Phytochemical Variations in Lemon Verbena (*Lippia citriodora* H.B.K.) Plantlets Affected by Propagation Methods and Soil Type

Moradi M (M.Sc.) [1], Mehrafarin A (Ph.D.) [2], Naghdi Badi H (Ph.D.) [2*]

1- Department of Horticulture, Science and Research Branch, Islamic Azad University, Karaj, Iran
2- Cultivation & Development Department of Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran
*Corresponding author: Institute of Medicinal Plants, ACECR, Karaj, Iran
P.O.Box: 31375-369, Tel: +98-26-34764010-8, Fax: +98-26-34764021
Email: Naghdibadi@yahoo.com

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Abstract

Background: Lemon verbena (*Lippia citriodora* H.B.K.) is an aromatic and medicinal plant of family Verbenaceae, which cultivated in North region of Iran.

Objective: Evaluation of phytochemical characters in *Lippia citriodora* H.B.K. plantlets affected by propagation methods (micro-propagation and stem cutting) cultivated in different soil type (peat moss and mixture soil).

Methods: This study was done on the base of factorial experiment in randomized complete block design with three replications and four treatments. The treatments were propagation methods (micro-propagation and stem cutting) and soil type (peat moss and mixture soil). The volatile oil was extracted by hydro-distillation using clevenger-type apparatus and analyzed by GC and GC/MS.

Results: The results indicated that the interaction effects of different soils and plantlets types had significant effect ($p<0.05$) on all studied parameters. The highest content of essential oil (0.79%), geranial (53.52%), neral (31.82%), limonene (11.29%), leaf dry weight (1.43 g) and SPAD value (20.85) were observed in treatment of MPP (micro-propagation plantlets cultivated in peat moss) and the lowest of that was obtained in treatment of SCM (stem cutting plantlets cultivated in mixture soil).

Conclusion: The peat moss was the best bed in respect of phytochemicals and morpho-physiological traits in both types of *in vitro* and stem cutting propagation. The highest essential oil content was observed in treatment of micro-propagation cultivated in peat moss.

Keywords: *Lippia citriodora* H.B.K., Micro-propagation, Peat moss, Soil type, Stem cutting

Introduction

The genus *Lippia*, (Verbenaceae) comprises approximately of 200 species which are indigenous to Southern and Central America and Africa [1]. Most of them are traditionally utilized as remedies for gastrointestinal and respiratory problems. Some species have shown antimalarial, antiviral and cytostatic properties. It is believed that their essential oils and phenolic compounds (flavonoids) are responsible for these properties [2].

Lippia citriodora is cultivated mainly due to the lemon-like aroma emitted from its leaves that are utilized for the preparation of herbal tea, which is reputed to have antispasmodic, antipyretic, sedative and digestive properties [3]. The essential oil from its leaves has been shown to exhibit antimicrobial activity [4]. The chemical composition of the essential oil from the leaves of *L. citriodora* has also been studied and reviewed [5]. Limonene, geranial, neral, 1.8-cineole are the main constituents of *L. citriodora* essential oil [5, 6].

The biotechnological tools are important to select, multiply, improve and analyze medicinal plants. *In vitro* production of secondary metabolites in plant tissue culture has been reported from various medicinal plants and also micro-propagation is the key step towards commercial production of secondary metabolites by plant biotechnology. Micro-propagation allows the controlled production of genetically uniform and pathogen-free plants, and makes studies on secondary metabolites feasible [7]. However, most of the micro-propagation processes are carried out on semi-solid media and several studies have reported the media effect on different medicinal plants culture [7, 8].

Secondary metabolites produced by plant tissue culture cultivated in peat moss probably have many differences in comparison with the compounds extracted from plants under natural conditions. Therefore, this study was done to identify effect of peat moss on essential oil content and its components in micro-propagated and stem cutting plantlets of *L. citriodora*.

Material and Methods

Plant materials

This study was done on the base of factorial experiment in randomized complete block design with three replications and four treatments at the research greenhouse of Institute of Medicinal Plants (MPI), Iranian Academic Centre for Education, Culture & Research (ACECR). The treatments were included propagation methods including micro-propagation (MP) and stem cutting (SC), and soil types consisting peat moss (P) and mixture soil (M). Peat moss is the least decomposed form of the peat types, typically tan to brown in color, lightweight, and high in moisture-holding capacity with pH 3.8 to 4.3. The physico-chemical properties of peat moss medium were presented in Table 1 [9].

The stem segments were excised from the shoot tip, and then cultured on multiplication medium containing MS basic salts (Murashige and Skoog, 1962) [10] supplemented with 30 $g.l^{-1}$ sucrose, 0.5 $mg.l^{-1}$ indole-3-butyric acids (IBA), 0.1 $mg.l^{-1}$ benzylaminopurine (BAP), and 1 $g.l^{-1}$ activated charcoal, and solidified with 7 $g.l^{-1}$ agar under aseptic conditions (Figure 1). The pH was adjusted to 5.87 followed by autoclaving at 121°C and 0.1 Mpa for 15 min. The cultures were incubated under a 16-h photoperiod (100 $\mu mol.m^{-2}.s^{-1}$) with a temperature of 24 °C [11] for 2 weeks, then

placed on adjacent benches in a plastic-covered greenhouse with mean maximum light intensity 660 $\mu mol.m^{-2}.s^{-1}$, and mean temperature 24 °C for pre-acclimation (Figure 1). Shoot tips were cut from the plants with a sterile surgical blade. The shoot tips cuts contained at least one axial bud and two leaves. The shoot cuts were rammed in 1% indole-3-butyric acid (IBA). For the similar stem cutting plantlets, nodal segments (10-15 cm) with armpit buds were taken from the apical portions of *L. citriodora* and then placed at the same culture conditions mentioned above.

Plantlets were transplanted in large pots containing 1:1:1 of clay: sand: peat moss in May 2012. All plantlets were grown in a non-shaded greenhouse under natural sunlight (that mean maximum light intensity during the experiment was 1200 $\mu mol.m^{-2}.s^{-1}$, relative humidity 85 to 90%, and mean of maximum/minimum temperature was approximately 28°C/20°C). The leaves of *L. citriodora* were harvested for determining the essential oil content and its components after 3 months in August 2012.

Table 1- Physico - chemical properties of peat moss medium

Elements	Content	Unit
N	93	g
P_2O_5	176	"
K_2O	198	"
NH_4^+	60	"
B	0.33	"
MgO	8.8	"
OC	50	"
Mo	2.2	"
Zn	0.44	"
Fe	0.99	"
Mn	1.76	"
Cu	1.32	"
Water holding capacity	563	**%**
Total porosity	87	"
Bulk density	0.13	**$g.cm^{-3}$**
CEC	165	**$meq.100g^{-1}$**
pH	4.3	-
EC	0.62	**$ds.m^{-1}$**

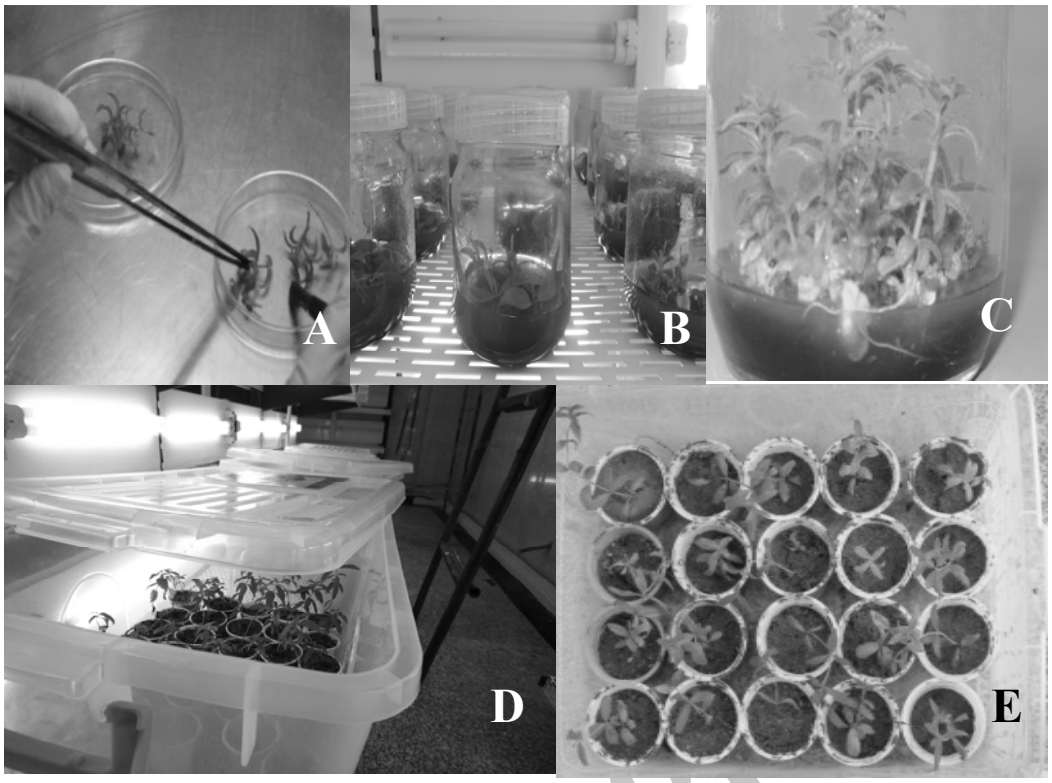

Figure 1- *In vitro* micro-propagation of *L. citriodora* induced by shoot multiplication. Sub-culturing (A, B), proliferation (C), pre-acclimation (D), and transfer of micro-propagated plantlet into greenhouse (E)

Essential oil content and composition

The essential oil of leaf plantlets were extracted by hydro-distillation for 3 h, using Clevenger-type apparatus [12]. The oils were dried over anhydrous sodium sulphate [13].

The volatile constituents were analyzed using an Agilent 6890 GC equipped with BPX5 capillary columns (30 m × 0.25 mm i.d. 0.25 µm film thicknesses) and a mass spectrometer Agilent 5973 as a detector. The carrier gas was helium, at a flow rate of 1 $ml.min^{-1}$. For GC-MS detection, an electron ionization system was used with ionization energy of 70 eV. Mass range was from 35-375 amu, emission current 150 mA. The injector and MS transfer line temperatures were set at 220 °C and 290 °C, respectively. One micro-liter of each sample was injected manually in splitless mode. C9-C20 n-alkanes were used as reference points in the calculation of the Kovats Indices (KI). Quantitative data were obtained from the electronic integration of the Flame Ionization Detector (FID) peak areas. Tentative identification of the compounds based on the comparison of their relative retention time and mass spectra with those of the NIST-98 and Wiley-275 library data of the GC-MS system and the literature data [14]. Each analysis was performed in triplicate.

Statistical analysis

The data were analyzed using SPSS (ver 17.0) and for schema tradition Excel (ver 2003) softwares. The differences among the means were determined using the Least Significance Differences (LSD) at $p \leq 0.05$ level.

Results

The results revealed that the essential oil content in the stem cutting plantlets cultivated in mixture soil (MPP) and the micro-propagated plantlets cultivated in peat moss (SCM) treatment was 0.6% and 0.5%, respectively. Analysis of variance for interaction effects of propagation methods and soil types on phytochemicals characters of *L. citriodora* was presented in Table 2. The maximum and minimum essential oil content was obtained in the MPP and SCM treatment, respectively. The MPP treatment had also significant effect ($p \leq 0.05$) on leaf dry weight (Figure 2), essential oil (Figure 4), 1.8-cineole (Figure 6), neral (Figure 7), geranial (Figure 8), 6-methyl-5-hepten-2-one (Figure 10), bicyclogermacrene (Figure 11), (*E*)-caryophyllene (Figure 12) and (*E*)-β-ocimene (Figure 13) and also, the effect of this treatment on SPAD value (Figure 3), limonene (Figure 5) and *ar*-curcumene was significant at 5% level (Figure 9).

The maximum amount of leaf dry weight (1.43 g), SPAD value (20.85), essential oil content (0.79%), limonene (11.29 %), 1.8-cineole (5.95%), neral (31.82%), geranial (53.52%), *ar*-curcumene (5.9%), 6-methyl-5-hepten-2-one (8.86%), bicyclogermacrene (5.97%), (*E*)-caryophyllene (5.01%) and (*E*)-β-ocimene (2.81 %) were obtained in the MPP treatment.

Schematic comparisons of the main components of essential oils in *L. citriodora* were showed in MPP and SCP treatments. In the MPP and SCP treatment, oxygenated monoterpenes were the prevailing group (91.4 and 46.74%, respectively) as compared to monoterpene hydrocarbons (14.6 and 11.96%, respectively). Also, the sesquiterpene hydrocarbons constituted only 16.68 and 10.48% of the essential oil in MPP and SCP treatment, respectively. In the MPP treatment as compared to SCP, the fraction of oxygenated monoterpene was higher, mainly due to an increase in geranial's percentage.

In MPP and MPM treatment, the major monoterpene hydrocarbons were represented by (E)-β-ocimene (2.81 and 1.91%), limonene (11.79 and 5.66%), respectively. Moreover, in SCP and SCM treatment, (E)-β-ocimene (1.94 and 1.12%) and limonene (10.02 and 8.04%) were also the major monoterpene hydrocarbons, respectively. The main oxygenated monoterpenes were demonstrated by geranial (53.52 and 30.01%), neral (31.81 and 24.15%), 1.8-cineole (5.87 and 3.91%) in MPP and MPM treatment, respectively. Also, the geranial (24.37 and 14.07%), neral (19.35 and 12.38%), 1.8-cineole (3.02 and 2.43 %) were the main oxygenated monoterpenes in SCP and SCM treatment, respectively.

The major sesquiterpene hydrocarbons were displayed by ar-curcumene (5.9 and 3.62%), (E)-caryophyllene (5.01 and 3.34%), bicyclogermacrene (5.77 and 4.23 %) in MPP and MPM treatment, respectively. Also, ar-curcumene (3.87 and 3.46%), (E)-caryophyllene (2.63 and 1.6%), bicyclogermacrene (3.98 and 2.25%) were the major sesquiterpene hydrocarbons in SCP and SCM treatment, respectively.

Table 2- Analysis of variance for effects of propagation methods and soil types and their interaction on phytochemicals characters of *L. citriodora*

S.O.V	df	Leaf dry weight	SPAD value	Essential oil	Limonene	1.8-Cineole	Neral	Geranial	Ar - Curcumene	6-Methyl-5-hepten-2-one	Bicyclogermacrene	(*E*) Caryophyllene	(*E*)-β-Ocimene
Block	2	0.01^{ns}	0.29^{ns}	0.01^{ns}	6.87^{ns}	0.02^{ns}	2.25^{ns}	38.46^{ns}	1.53^{ns}	2.12**	0.76^{ns}	1.37^{ns}	1.58**
P	1	0.1*	0.02^{ns}	0.06*	0.94^{ns}	14.58**	424.23**	1494.32**	3.63^{ns}	69.50**	12.58**	12.44**	2.03**
S	1	0.01^{ns}	5.46^{ns}	0.01**	23.57*	5.16*	150.87**	816.58**	5.38*	16.47**	8.38**	5.6**	2.21**
P×S	1	0.41**	18.9*	0.09**	2.03*	1.51*	1.003*	139.87**	2.61*	11.05**	0.01*	0.27*	0.1*
Error	6	0.01	3.67	0.01	2.98	0.63	5.34	12.51	0.81	0.24	0.21	0.38	0.06
CV (%)	-	9.96	10.15	11.07	19.72	20.86	10.48	11.25	21.41	11.49	11.28	19.57	13.13

ns: Non-significant, *: significant at 5% level, **: significant at 1% level

P: propagation methods (micro- propagation and stem cutting), S: Soil types (peat moss and mixture soil)

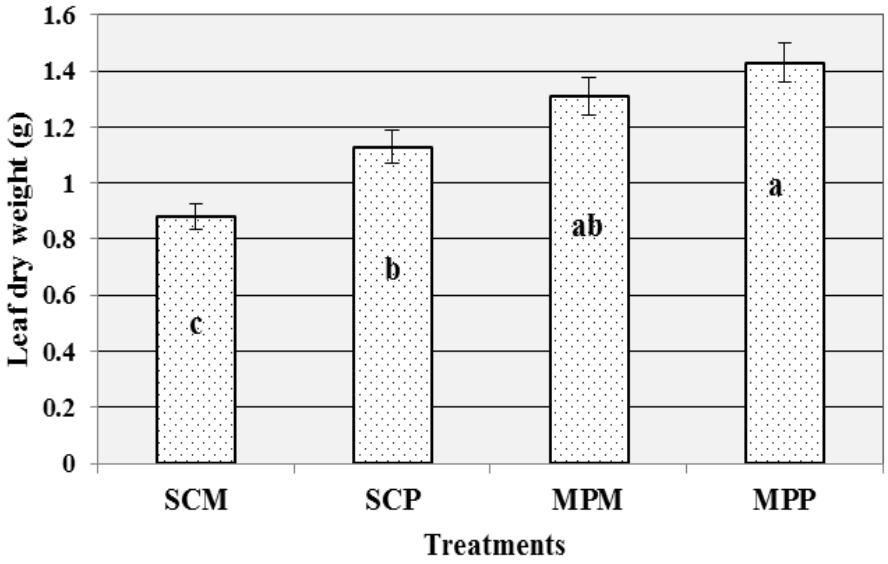

Figure 2- Mean comparisons for interaction effects of propagation methods and soil types on leaf dry weight of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

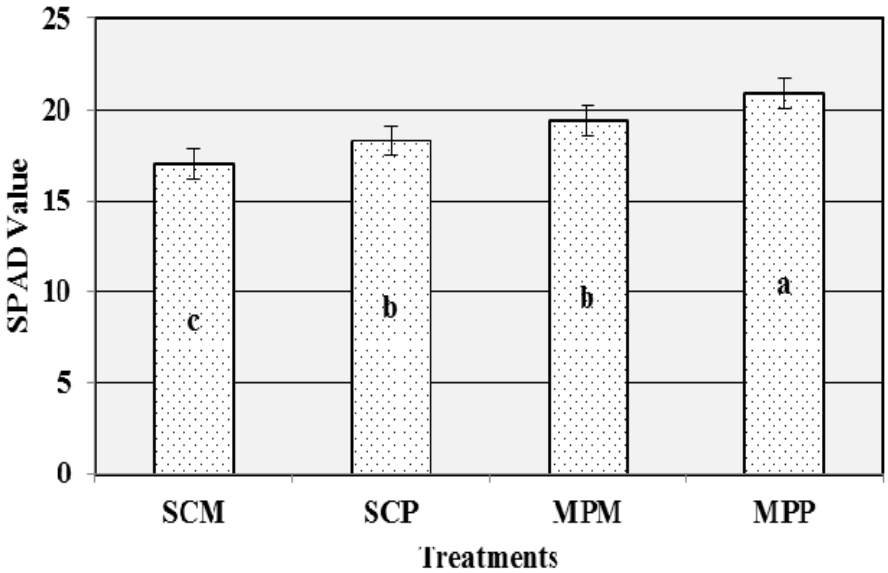

Figure 3- Mean comparisons for interaction effects of propagation methods and soil types on SPAD Value of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

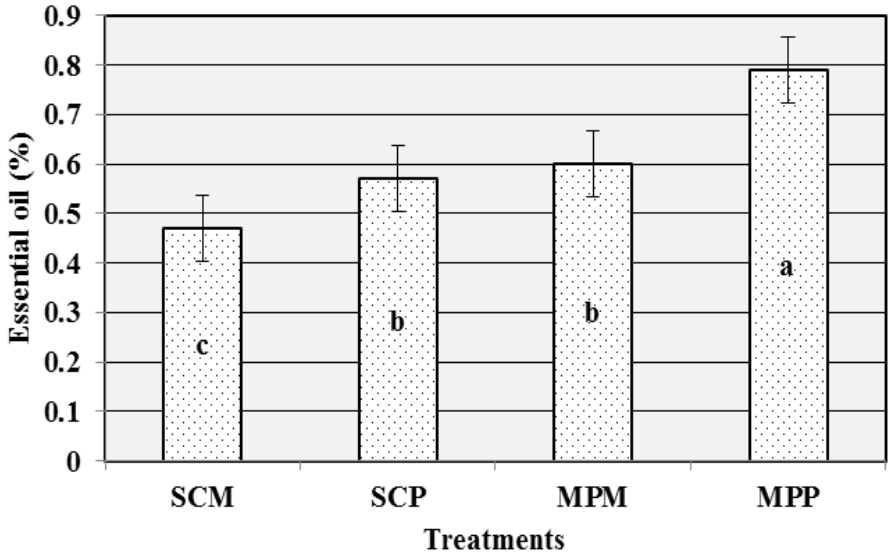

Figure 4- Mean comparisons for interaction effects of propagation methods and soil types on essential oil of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

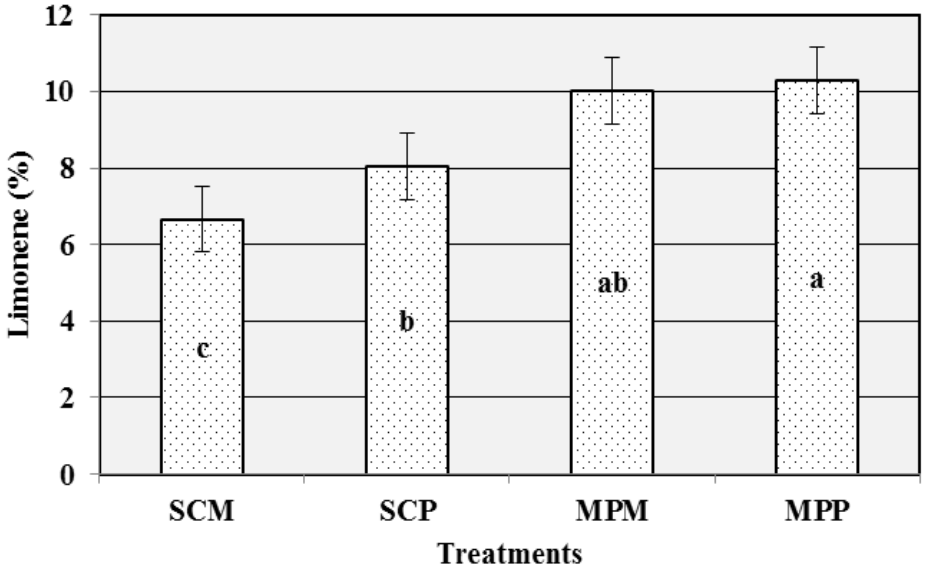

Figure 5- Mean comparisons for interaction effects of propagation methods and soil types on limonene of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

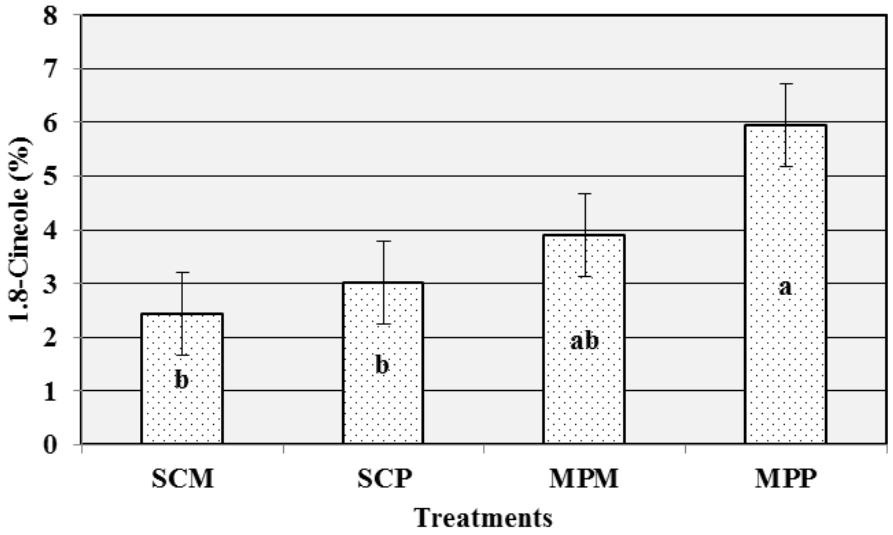

Figure 6- Mean comparisons for interaction effects of propagation methods and soil types on 1,8-cineole of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

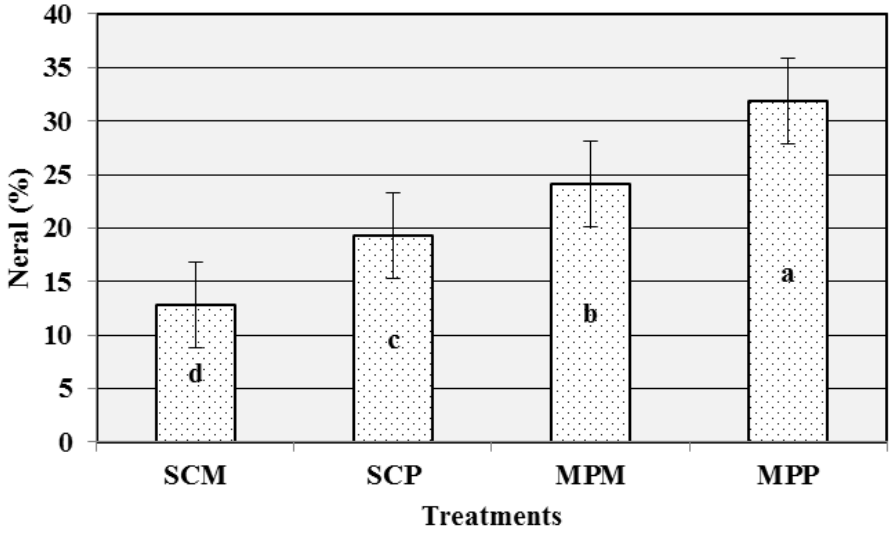

Figure 7- Mean comparisons for interaction effects of propagation methods and soil types on neral of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

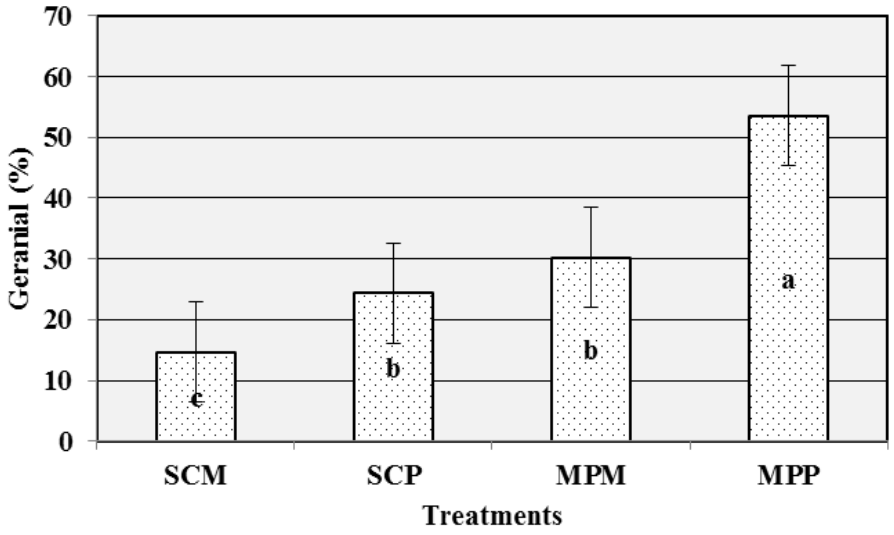

Figure 8- Mean comparisons for interaction effects of propagation methods and soil types on geranial of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

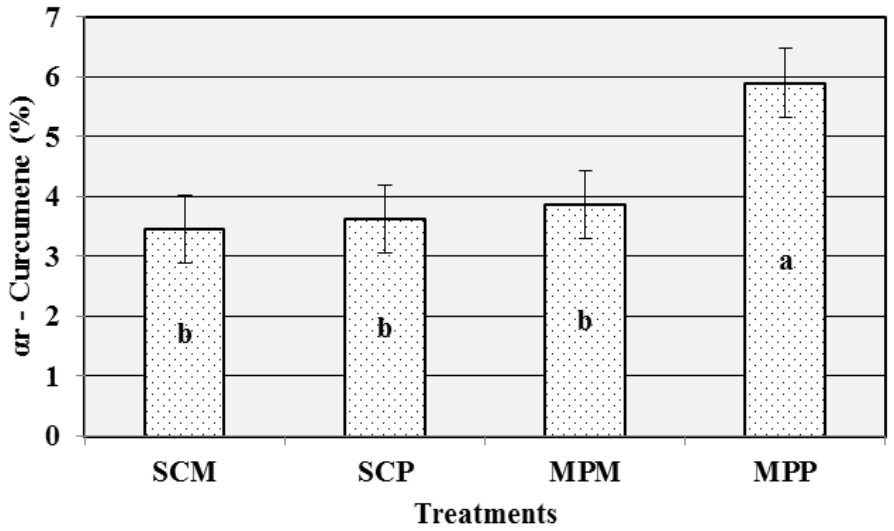

Figure 9- Mean comparisons for interaction effects of propagation methods and soil types on αr-curcumene of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

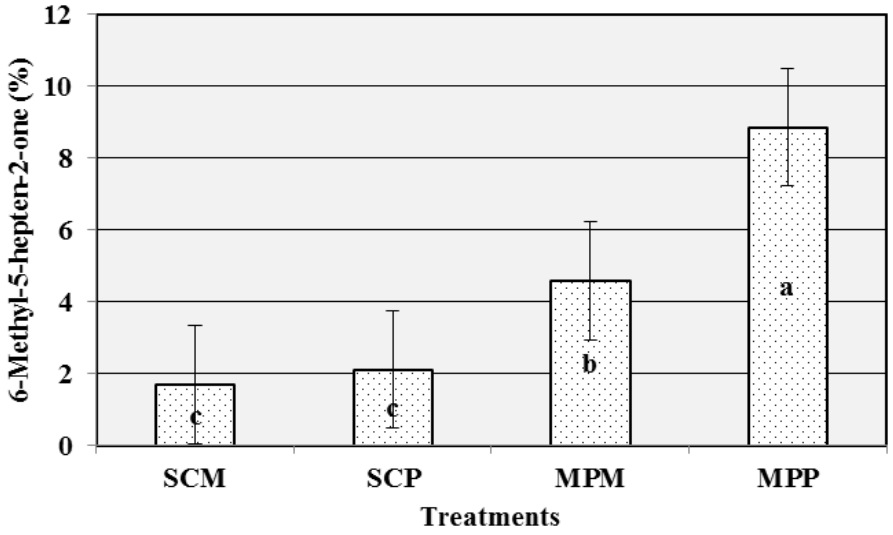

Figure 10- Mean comparisons for interaction effects of propagation methods and soil types on 6-methyl-5-hepten-2-one of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

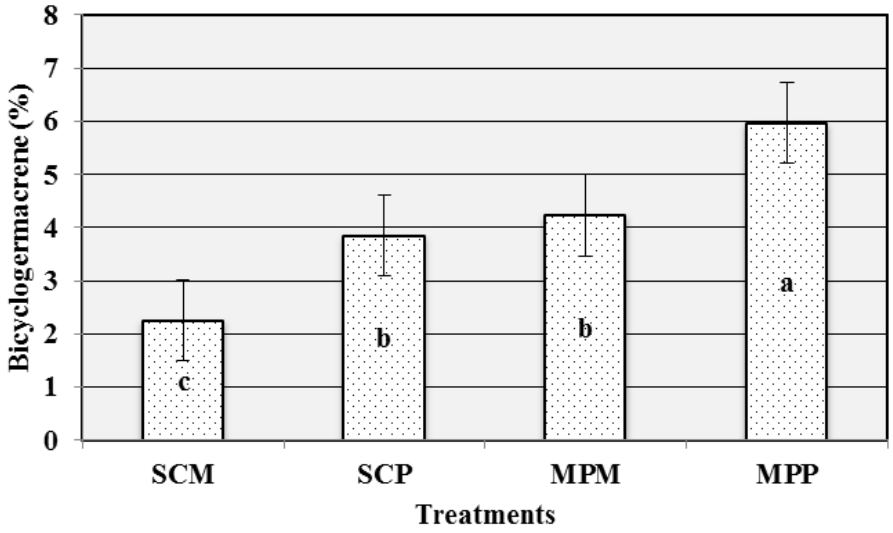

Figure 11- Mean comparisons for interaction effects of propagation methods and soil types on bicyclogermacrene of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

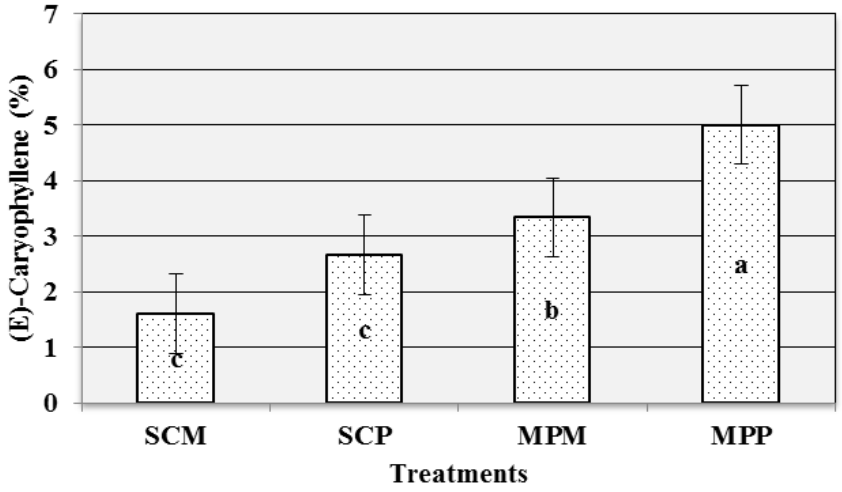

Figure 12- Mean comparisons for interaction effects of propagation methods and soil types on (*E*)-caryophyllene of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

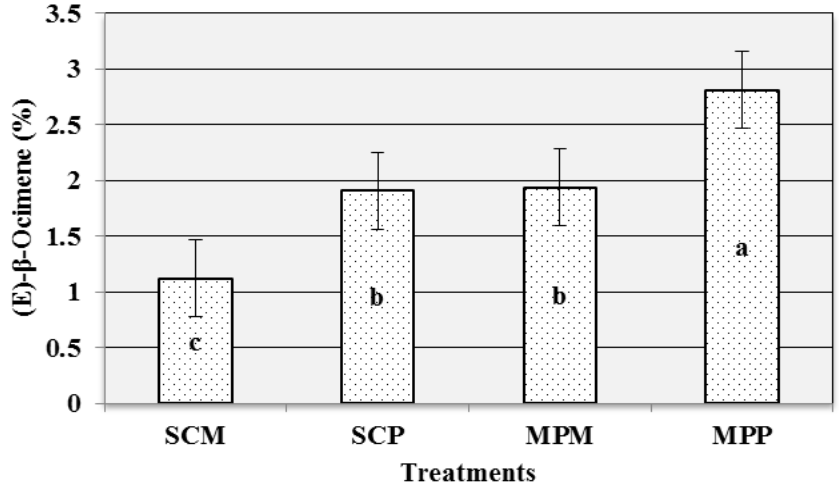

Figure 13- Mean comparisons for interaction effects of propagation methods and soil types on (*E*)-β-ocimene of *L. citriodora.* Abbreviations: SCM, SCP, MPM, and MPP was used for stem cutting plantlets cultivated in mixture soil, stem cutting plantlets cultivated in peat moss, micro-propagated plantlets cultivated in mixture soil, and micro-propagated plantlets cultivated in peat moss, respectively

However, the essential oil was characterized by the presence of terpenoids (monoterpenes and sesquiterpenes), except for 6-methyl-5-hepten-2-one (component group compound others), whose contribution is very low (Table 3).

Table 3- Compound groups and their percentages of the essential oil of *L. citriodora*

Compounds group	Percentage in MPP[1]	Percentage in MPM[2]	Percentage in SCP[3]	Percentage in SCM[4]
Monoterpene hydrocarbons	14.6	8.57	11.96	8.16
Oxygen-containing monoterpenes	91.4	58.07	46.74	29.3
Sesquiterpene hydrocarbons	16.68	11.19	10.48	7.31
Others	8.86	4.59	2.13	1.7

[1]**MPP:** micro-propagated plantlets cultivated in peat moss.
[2]**MPM:** micro-propagated plantlets cultivated in mixture soil.
[3]**SCP:** stem cutting plantlets cultivated in peat moss.
[4]**SCM:** stem cutting plantlets cultivated in mixture soil.

Discussion

Plant tissue culture is one of the important biotechnological tools that could be applied in various ways, especially in micro-propagation and secondary metabolite production [15]. The comparison of essential oil content in four treatments (MPM, MPP, SCM, and SCP) represented that MPP is the best treatment. This study indicated that the medium and peat moss increased the phytochemical characters of *L. citriodora*. The results of present research are in accordance with findings of Samiei et al. [16] on *Aglaonema commutatum* Cv. *Silver Queen*. Also, with scrutiny effect peat moss in *Aglaonema commutatum* plant obtained the highest number and surface of leaves per plant [16]. Production of secondary metabolites in tissue culture may be affected by several factors such as medium components, explants type, and physical conditions [17]. The literature emphasizes that a variety of media and environmental factors can lead to qualitative and quantitative changes in the essential oil production. The photosynthesis rate may be increased the essential oil content. Although, the essential oil biosynthesis is generally more related to the primary photosynthetic process [18], the composition of the essential oil can be changed by the proliferation method [19]. Today, *in vitro* propagation plays important roles in production of the secondary metabolites. In a research, the highest efficacious materials (as vincristine and vinblastine) were obtained in the *in vitro* micro-propagation of *Catharanthus roseus* in comparison with field culture. Also, cell cultures of *Dioscorea deltoidea* increased production of secondary metabolites in comparison with field culture. Therefore, the medium conditions can be affected on production of secondary metabolites [20].

The profiling of metabolites in the *L. citriodora* plantlets indicted that oxygenated monoterpenes were the prevailing group of essential oil as compared to the other compounds. Also, the monoterpen compounds such as geranial, neral and limonene were the main components of the essential oil. The Methyl Erythritol Phosphate (MEP) pathway is an important cellular metabolic pathway, which present in plants plastid. The MEP pathway is responsible for the biosynthesis of numerous essential molecules among monoterpenes groups. MEP pathway is the key precursor for synthesis of monoterpenes and related isoprenoid compounds. Monoterpenes is one of the isopernoids, synthesis of which start from combination pyruvat and glycire-3-phosphat. In this study, the monoterpenes increased with increasing carbon which can be due effect on MEP pathway [21].

Conclusion

Propagation methods and soil type could be controlled essential oil production and increase their useful compounds. Therefore, the *in vitro* micro-propagation of *L. citriodora* and cultivated in peat moss is an important tool for increasing secondary metabolites such as geranial and neral. In general, the highest content of the essential oil was obtained in treatment of MPP (micro-propagated plantlets cultivated in peat moss) and followed by MPM (micro-propagated plantlets cultivated in mixture soil), SCP (stem cutting plantlets cultivated in peat moss), and SCM (stem cutting plantlets cultivated in mixture soil). Overall, treatment of the *in vitro* micro-propagation and peat moss cultivation was suitable method to quickly proliferate and increase production the essential oil content of *L. citriodora*.

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