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Original Contribution

CALLUS INDUCTION AND PLANT REGENERATION IN LEMON VERBENA (*LIPPIA CITRODORA* L.), AN IMPORTANT MEDICINAL PLANT

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ABSTRACT

In order to investigate the optimization of *Lippia citriodora* L., a richly scented medicinal plant, in different aspects of tissue culture; young stem cuttings were cultured on Murashige and Skoog (MS) medium containing 0.01 mg l^{-1} indole-3-butyric acid (IBA) and 0.5 mg l^{-1} N6-benzyladenine (BA) to produces sterilized seedling. After 21 days, three types of explants including apical meristem, young stems carrying buds and young petioles with part of leaf were excised from the growing seedling and cultured on MS medium containing various combinations of α-naphthalene acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2,4-D) and BA for callus induction. Callus formation was occurred in several combinations of growth regulators among them 5 treatments have found as the most effective mediums for callus formation percentage. The highest callus wet weight was observed in young petiole explants with MS medium supplemented with 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} BA, and the best callus dry weight was obtained in meristem explants which were placed on MS medium, supplemented with 0.5 mg l^{-1} NAA and 2 mg l^{-1} BA. Visible calli were transferred to MS and quarter MS medium supplemented with different concentrations of 2,4-D, BA, NAA and GA3 to shoot regeneration. Only calli derived from young stems were regenerated. Furthermore, MS medium with 2 mg l^{-1} BA alone was the most effective treatment for regeneration percentage. Many of regenerated calli were rooted in their shoot organogenesis medium and the rest of them were sub-cultured in MS medium supplemented with activated carbon thus root emergence was induced in all calli samples. The rooted shoots were acclimatized and transferred into soil successfully.

Key word: *Lippia citriodora* L., plant regeneration, medicinal plant, Callus induction, root induction, N6-benzyladenine

INTRODUCTION

Herbal medicines have been the basis of treatment and cure for various diseases in traditional methods. Lemon verbena **(***Lippia citriodora* L.) is an aromatic plant belonging to the *Verbenaceae* family, Common names for this plant include *Lippia triphylla*; *Aloysia triphylla*; *Aloysia citriodora* and *verbena triphylla* [1]. *Lippia* genus has more than 200 species which *L. citriodora* is the most important species [2]. It is a shrub perennial medicinal plant, indigenous to South American countries and acts mainly on nervous system. Citral is the main chemical component found in *L.citriodora* leaves extract [3]. The leaves and vegetative parts of this plant are refrigerant, anticonvulsant and gastrointestinal strengthening [1, 4]. Also some antibacterial and antioxidant effects have found in this plant [3]. So the mentioned properties of this plant were clarified the importance of it.

Propagation of *L.citriodora* is possible by stem cutting during spring days [2]. However few numbers of plants would be survived and produced through this method. From the other side proliferation of *L.citriodora* by seeds is not proposed due to allogamy and genetic trait segregation. So it seems that propagation of this plant by tissue culture methods would be appropriated to overcome the problems of proliferation. These methods help us producing large number of plant without any pollution. Consider to importance of this plant in various industries the first step in breeding based on

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molecular methods is optimization of Lemon verbena tissue culture.

In recent years, there has been increasing interest in tissue culture of medicinal plants. In spite of the importance of *Lippia* genus and specially *L.citriodora* species, there is no enough information on *L.citriodora* tissue culture. The effects of plant growth regulators to generate a method of propagation were investigated in a study about Lemon Verbena Tissue Culture [5]. In a research about *L.citriodora*, nodal segments were offered as the best explant for in vitro proliferation and half MS with no growth regulator has been the best medium in order to form sprouts and roots from buds [6]. In another study about evaluation in vitro propagation of endangered *L. filifolia* researchers have detected that, nodal segments cultivation in MS medium supplemented with BA; 4.5 μ*M* / NAA; 54 n*M* induced multiple shoots [7]. There has been a report about in vitro micro propagation of *Lippia alba*, The study has shown that multiple shoots were induced in vitro from shoot tips of *L.alba* on MS medium containing 2 mg ml^{-1} [8].

The present study was aimed to evaluate influence of varied concentrations of hormones and different type of explants on callus induction and regeneration of *L.citriodoria*. Also the regenerated calli were rooted and acclimatized to soil, so complete plantlets were obtained.

MATERIAL AND METHODS

1. Preparation of plant materials

Stem cutting provided from Five year old plants and were cultured in greenhouse for 2 months, and then young stem cuttings in length of 10-15 cm were obtained from 2 month old plants. First of all, stem cuttings were washed thoroughly under running tap water with dish washing liquid for 30 minutes. Then stem cuttings were surface sterilized with 70% ethanol for 45 seconds, then rinsed with sterile water three times. After that samples were surface sterilized again using 2.5% sodium hypochlorite solution and twin 20 for 20 min and followed by three time washes in succession with sterile water. The leaves of sterilized cuttings were removed and then divided into nodal segments with one or two buds. The nodal segments were cultured on MS medium, supplemented with 30 g l^{-1} sucrose, 7 gr l^{-1} agar, 0.5 mg l^{-1} BA and 0.01 mg l^{-1} IBA. The cultures were incubated at 23 ± 2 ºc under 16/8 h day/night photoperiod subjected to cool fluorescent light. After 21 days germinated sterilized seedlings were used as source for explants. It is worth mentioning that the cultures were sub cultured every 21 days.

2. Callus induction

When the seedlings were about 9 cm height, three types of explants (apical meristem, young stems with buds and young petiole with a part of leaf) were excised. These explants (3-6 mm long) were cultured on MS medium fortified with various concentration of three growth regulators i.e. NAA (0, 0.5, 1, 2 mg l^{-1}), 2, 4-D (0, 0.5, 1, 2 mg l^{-1}) and BA (0, 1, 2 mg l^{-1}) to callus induction. After 45 days, culture data were recorded for percent of callus formation, fresh and dry weight of callus.

3. Regeneration

After callus production, the shoot regeneration was attempted by transferring seven week old calli on MS medium and quarter MS in combination with different levels of growth regulators i.e. NAA (0, 0.05, 0.1, 0.5, 1 mg l^{-1}), 2, 4-D (0, 0.05, 0.1, 0.5, 1 mg l^{-1}), GA_3 (0, 1 mg l^{-1}) and BA (0, 0.1, 0.5, 1, 2, 5 mg l^{-1}). After 30 days, data were recorded for percent of shoot formation, number and length of shoots.

4. Root formation and acclimatization

Healthy shoots (10 cm height) were excised and transferred onto rooting medium consisting of MS medium supplemented with 2 gr l^{-1} activated carbon without growth regulators. In acclimatization part, the plantlets were gently washed in water to get rid of agar from their roots and were planted in plastic cups containing perlite, fertile soil and vermiculite (1:1:1; V/V/V). The cups were irrigated with normal water and in order to keep humidity at high level, cups were covered with another transparent plastic cup and placed in glasshouse.

5. Culture conditions

All media were supplemented with 30 g l^{-1} sucrose and medium gelled with 7 gr l^{-1} agar. The pH of all media was adjusted to 5.8 before the addition of agar. The media were sterilized by autoclave under 1.2 bar pressure and 121º C for 20 min. All cultures were grown at 23± 2 º C under 16/8 h day/night photoperiod subjected to cool fluorescent light.

6. Experimental design and data analysis

The treatments were arranged in factorial experiment based on completely randomized design (CRD) with three replicates per treatment and five explants per replicate. Data were given in percentage. Raw data were analyzed statistically using MSTAT-C software. The mean values of different

treatments were compared using Duncan's multiple range test.

RESULTS

The earliest visible signs of callus growth were observed after about 4 weeks. This sign varied from treatment to treatment and explants to explants **(Fig 1, A-F**). Callus formation was significantly influenced by the explants type, type and concentration of growth regulators **(Table 1).** In general, the frequency of callus induction from apical meristem was higher rather than young stems and young petioles. Apical meristem in combination with MS medium consist of (0.5 mg l^{-1} NAA, 1 mgl^{-1} BA), (1 mg l^{-1} NAA, 1 mg l^{-1} BA) and (2 mg l^{-1} NAA, 2 mg l^{-1} BA), young petioles in combination with MS medium containing 1 mg l^{-1} NAA, 1 mg l^{-1} BA, 0.5 mg l^{-1} 2, 4-D, 1 mg l^{-1} BA and 0.5 mg l^{-1} 2, 4-D, 2 mg l^{-1} BA and young stems in combination with MS media supplemented with (0.5 mg l^{-1} 2, 4-D , 2 mg l^{-1} BA were the most effective treatments about frequency of callus induction (100%) **(Table 1).**

In addition, evaluation of mean values showed that callus fresh weight was significantly influenced by explants type, type and concentration of growth regulators **(Table 2).** Generally, the callus fresh weight derived from young petiole was higher than that produced callus from young stem and apical meristem. Young petioles in combination with MS medium consist of 0.5 mg l^{-1} 2, 4-D, 1 mg l^{-1} BA were the best treatment as for callus fresh weight **(Table 2).**

Comparison of means indicated that callus dry weight was significantly affected by kind of explants, type and concentration of growth regulators **(Table 3).** In general, callus dry weight in apical meristem treatment was higher than produced callus from young stems and young petioles treatments. The maximum dry weight was observed on MS medium consist of 0.5 mg l^{-1} NAA and 2 mg l^{-1} BA in combination with Apical meristem explants**. (Table 3).**

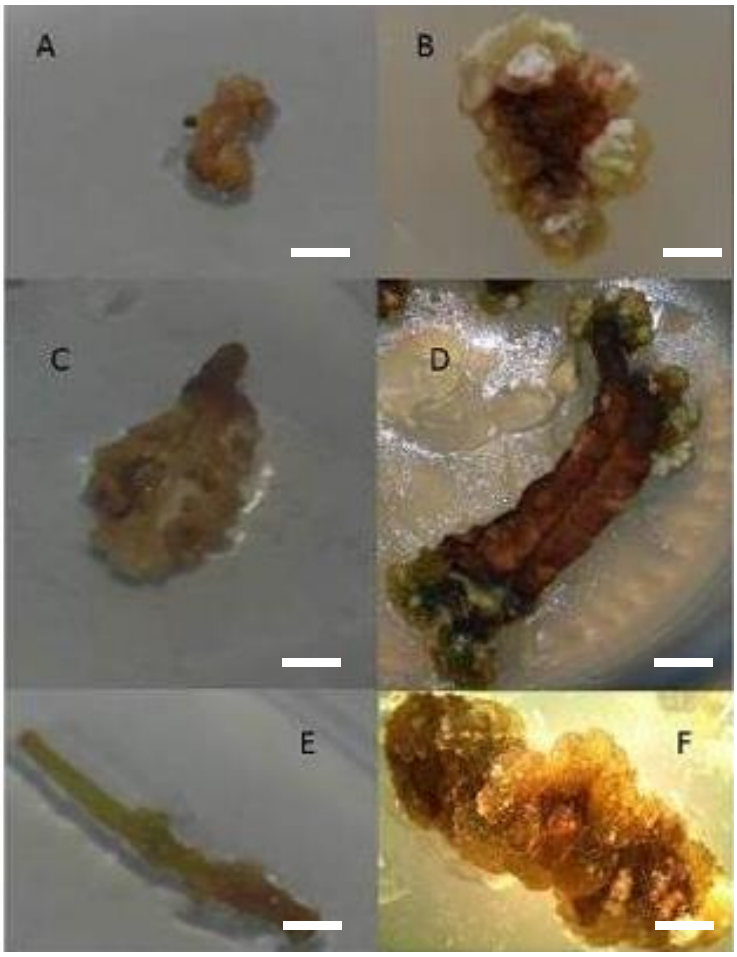

Figure 1. Callus induction in *Lippia citriodora* L.; A: initiation of callus formation in apical meristem explants after 4 weeks; B: callus cells in apical meristem explants after 7 weeks; C: initiation of callus formation in young petiole with part of leaf explants after 4 weeks; D: callus cells in petiole explants after 7 weeks; E: initiation of callus formation in young stems with buds explants after 4 weeks; F: callus cells in stem explants after 7 weeks (bar= 0.1 cm).

Table 1. *Influence of different concentrations of growth regulators and effect of some explants on callus formation (%)*

Growth regulators (mg l^{-1})			Explants		
NAA	2,4-D	BA	Young stem with buds	Apical meristem	Young petiole with part of leaf
0		1	0 ^{i}	20 ^{gh}	0 ^{i}
0.5		0	0 ^{i}	93.33 ^{ab}	26.67 ^{g}
0.5		1	26.67 ^{g}	100 ^{a}	86.67 ^{ab}
0.5		2	33.33 ^{fg}	93.33 ^{ab}	93.33 ^{ab}
1		0	6.6667 ^{hi}	0 ^{i}	6.667 ^{hi}
1		1	66.67 ^{cd}	100 ^{a}	100 ^{a}
1		2	26.67 ^{g}	93.33 ^{ab}	93.33 ^{ab}
2		0	33.33 ^{fg}	66.67 ^{cd}	6.667 ^{hi}
2		1	93.33 ^{ab}	93.33 ^{ab}	93.33 ^{ab}
2		2	86.67 ^{ab}	100 ^{a}	86.67 ^{ab}
	0.5	0	46.67 ^{ef}	46.67 ^{ef}	46.67 ^{ef}
	0.5	1	66.67 ^{cd}	93.33 ^{ab}	100 ^{a}
	0.5	2	100 ^{a}	93.33 ^{ab}	100 ^{a}
	1	0	46.67 ^{ef}	86.67 ^{ab}	93.33 ^{ab}
	1	1	26.67 ^{g}	93.33 ^{ab}	93.33 ^{ab}
	1	2	0 ^{i}	93.33 ^{ab}	53.33 ^{de}
	2	0	0 ^{i}	73.33 ^{bc}	93.33 ^{ab}
	2	1	6.667 ^{hi}	26.67 ^{g}	60 ^{cde}
	2	2	0 ^{i}	20 ^{gh}	0 ^{i}

Means followed by the same letters within a column are not significantly different using Duncan's multiple range test.

Table 2. *Effect of different concentrations of growth regulators and effect of some explants on wet weight of Lemon verbena (Lippia citriodora)*

Growth regulators (mg l^{-1})			Explants		
NAA	2,4-D	BA	Young stem with buds	Apical meristem	Young petiole with part of leaf
0		1	0 ^{y}	113.5 $^{v-x}$	0 ^{y}
0.5		0	0 ^{y}	363 ^{klm}	0 ^{y}
0.5		1	230.7 $^{o-t}$	259 $^{n-r}$	643.1 ^{bcd}
0.5		2	152.9 $^{t-w}$	482.5 $^{g-j}$	683.2 ^{abc}
1		0	40.07 ^{xy}	0 ^{y}	43.97 ^{xy}
1		1	161.9 $^{s-w}$	516.9 $^{f-i}$	594 $^{c-f}$
1		2	171.6 $^{q-v}$	562.4 $^{d-g}$	424.1 $^{i-k}$
2		0	198.5 $^{p-v}$	397.5 $^{j-l}$	44.73 ^{xy}
2		1	225.6 $^{p-t}$	534.8 $^{f-h}$	597.8 $^{c-f}$
2		2	229.4 $^{o-t}$	567 $^{d-g}$	490.2 $^{g-i}$
	0.5	0	209.8 $^{p-u}$	75.17 $^{w-y}$	277.3 $^{m-p}$
	0.5	1	222.1 $^{p-t}$	492.1 $^{g-i}$	740.8 ^{a}
	0.5	2	264.1 $^{n-p}$	251.9 $^{n-s}$	694.7 ^{ab}
	1	0	172.3 $^{q-v}$	453.6 $^{h-j}$	628 $^{b-e}$
	1	1	156.8 $^{s-w}$	477 $^{g-j}$	544.6 $^{e-h}$
	1	2	0 ^{y}	458.1 $^{h-j}$	290.2 $^{m-p}$
	2	0	0 ^{y}	339.1 $^{k-n}$	524.1 ^{fgh}
	2	1	47.93 ^{xy}	123 $^{u-x}$	322.2 $^{l-o}$
	2	2	0 ^{y}	75.47 $^{w-y}$	0 ^{y}

Means followed by the same letters within a column are not significantly different using Duncan's multiple range test.

Proliferated calli were transferred to regeneration media and after 21 days. Meristematic regions in callus for initiation of regeneration were identified by small white buds on the callus **(Figure 2-A, B).** Primary shoots were emerged on 23rd and 25th days after culturing in regeneration medium **(Figure 2-C).** Among different treatments, 11 treatments have regenerated and all of them were originated from young stem explants. There were no regenerated calli obtaining from young petiole or apical meristem so they were getting dark and necrosis.

Table 3. *Effect of different concentrations of growth regulators and effect of some explants on dry weight of Lemon verbena (Lippia citriodora)*

Growth regulators (mg l^{-1})			Explants		
NAA	2,4-D	BA	Young stem with buds	Apical meristem	Young petiole with part of leaf
0		1	0 ^{r}	4.400 ^{qr}	0 ^{r}
0.5		0	0 ^{r}	25.57 $^{j-m}$	16.77 $^{m-o}$
0.5		1	20.43 $^{l-n}$	14.83 $^{n-p}$	62.57 ^{d}
0.5		2	23.97 $^{j-n}$	93.87 ^{a}	59.70 ^{de}
1		0	7.400 $^{p-r}$	0 ^{r}	4.700 ^{qr}
1		1	10.57 $^{o-q}$	76.80 ^{bc}	81.10 ^{bc}
1		2	29.47 $^{i-l}$	44.83 ^{fg}	56.63 ^{de}
2		0	31.57 $^{h-k}$	51.70 ^{ef}	4.767 ^{qr}
2		1	40.33 ^{gh}	78.77 ^{bc}	72.93 ^{c}
2		2	37.70 $^{g-i}$	82.83 ^{b}	51.57 ^{ef}
	0.5	0	30.20 $^{i-l}$	5.553 $^{p-r}$	23.60 $^{k-n}$
	0.5	1	32.87 $^{h-k}$	77.50 ^{bc}	62.90 ^{d}
	0.5	2	41.30 ^{gh}	32.47 $^{h-k}$	58.60 ^{de}
	1	0	25 $^{j-m}$	58.17 ^{de}	51.53 ^{ef}
	1	1	24.13 $^{j-n}$	56.90 ^{de}	36.90 $^{g-i}$
	1	2	0 ^{r}	54.77 ^{de}	20.63 $^{l-n}$
	2	0	0 ^{r}	41.33 ^{gh}	34.10 $^{h-j}$
	2	1	5.967 $^{p-r}$	5.900 $^{p-r}$	18.87 $^{m-o}$
	2	2	$^{0\ r}$	4.443 ^{qr}	0 ^{r}

Means followed by the same letters within a column are not significantly different using Duncan's multiple range test.

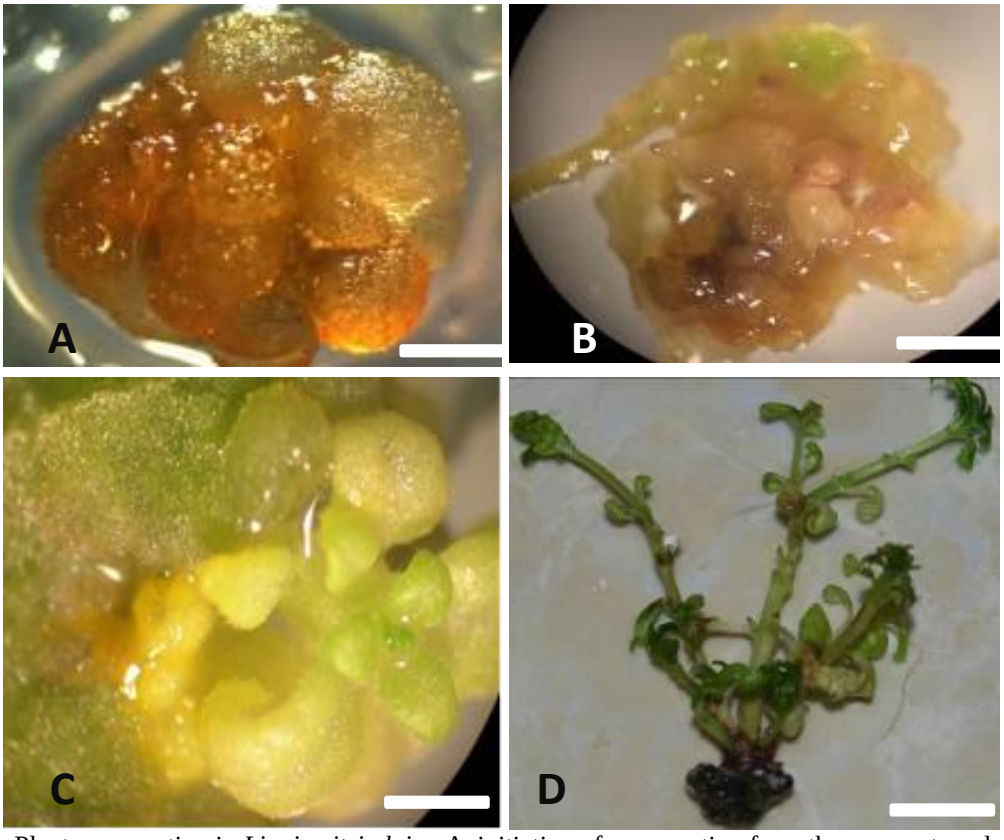

Figure 2. Plant regeneration in *Lippia citriodoia* , A. initiation of regeneration from the young stem derived callus after 21 days in MS media supplemented with 0.5 mg l^{-1} BA (bar= 0.1 cm), B. regeneration steps of young stem explant on MS media containing 0.1 mg l^{-1} NAA and 0.5 mg l^{-1} BA (bar= 0.1 cm), C. Initiation shoot regeneration of young stem explant on MS medium containing 2 mg l-1 BA alone (bar= 0.1 cm), D. Multiple regenerated callus derived from young stem explant in combination with MS media fortified with 1mg l^{-1} BA alone (bar= 0.5 cm)

Evaluation of mean values demonstrated that regeneration of calli was strongly influenced by type and concentration of growth regulators **(Table 4).** Among different concentrations of growth regulators, MS medium supplemented with 2 mg l^{-1} BA was the most effective medium for the percentage of regeneration.

Table 4. *Effect of different concentrations of growth regulators and effect of some explants on regeneration percentage (%) of Lemon verbena (Lippia citriodora)*

Growth regulators (mg l^{-1})			Regeneration percentage (%)
NAA	2,4-D	BA	
0	0	0.5	20 [c]
0	0	1	20 [c]
0	0	2	100 [a]
0.05	0	0.1	20 [c]
0.1	0	0.1	40 [b]
0	0.05	0.1	20 [c]
0.05	0	0.5	40 [b]
0.1	0	0.5	40 [b]
0.1	0	1	40 [b]
1	0	1	20 [c]
1	0	2	20 [c]

Means followed by the same letters within a column are not significantly different using Duncan's multiple range test.

Study on mean values revealed that kind and concentration of growth regulators had a deep affection for number of shoots **(Table 5).** MS medium supplemented with 0.5 mg l^{-1} BA plus 0.05 mg l^{-1} NAA was the best medium for increasing shoot production from callus **(Figure 2-D).**

The results showed that there were a significant relationship between shoot length and combinations of growth regulators. Maximum shoot length was obtained from MS medium supplemented with 0.5 mg l^{-1} BA plus 0.1 mg l^{-1} NAA **(Table 6).**

Table 5. *Effect of different concentrations of growth regulators and effect of some explants on number of shoot in Lemon verbena (Lippia citriodora)*

Growth regulators (mg l^{-1})			NO. of shoot
NAA	2,4-D	BA	
0	0	0.5	1 [d]
0	0	1	2 [b]
0	0	2	2 [b]
0.05	0	0.1	2 [b]
0.1	0	0.1	1.5 [c]
0	0.05	0.1	1 [d]
0.05	0	0.5	2.5 [a]
0.1	0	0.5	1.5 [c]
0.1	0	1	1.5 [c]
1	0	1	1 [d]
1	0	2	2 [b]

Means followed by the same letters within a column are not significantly different using Multiple Ranges Test.

Table 6. *Effect of different concentrations of growth regulators as well as effect of some explants on shoots length in Lemon verbena (Lippia citriodora)*

Growth regulators (mg l^{-1})			Shoot length
NAA	2,4-D	BA	
0	0	0.5	5.70 [b]
0	0	1	4.10 [c]
0	0	2	3.20 [d]
0.05	0	0.1	4.25 [c]
0.1	0	0.1	5.35 [b]
0	0.05	0.1	0.70 [g]
0.05	0	0.5	1.70 [f]
0.1	0	0.5	6.30 [a]
0.1	0	1	2 [ef]
1	0	1	4.10 [c]
1	0	2	2.35 [e]

Means followed by the same letters within a column are not significantly different using Duncan's Multiple Range Test.

Most of the regenerated shoots were rooted in their relevant regeneration media **(Figure 3-A).** Only those treatments which were supplemented just with BA, without combination of auxin, were not rooted and only shoot and leaves were produced from the callus. The non-rooted callus was transferred to MS media plus activated carbon without any growth regulators so all of them were rooted after 5 days **(Figure 3-B).**

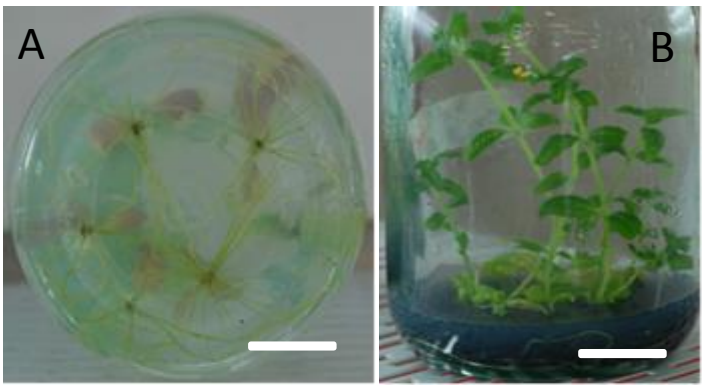

Figure 3. Root induction in *L.citriodora*. A. Root induction from multiplied shoots on MS medium supplemented with 0.05 mg l^{-1} NAA and 0.1 mg l^{-1} BA, B**.** Root induction from multiplied shoots on MS medium with activated carbon

Plantlets with well-developed roots were transferred into small plastic cups containing sand, fertile soil and vermiculite (1:1:1; V/V/V). Cups were watered twice a day. At the end 85% of the plantlets were acclimatized **(Figure 4).**

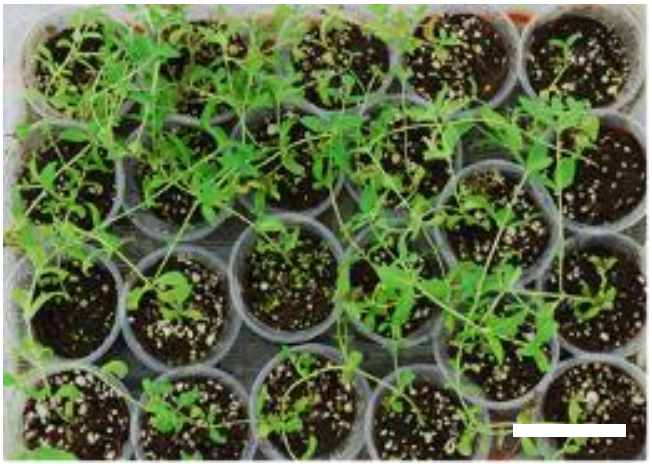

Figure 4. Healthy acclimatized plants in combination with sand, fertile soil and vermiculite (1:1:1)

DISCUSSION

Although lemon verbena is an important medicinal plant, there are a few studies about in vitro propagation of this plant and no research in case of callus induction. So the present investigation evaluates the effect of explant type, media, growth regulator and interaction of them to develop an in vitro culture protocol. According to importance of this plant in various industries the first step in genetic breeding, using molecular approaches, is optimization of lemon verbena tissue culture.

We found that meristem was the most effective explant to callus formation. However induced callus from young stems were regenerated, suggesting that stems with buds are better source to produce callus with regeneration capability. In a study, stems of *Vitex leucoxylon* were introduced as the best explant in order to callus induction [9] which was against present study. In addition, this type of explant was reported as the best explant in evaluation of *Aloysia citriodora* (Syn: *L. citriodora*) [6]. Stem segments were reported by some other researchers as an appropriate explant to in vitro culture of the plants being in same family and genus with *L.citiodora* [10; 7; 3; 11]. Therefore stem segments carrying bud were proposed for doing use in vitro trials and evaluations of *Lippia* genus.

In an investigation aimed to find out the effect of plant growth regulators to generate a method of propagation of lemon verbena, callus induction was observed in treatments which were supplemented with auxin [5]; this result was in accordance with present work, as there were no callus formation in free auxin media but only callus induction was occurred from meristem explants, these results suggest that auxin is necessary for callus induction; since the mentioned hormone is cause to an increase in protein synthesis through enhancing transcription of RNA [12], so it would be related to proliferation in cells and forming a callus.

It has been reported that *Vitex leucoxylon* callus induction rised by increasing concentration of NAA along with stable concentration of BA in the media [9]. These results were in agreement with our study, so that increase in callus formation was observed at stable concentration of BA by increasing NAA from 0 to 2 mg l^{-1} in combination with stem explants. Study on *Lippia dulcis* has indicated that MS media supplemented with lower concentration of 2, 4-D would have appropriate response to callus induction [13], so that this finding is similar to our study, thus increase in 2, 4-D concentration to more than 0.5 mg l^{-1} leads to reduction of callus formation in stem explants.

In the current study, callus fresh weight was raised by increasing 2, 4-D concentration up to 1 mg l^{-1} and more hormones led to a significant decrease in callus fresh weight (Table 2). Our observations are in agreement with Nisha et al (2005) about *vitex negundo* [14].

The results of this study indicate that existence of auxin and cytokinin in optimum concentration in medium is necessary for callus formation of various explants of *L.citriodora.*

In case of *verbena officinalis*, it was reported that, increase in BA till defined concentration led to increase in shoot regeneration percentage but more increase resulted to significant reduction in regeneration [15]; these findings are approximately similar to our observations in the current study **(Table 4).**

In case of relationships between growth regulators and shoot numbers our evaluations indicated that increase in BA with stable concentration of auxin led to increase in shoot regeneration percentage **(Table 5)**, while Mosavi (2012) observed that application of MS media supplemented with cytokinin was not appropriate for *L. citriodora* regenerating. In the present study reduce in shoot numbers was observed at stable concentration of BA and increased NAA **(Table 5)**. The best shoot regeneration percentage was obtained at low level of auxin [5]. *In vitro* micro propagation of *Lippia alba* have shown that increase in BA concentration leads to increase in shoot number [8], this claim is quite similar to our observations **(Table 5).**

Mean values indicated that shoot length of regenerated callus was significantly raised at stable concentration of BA by increasing of NAA, and stable concentration of NAA by increasing of BA led to decreasing of shoot length **(Table 6)**. While in a study about Pepper-rosmarin (*Lippia sidoides*) it was reported that medium supplemented with lower concentration of NAA resulted in larger shoot length [16].

There are lots of researches about Verbenaceae family which report that root development of plants from foresaid family are very easy, for instance, there is appropriate root induction about *Tectona philippinensis* [17] and also an excellent root development about micro propagation of *Lippia junelliana* [10]. As it was mentioned before, 100% of regenerated

shoots were rooted in our work, so this result supports the above researches.

In another report about micro propagation of *Lippia junelliana* it was reported that root induced plantlets were acclimatized and transferred in to soil, successfully [10]. This conclusion was completely similar to the present trial about *L.citriodora*.

In conclusion, we report for the first time a protocol for callus induction and successful plant regeneration through callus cultures derived from stem segments. Consider to importance of secondary metabolites in this plant, further research on hairy root culture in order to callus induction and evaluation of suspension culture of *L.citriodora* is suggested.

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