded for leaf disc and shoot tip explants which cultured on medium supplemented with NAA. While, it was obvious that IBA at the different concentrations significantly possess the lowest value of callus induction.

Data in the same table cleared that culturing leaf disc and shoot tip explants on MS media supplemented with 2,4-D at the concentration of 1.5 mg/L proved to be the best media for callus formation percentage of 91.7 and 83.3 %, respectively and callus fresh weight of 7.85and 6.94 g, respectively and increased concentration to 2 mg/L decreased the frequency of callus production. This result was similar with the findings of Urrea *et al.* (2009) on *Lippia dulcis* and Boustani *et al.* (2016) on *Lippia citriodora*.

Table 1. Effect of explant type, auxin type, auxin concentration and their interactions on callus induction of lemon verbena.

Treatments		callus formation %		callus fresh weight (g)	
Auxin type	**Auxin conc. mg/L**	**Shoot tip**	**Leaf disc**	**Shoot tip**	**Leaf disc**
	0.00	0.0	0.0	0.00	0.00
	0.5	25.0	33.3	0.65	1.53
NAA	1.0	41.7	50.0	2.26	2.66
	1.5	66.7	75.0	4.86	5.79
	2.0	50.0	58.3	3.39	4.30
	0.00	0.0	0.0	0.00	0.00
	0.5	0.0	0.0	0.00	0.00
IBA	1.0	16.7	33.3	0.33	1.25
	1.5	41.7	50.0	1.79	2.43
	2.0	33.3	33.3	1.23	1.85
	0.00	0.0	0.0	0.00	0.00
	0.5	41.7	50.0	3.53	4.73
2,4-D	1.0	66.7	75.0	5.11	6.33
	1.5	83.3	91.7	6.94	7.85
	2.0	66.7	83.3	5.81	6.23
L.S.D. at 5 %		18.7		0.28	

Generally depending on the species, callus can be initiated from a variety of tissues by employing the appropriate growth medium. However, rapid cell division can be more easily induced in some tissue than in others (Yadav and Tyagi, 2006). Also, auxin is necessary for callus induction; since the mentioned hormone is cause to an increase in protein synthesis through enhancing transcription of RNA (Yang *et al.*, 2012), so it would be related to proliferation in cells and forming a callus. In this experiment incorporation of 2,4-D in MS media was found to play a big role in callus induction and this is with the agreements of Yadav and Tyagi (2006) and Ge *et al*. (2016) whom reported that 2,4-D is one of the most important synthetic auxin and usually considered to be one of the strongest and most commonly used auxins for callus induction in plant tissue culture studies. This may be refer to that 2,4-D had a positive impact on the molecular and physiological process of callus by inducing specific proteins, regulates the endogenous IAA metabolism, and control DNA methylation (Pan *et al.*, 2010).

2- Effect of 2,4-D, explant type and cytokinin type at different concentrations on callus induction.

In addition to improve callus induction from the two explants types (shoot tips and mature leaves) the best result obtained from Auxins (2,4-D at 1.5 mg/L) was further combined with three cytokinins; Kin, BA and TDZ at different concentrations of 0.25, 0.5, 1 and 1.5 mg/L.

Data in Table (2) showed that culturing leaf disc explants in that interaction always had the upper hand in the respect of callus formation percentage and recorded the highest results, when compared with the shoot tip culturing at the same cytokinin type and the same concentrations. It could be noticed that, there was no significant differences were found among the all treatments, but the percentage of 100 % callus formation was obtained with leaf disc culturing on MS media supplemented with Kin , BA and TDZ at 1 mg/L. Also, the same media gave the highest callus formation percentage (91.7 %) for the shoot tip explant. While, the lowest callus formation percentage 66.7 % for shoot tip and 75 % for leaf disc were obtained on MS media fortified with Kin at 0.25 mg/L.

For callus fresh weight it was obvious from data in the same table that all treatments using leaf disc explant always had the upper hand in that respect when compared with the shoot tip explant at the same media. In this concern, the heaviest callus fresh weight of 10.66 g was obtained when the leaf disc explant was cultured on media supplemented with 2,4-D at 1.5 mg/L in combination with BA at 1 mg/L (Fig. 1B). The next positive effect of 9.41 g was obtained with the same media when culturing shoot tip (Fig. 1A) but, a significant difference was found between them. These results were in agreement with Zang *et al.* (2016) on *Dendrocalamus hamiltonii* and Hesami *et al.* (2017) on *Ficus religiosa*. On the other hand, data clarified that medium received 0.25 mg/L Kin and cultured with shoot tip significantly tabulated the lowest value of callus fresh weight 4.60 g only, when compared with all of the other treatments.

Table 2. Effect 2,4-D, explant type and cytokinin type at different concentrations on callus induction of lemon verbena.

Treatments		callus formation %		callus fresh weight (g)	
Cytokinin type	**Cytokinin conc. mg/L**	**Shoot tip**	**Leaf disc**	**Shoot tip**	**Leaf disc**
Kin	0.25	66.7	75.0	4.60	5.16
	0.5	75.0	83.3	5.80	6.24
	1.0	91.7	100	6.88	8.52
	1.5	83.3	91.7	6.23	7.31
BA	0.25	83.3	91.7	6.13	7.23
	0.5	83.3	91.7	7.49	8.00
	1.0	91.7	100	9.71	10.66
	1.5	83.3	91.7	8.04	9.25
TDZ	0.25	75.0	83.3	5.18	6.35
	0.5	83.3	91.7	7.15	7.75
	1.0	91.7	100	8.21	8.90
	1.5	83.3	91.7	7.57	8.23
L.S.D. at 5 %		22.25		0.45	

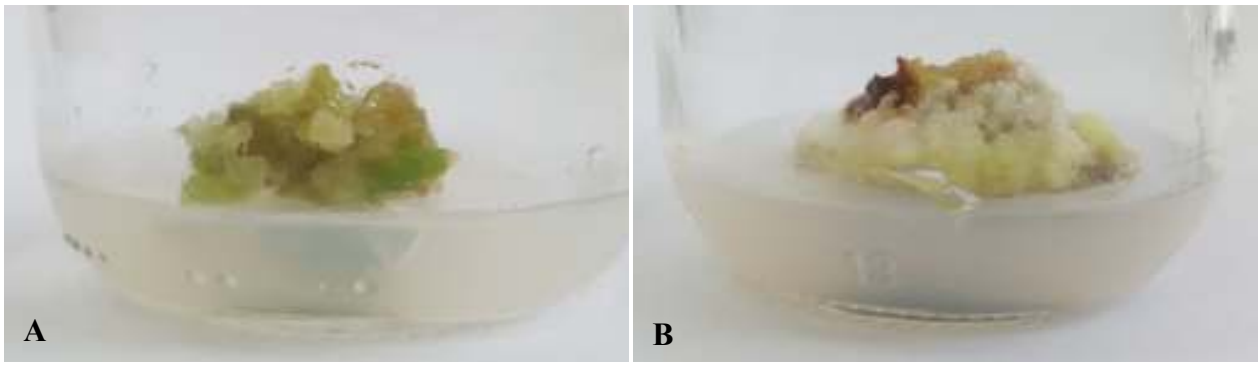

Fig. 1. Callus formation of *Aloysia triphylla* explants obtained on MS medium supplemented with 2,4-D at 2 mg/L and BA at 1 mg/L . A) By culturing shoot tip B) By culturing leaf disc

Dealing with the interaction between auxin and cytokinin, Mineo (1990) mentioned that hormone balance is apparently more important than the absolute concentration of any one hormone for both cell division and cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue. For callus induction Yadav and Tyagi (2006) reported that callusing of dicotyledonous plants is most readily induced by an approximately equal amount of both auxin and cytokinin. The role of cytokinin in callus induction may be due to that Cytokinins are derived from adenine and produce two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increased cell division (Mineo 1990).

II- Callus differentiation stage:

1- Effect of callus source, cytokinin type, cytokinin concentration and their interactions on callus differentiation.

For shoot regeneration from callus, the callus formed from shoot tip and leaf disc were transferred to MS medium supplemented with different cytokinins (BA, Kin or TDZ) at various concentrations of 1.0, 1.5, 2.0 and 2.5 mg/L. After 30 days of culture the data were registered and were presented in Tables (3, 4 and 5).

1- Effect of callus source (A) on callus differentiation:

Concerning the effect of callus source on callus differentiation percentage, shoots number and shoot length, data in Tables (3, 4 and 5) showed that significant differences were detected between using callus derived from shoot tip and leaf disc. it was obvious that callus derived from shoot tip gave the highest callus differentiation percentage (73.6 %), shoots number (6.87 shoots) along with the highest shoot length of 3.0 cm compered to callus derived from leaf disc which recorded 34.7 % of callus differentiation and 3.63 shoots number with average shoot length of 1.78 cm (Fig. 2 A&C).

2- Effect of cytokinin type (B) on callus differentiation:

It was obvious from data presented in Tables (3, 4 and 5) that media supplemented with TDZ significantly promoted the highest callus differentiation % and shoots number, since it was 66.7 % and 7.43 shoots, respectively. But it significantly gave the lowest shoot length (1.76 cm). The next positive effect for callus differentiation was recorded with BA while, it was quite clear that Kin significantly recorded the lowest callus differentiation.

Table 3. Effect of callus source, cytokinin type, cytokinin concentration and their interactions on callus differentiation percentage of lemon verbena.

Callus source (A)	Cytokinin type (B)	Cytokinin conc. (mg/L) (C) 1.0	1. 5	2.0	2. 5	Mean of (A)	Mean of (B)	Mean of (A×B)
Shoot tip	BA	50.0	66.7	100	83.3	73.6	57.3	75.0
	Kin	41.7	58.3	75.0	66.7			60.4
	TDZ	66.7	83.3	100	91.7			85.4
Mean of (A×C)		52.8	69.4	91.7	80.6			
Leaf disc	BA	16.7	33.3	41.7	66.7	34.7	38.5	39.6
	Kin	0.0	0.0	25.0	41.7			16.7
	TDZ	25.0	41.7	58.3	66.7			47.9
Mean of (A×C)		13.9	25.0	41.7	58.3			
Mean of (C)		33.3	47.2	66.7	69.4		66.7	
Mean of (B×C)	BA	33.3	50.0	70.8	75.0	LSD at 5%		
	Kin	20.8	29.2	50.0	54.2	A 6.3	AC 10.3	
	TDZ	45.8	62.5	79.2	79.2	B 5.9	BC 12.8	
						C 7.8	ABC 18.0	
						AB 7.9		

Table 4. Effect of callus source, cytokinin type, cytokinin concentration and their interactions on shoots number differentiation from callus of lemon verbena.

Callus source (A)	Cytokinin type (B)	Cytokinin conc. (mg/L) (C) 1.0	1. 5	2.0	2. 5	Mean of (A)	Mean of (B)	Mean of (A×B)
Shoot tip	BA	4.38	7.75	10.41	8.46	6.87	6.02	7.75
	Kin	2.50	2.75	3.92	5.63			3.70
	TDZ	7.38	8.29	11.75	9.29			9.18
Mean of (A×C)		4.75	6.26	8.69	7.79			
Leaf disc	BA	0.75	4.75	5.38	6.25	3.63	2.30	4.28
	Kin	0.00	0.00	1.00	2.63			0.91
	TDZ	2.75	5.50	6.75	7.75			5.69
Mean of (A×C)		1.17	3.42	4.38	5.54			
Mean of (C)		2.96	4.84	6.53	6.67		7.43	
Mean of (B×C)	BA	2.56	6.25	7.89	7.35	LSD at 5%		
	Kin	1.25	1.38	2.46	4.13	A 0.35	AC 0.53	
	TDZ	5.06	6.90	9.25	8.52	B 0.14	BC 0.60	
						C 0.39	ABC 0.86	
						AB 0.32		

Table 5. Effect of callus source, cytokinin type, cytokinin concentration and their interactions on length of shoot (cm) differentiation from callus of lemon verbena.

Callus source (A)	Cytokinin type (B)	Cytokinin conc. (mg/L) (C) 1.0	1. 5	2.0	2. 5	Mean of (A)	Mean of (B)	Mean of (A×B)
Shoot tip	BA	2.66	2.99	3.12	3.02	3.00	2.58	2.95
	Kin	3.15	3.66	4.63	4.93			4.09
	TDZ	1.20	1.66	2.36	2.64			1.96
Mean of (A×C)		2.34	2.77	3.37	3.53			
Leaf disc	BA	1.05	2.35	2.86	2.61	1.78	2.84	2.22
	Kin	0.00	0.00	2.50	3.83			1.58
	TDZ	0.98	1.59	1.81	1.84		1.76	1.55
Mean of (A×C)		0.68	1.31	2.39	2.76			
Mean of (C)		1.51	2.04	2.88	3.14			
Mean of (B×C)	BA	1.86	2.67	2.99	2.82			
	Kin	1.58	1.83	3.57	4.38			
	TDZ	1.09	1.62	2.08	2.24			

LSD at 5%

A	0.54	AC	0.53
B	0.19	BC	0.45
C	0.28	ABC	0.72
AB	0.50		

Fig. 2. Shoot regeneration from callus of Aloysia triphylla. A) By culturing callus obtained from shoot tip on MS medium supplemented with TDZ at 2 mg/L. B) By culturing callus obtained from shoot tip on MS medium supplemented with BA at 2 mg/L. C) By culturing callus obtained from leaf disc on MS medium supplemented with TDZ at 2.5 mg/L.

3- Effect of cytokinin concentration (B) on callus differentiation:

Data dealing with the effect of cytokinin concentration on percentage of callus differentiation were illustrated in Table (3), the obtained results showed a positive relationship between cytokinin concentrations and callus differentiation percentage, since every increase in cytokinin concentrations from 1.0 mg/L up to 2.5 mg/L was followed by increase in callus differentiation percentage. The highest callus differentiation percentage (69.4 %) was achieved with cytokinin at 2.5 mg/L while, the lowest callus differentiation percentage (33.3 %) was achieved with cytokinin at 1.0 mg/L.

Considering the effect of cytokinin concentration on shoots number and shoot length, the highest value of shoots number (6.67shoots) and the highest value of the shoot length (3.14 cm) was obtained when MS medium was supplemented with cytokinin at 2.5 mg/L (Tables 4 and 5). Also, the low concentration of cytokinin (1.0 mg/L) gave the lowest value of shoots number and shoot length (2.96 shoots and 1.51 cm, respectively).

4- Effect of callus source and cytokinin type (A×B) on callus differentiation:

Data in Tables (3 and 4) showed that culturing callus derived from shoot tip on medium fortified with TDZ significantly recorded the highest callus differentiation percentage (85.4%) and shoots number (9.18 shoots). While, the least value obtained with callus derived from leaf disc explant cultured on medium supplemented with Kin.

As for shoot length, data in Table (5) revealed that callus derived from shoot tip on medium fortified with Kin significantly recorded the highest shoot length (4.09 cm). It was matter of importance to note that callus derived from both shoot tip and leaf disc with TDZ resulted in the lowest value in these respect.

5- Effect of callus source and cytokinin concentration (A×C) on callus differentiation:

It appeared from data in Tables (3 and 4) that the highest percentage of callus differentiation (91.7 %) and shoots number (8.69 shoots) were achieved when callus derived from shoot tip was planted on nutrient MS medium augmented with 2 mg/L cytokinin.

In regard to shoot length as illustrated in Table (5), it was found that cytokinin at 2 and 2.5 mg/L with callus derived from shoot tip recorded the highest significant

values of 3.53 and 3.37cm, respectively and no significant difference was found between them

6- Effect of cytokinin type and cytokinin concentration (B×C) on callus differentiation:

It was cleared that TDZ concentrations increased the callus differentiation percentage than BA and Kin concentrations as shown in Table (3). Also, it was obvious from data in Table (2) that TDZ at the concentration of 2 mg/L significantly produced the highest shoots number (9.25 shoots). But, the highest significant shoot length (4.38 cm) was obtained with Kin at 2.5 mg/L (Table 3).

7- Effect of callus source, cytokinin type and cytokinin concentration (A×B×C) on callus differentiation:

Considering the effect of this interaction on callus differentiation percentage, the recorded results in Table (3) indicated that callus differentiation percentage of 100 % was achieved with nutrient MS medium was fortified with BA or TDZ at 2 mg/L with callus derived from shoot tip. Also, it was clear that culturing leaf disc on nutrient medium fortified with low concentrations 1.0 and 1.5 mg/L of Kin failed absolutely to induce shoots from callus. Dealing with the effect of this interaction on shoots number, data in Table (4) cleared that culturing callus derived from shoot tip on medium fortified with TDZ at 2 mg/L significantly tabulated the greatest value of shoots number (11.75 shoots) followed by 10.41 shoots, when medium was supplemented with BA at 2 mg/L (Fig. 2 A&B).

Regarding the effect of this interaction on shoot length as shown in Table (5), it was cleared that callus derived from shoot tip cultured on MS medium supplemented with different cytokinin types at various concentrations significantly measured the tallest shoots length, when compared with callus derived from leaf disc cultured at the same media. The callus derived from shoot tip explant cultured on medium supplemented with Kin at 2.5 mg/L significantly measured the tallest shoots of 4.93 cm. While, the shortest shoots length of 0.98 cm was recorded for callus derived from leaf disc explant cultured on media supplemented with TDZ at 0.5 mg/L.

Generally, callus is often used as the target tissue for genetic transformation and callus formation is initiated for the regeneration of plants after transformation of other tissue. Therefore, formation of callus is a fundamental step in the in vitro culture of many types of plant cells and tissues because in vitro callus provides the most frequently used totipotent cells from which whole plants are regenerated via organogenesis (Yadav and Tyagi, 2006). Also, callus differentiation is a very important stage which based on many factors like the degree of cell sensitivity towards growth regulators due to the origin of the explant, the type of cytokinin and auxin used and their mode of action (Bhojwani and Razdan, 1996). In the present study, the highest callus differentiation percentage and number of shoot regeneration was obtained from callus derived from shoot tip cultured on MS medium supplemented with TDZ at 2 mg/L which was similar to the reports of Rani and Nair (2006) on *Vitex negundo* L. and Kasem (2011) on *lavandula angustifolia*. The role of TDZ was reported by Thinh (1997) who suggested that TDZ either increases the levels of nucleoside or the accumulation and synthesis of purine cytokinins as well as promoting the conversion of adenine to adenosine. Murthy *et al.* (1995) illustrated that TDZ intimately related to the metabolism of endogenous growth regulators, specifically cytokinins and auxins, so its effect appears clearly in the proliferation process.

1- Effect of media strength and GA_3 concentrations on callus differentiation.

This experiment was carried out in order to improve callus differentiation and characteristics of shoot regeneration like increasing the length of shoots which is very important for the step of in vitro rooting. Also, reduce the frequency of vitrification percentage. Friable callus obtained from shoot tip inoculated on MS medium at full, ¾ and ½ strength in combination of GA_3 at different concentrations (0.0, 0.5 and 1.0 mg/L) and all treatments were provided with TDZ at 2.0 mg/L (the best result in the previous experiment).

Data presented in Table (6) cleared that no significant difference was obtained for callus differentiation percentage between all treatments, since it range from 83.3 to 100 %. But, it was obvious that using half strength media supplemented with 0.0 or 0.5 mg/L GA_3 significantly prevented entirely occurrence of vitrification percentage, as they recorded 0.00. While, it was clear that increasing GA_3 up to 1.0 mg/L with half strength media significantly enhanced the vitrification percentage (25 %). The worst vitrification value of 58.3 % was recorded for full strength medium which fortified with GA_3 at 1.00 mg/L. These results were in agreement with Bhalla *et al.* (2009) on *Hibiscus rosa-sinensis* and Kasem (2011) on *lavandula angustifolia* who reported that the high concentrations of the micro and macro-elements in the full strength media play as a compressor with the plant cell which make its walls thick and feeble and may led to absorb a high amounts of salts by the plant cell this confirm the result of Kozai *et al.* (1995) they cleared that the uptake rate per plantlet from NO3, NH4, P, K, Ca and Mg generally increased with increasing volume and initial strength of the nutrient medium. So, the osmotic stress increase inside the plant cell which makes it absorb a high percentage of the medium water and in the end the hyperhydricity appears.

As for shoots number and shoot length, data presented in Table (6) revealed that the highest significant value of shoots number regeneration (12.92 shoots) was obtained when half strength media was supplemented with GA_3 at 0.5 mg/L (Fig. 3A), while the highest significant value of the shoot length (4.68 cm) was recorded with ¾ strength media supplemented with GA_3 at 1.0 mg/L (Fig. 3B). But, it was a matter of importance to notice that the best treatment for shoots number regeneration gave suitable shoot length (3.88 cm).

Vitrification can be a problem in micropropagation because shoots that are in this condition are difficult to establish as plants. In vitrification, tissue becomes water soaked and translucent, which is mainly caused by various culture conditions such as the type of explants utilized, the concentrations of microelement and hormonal imbalances, high ammonium concentration, oxidative stresses as a result of high salt concentration and low light intensity (Bhatia *et. al.,* 2015). Hence, in this work there was appositive relationship between reducing MS strength and vitrification percentage and this may due to decreasing the salt concentration in the medium which has major roles in excessive water uptake and osmotic regulation (Yadav and

Tyagi, 2006). Also, they reported media that have high concentrations of ammonium ions can also cause the formation of vitrescent shoots during the culture of some plants and vitrification seems less prevalent on media containing little or no ammonium.

III- Induction of rooting and acclimatization.

Individual shoots were carefully separated from shoots Clusters and transferred on to MS media containing IBA at concentration of 0.5 mg/L for rooting. Plantlets with well-established root system was developed in 4 weeks with high rooting frequency (100 %), high number of roots per shoot (6 roots/ shoot) and the roots were long. The survival rate of hardening plantlets was 97 % after they were transferred to an equal ratio mixture of soil, sand and peat moss (1: 1: 1).

Table 6. Effect of the interactions between media strength and GA_3 concentrations on callus differentiation from shoot tip of lemon verbena.

Treatments		callus differentiation %	Vitrification %	Shoots number	Shoots length (cm)
Media strength	GA_3 conc. mg/L				
Full strength	0.0	100	41.7	11.50	2.28
	0.5	91.7	50.0	10.29	3.71
	1.0	83.3	58.3	9.37	3.93
3/4 strength	0.0	100	25.0	11.92	3.76
	0.5	91.7	25.0	9.46	4.14
	1.0	91.7	41.7	8.46	4.68
1/2 strength	0.0	100	0.0	12.17	3.21
	0.5	100	0.0	12.92	3.88
	1.0	91.7	25.0	10.50	4.22
L.S.D. at 5 %		18.6	21.83	0.34	0.27

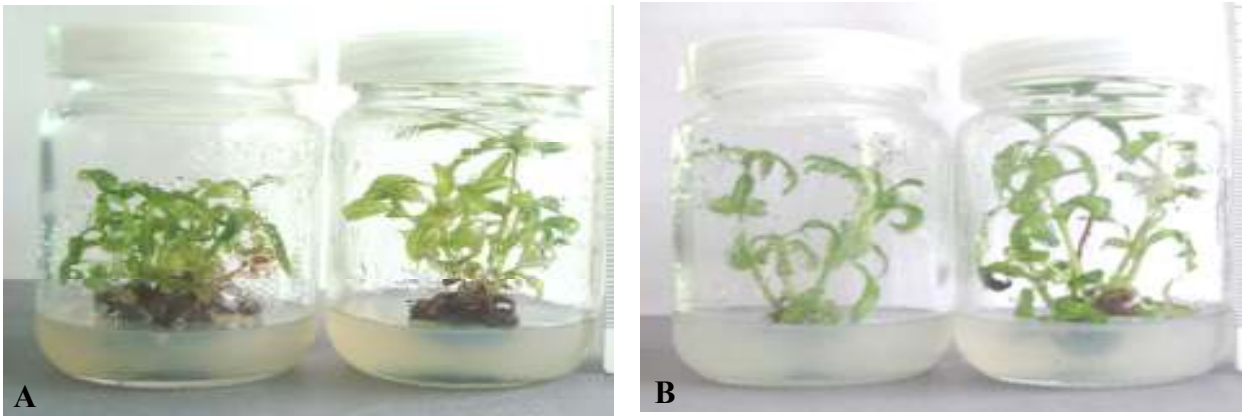

Fig. 3. Shoot regeneration from callus of *Aloysia triphylla*. A) By culturing callus on ½ strength MS medium supplemented with TDZ at 2 mg/L and GA3 at 0.5 mg/L B) By culturing callus on ¾ strength MS medium supplemented with TDZ at 2 mg/L and GA3 at 1 mg/L.

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انتاج الكالس و تخليق النباتات معمليا لنبات طبي و عطري هام (اللويزا)

هبة يوسف البنا

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يهدف البحث لانشاء بروتوكول لانتاج الكالس و تخليق نباتات لنبات طبي و عطري هام(اللويزا) باستخدام القمم النامية و قرص من الورقة كأجزاء نباتية. استخدمت بيئة موراشيج و سكوج محتوية على تركيزات مختلفة من الاوكسينات (بنفثالين حامض الخليك، أندول اسيتك اسيد و ٢،٤ داى كلوروفينوكسى حامض الخليك وحدها او في تداخل مع سيتوكينينات مختلفة (البنزيل ادينين و الكينيتين) لانتاج الكالس. زراعة القمم النامية و قرص من الورقة على بيئة موراشيج و سكوج محتوية علي ٢،٤ داى كلوروفينوكسى حامض الخليك بتركيز ١,٥ مللجم / لتر في تداخل مع البنزيل ادينين بتركيز ١ مللجم / لتر اعطت اعلى نسبة تكون للكالس ٩١,٧ و ١٠٠ % على التوالي مرتبطة باكبر وزن طازج للكالس ٩.٧١ و ١٠,٦٦ جرام على التوالي. لإعادة تكشف الكالس، زراعة الكالس الناتج من القمم النامية علي بيئة موراشيج و سكوج متحتوية علي ٢ مللجم / لتر و حامض الجبريللك بتركيز ٠,٥ مللجم/ لتر سجلت أعلى نسبة مئوية لإعادة التكشف ١٠٠% و اعلى عدد افرع ١٢.٩٢ فرع. تم تجذير الافرع معمليا على بيئة موراشيج و سكوج مزودة باندول حمض البيوتيرك بتركيز ٠,٥ مللجم/لتر .وقد تم اقلمة النبيتات بزراعتها علي بيئة مكونة من التربة، الرمل و البيتموس بنسبة ١: ١ :١ و قد ادت الي نجاح الاقلمة بنسبة ٩٧% .