Comparison in Chemical Compositions of the Essential Oil from Leaves of *Lippia citriodora* H.B.K. Plantlets Produced from Micro-propagation and Stem Cutting

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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 the North region of Iran.**

Objective: **This comparative study was carried out on essential oil content and composition of *Lippia citriodora* H.B.K. from micro-propagated and rooted apical stem cutting plantlets.**

Methods: **The micro-propagated plantlets were obtained by use of synthetic medium condition containing basal MS medium supplemented with 1 mg.l^{-1} BAP, 0.5 mg.l^{-1} IBA, 1 g.l^{-1} activated charcoal, 30 g.l^{-1} sucrose, and 7 g.l^{-1} agars. Pre-acclimated plantlets were transferred to the research greenhouse. To determine the essential oil content and its components in two types of plantlets, leaves of *L. citriodora* were collected in August 2013 before flowering stage. The essential oils were extracted by Clevenger apparatus and their constituents were determined by GC-MS.**

Results: **The results indicated that thirty-two components were determined in the stem cutting derived plantlets. The main components of stem cutting plantlets were geranial (13.81%), limonene (11.41%), spathulenol (11.22%), and neral (11.21%). Thirty-five components were also obtained in the micro-propagated plantlets. The main components of micro-propagated plantlets were consisted of geranial (17.26%), neral (14.48%), 6-methyl-5-hepten-2-one (10.75%), and limonene (8.93%).**

Conclusion: **In general, the essential oil content of stem cutting plantlets was higher than micro-propagated plantlets, while the number of compounds and amount of geranial and neral in stem cutting plantlets was lower than micro-propagated plantlets.**

Keywords: ***Lippia citriodora* H.B.K., Essential oil, Geranial, Micro-propagated plantlets, Neral**

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Introduction

Lemon verbena (*Lippia citriodora* H.B.K.) is an important medicinal species of Verbenaceae family and native to South America (Chile and Peru). It grows exclusively in the north of Iran, especially in Golestan province [1]. This plant has a long history of folk uses in treating asthma, spasms, cold, fever, flatulence, colic, diarrhea, indigestion, insomnia and anxiety [2]. From each 100 kg of lemon verbena leaves, 510 g of essential oil is extracted [3]. The essential oil of the fragrant leaves with various levels and types of effective ingredients and constituents is used to make beverages in different countries. In Iran, the essential oil content of the leaves is 0.6% and usually includes 24 different main components [4]. Limonene and 1.8-cineole are the main components. Moreover, Geranial, neral, mega geraniol and phenyl carmen oxidized are other major components of essential oil [4, 5].

Secondary metabolites can be changed in the *in vitro* by using micro-propagation as a new method. Due to the importance of *L. citriodora* in various industries, optimization of secondary metabolites production of this plant by micro-propagation is very important as the first step. Secondary metabolites production of plant tissue culture probably has many differences in comparison with the compounds extracted from plants under natural conditions. These differences can be accurately controlled by various parameters such as enabling stabilization during various stages of tissue culture conditions, handling, transportation, ease of culture, and rapid proliferation of cells [6].

Knowledge of biosynthetic pathways of desired compounds in plants as well as plant cultures is often still rudimentary, and strategies are consequently needed to develop information. To the best of our knowledge, only few comparative studies on the essential oil compounds in the *in vitro* and stem cutting plantlets have been undertaken. Given the importance of changes in essential oil compounds, the objective of this research was to identify the essential oil components of the *L. citriodora* in micro-propagated and stem cutting plantlets.

Materials and Methods

In vitro and *in vivo* culture preparation

The experiment was conducted at the research greenhouse of Medicinal Plants Institute (MPI), Iranian Academic Centre for Education, Culture and Research (ACECR). Micro-propagated and stem apical cutting-derived plantlets of *L. citriodora* were used as planting materials.

Stem segments were excised from the shoot tip, and then were cultured on multiplication medium containing MS basic salts [7] supplemented with 30 $g.l^{-1}$ sucrose, 0.1 $mg.l^{-1}$ benzylaminopurine (BAP), 0.5 $mg.l^{-1}$ indole-3-butyric acids (IBA), and 1 $g.l^{-1}$ activated charcoal, and solidified with 7 $g.l^{-1}$ agar under sterile conditions in the laboratory. The pH was adjusted to 5.8 followed by autoclaving at 121 ^{o}C and 0.1 Mpa for 20 min. The cultures were incubated under a 16-h photoperiod (110 $\mu mol.m^{-2}.s^{-1}$) with a temperature of 23 °C [8] in 2 weeks, and then were placed on adjacent benches in a plastic-covered greenhouse (with mean maximum light intensity 650 $\mu mol.m^{-2}.s^{-1}$, and mean temperature 23 °C) in 2 weeks again for pre-acclimation. Apical stem cuttings were cut from the plants with a sterile surgical blade. The apical stem cutting was composed of at least one axial bud and two leaves. The apical stem cuttings were dipped in 1% indole-3-butyric acid (IBA). For the similar stem

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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.

Stem cutting and pre-acclimated plantlets were transplanted in large pots (with a volumetric ratio by 1:1:1:1 of soil:cocopeat:sand:perlite) at the research greenhouse in early May 2013. All plantlets were grown in a non-shaded greenhouse under natural sunlight (that mean maximum light intensity during the experiment was 1000 $\mu mol.m^{-2}.s^{-1}$, relative humidity 80 to 85%, and mean maximum and minimum temperatures approximately 28 °C/20 °C). To determine the essential oil content and its components in plantlets, leaves of *L. citriodora* were harvested after 3 months (in August 2013).

Oil extraction

The leaves samples of *L. citriodora* were dried in a shaded area, and then the essential oils content were extracted by hydro-distillation for 3 h, using Clevenger-type apparatus [9]. The oils were dried over anhydrous sodium sulphate and kept at 4°C until it was analyzed [10].

Identification of the volatile oil constituents

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. The column temperature was gradually increased from 60 °C to 220 °C with a rate of 2 °C/min for the polar column and from 60 °C to 240 °C with a rate of 3 °C/min for the non-polar column. For GC-MS detection, an electron ionization system was used with ionization energy of 70 eV. The injector and MS transfer line temperatures were set at 220 °C and 290 °C, respectively. One micro-liter of the sample was injected manually in splitless mode. C_9- C_{20} *n*-alkanes were used as reference points in the calculation of the Kovats Indices (KI). 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 [11].

Results

The results revealed that the essential oil content in the micro-propagated and stem cutting plantlets was 0.4% and 0.6%, respectively. Also, the percentages of the main components were different between the micro-propagated and stem cutting plantlets. The amounts distribution of the major components in the essential oils of different micro-propagated and stem cutting plantlets were presented in Table 2, while the changes in concentration of individual constituents were expressed in Table 1. In the essential oil of the stem cutting plantlets, a complex mixture of 34 compounds representing 91.73% of the total oil was detected. The major components were geranial (13.81%), limonene (11.41%), spathulenol (11.22%), neral (11.21%), and *ar*-curcumene (8.41%). In the essential oil of the micro-propagated plantlets, a complex mixture of 35 compounds representing 95% of the total oil was identified. The major components were geranial (17.26%), neral (14.48%), 6-methyl-5-hepten-2-one (10.75%), limonene (8.93%), spathulenol (6.21%), and *ar*-curcumene (6.02%).

The fluctuations in the percentage composition of the major compounds were rather complex. In the micro-propagated and

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Table 1- Chemical compositions of the essential oils of *L. citriodora* in micro-propagated and stem cutting plantlets

Compounds	Chemical formula	Composition in micro-propagated plantlet (%)	Micro-propagated plantlet (KI)	Composition in stem cutting plantlet (%)	Cutting plantlet (KI)
α-Pinene	$C_{10}H_{16}$	0.23 ± 0.1	933	0.44 ± 0.2	933
Sabinene	$C_{10}H_{16}$	0.84 ± 0.2	975	0.55 ± 0.04	975
1-Octan-3-ol	$C_8H_{16}O$	0.08 ± 06	986	1.55 ± 0.1	986
6-Methyl-5-hepten-2-one	$C_8H_{14}O$	10.75 ± 0.5	997	3.97 ± 0.1	996
Limonene	$C_{10}H_{16}$	8.93 ± 0.1	1037	11.41 ± 0.5	1037
1.8-Cineole	$C_{10}H_{18}O$	3.82 ± 0.1	1040	5.29 ± 0.6	1040
cis-Sabinene hydrate	$C_{10}H_{18}O$	0.38 ± 0	1076	0.60 ± 0	1076
Linalool	$C_{10}H_{18}O$	0.63 ± 0	1106	0.68 ± 0	1107
trans-Limonene oxide	$C_{10}H_{16}O$	-	-	0.31 ± 0.2	1145
Chrysannthenol	$C_{10}H_{16}O$	-	-	0.46 ± 0.01	1170
Rosefuran epoxide	$C_{10}H_{14}O$	0.24 ± 0	1178	1.64 ± 0.1	1179
α-Terpineol	$C_{10}H_{18}O$	1.07 ± 0.1	1206	1.63 ± 0	1207
Nerol	$C_{10}H_{16}O$	0.86	1232	0.51 ± 0	1232
Neral	$C_{10}H_{16}O$	14.48	1256	11.21 ± 0.6	1256
Geraniol	$C_{10}H_{18}O$	0.99 ± 0.6	1260	0.37 ± 0.1	1263
Geranial	$C_{10}H_{18}O$	17.26 ± 2.1	1288	13.81 ± 0.7	1288
α-Copaene	$C_{15}H_{24}$	0.49 ± 0	1379	0.93 ± 0	1380
Geranyl acetate	$C_{12}H_{20}O_2$	2.58 ± 0.03	1388	3.64 ± 0.2	1387
(*E*)-Caryophyllene	$C_{15}H_{24}$	3.74 ± 0	1426	1.62 ± 0	1425
β-Cedrene	$C_{15}H_{24}$	0.22 ± 0	1431	0.39 ± 0	1431
Aromadendrene	$C_{15}H_{24}$	0.41 ± 0.07	1465	0.91 ± 0.1	1466
Geranyl propionate	$C_{13}H_{22}O_2$	0.31 ± 0	1476	0.40 ± 0	1478
ar-Curcumene	$C_{15}H_{22}$	6.02 ± 0.2	1488	8.41 ± 1.2	1488
α-Zingiberen	$C_{15}\ H_{24}$	1.02 ± 0.4	1500	0.38 ± 0.19	1500
Bicyclogermacrene	$C_{15}H_{24}$	2.47 ± 0.6	1503	0.98 ± 0.02	1503
β-Curcumene	$C_{15}H_{24}$	1.09 ± 0	1515	0.48 ± 0	1515
β-Sesquiphellandrene	$C_{15}H_{24}$	0.65 ± 0.09	1523	0.81 ± 0.14	1524
(*Z*)-Nerolidol	$C_{15}H_{26}O$	0.38 ± 0	1566	0.57 ± 0	1564
(*E*)-Nerolidol	$C_{15}H_{26}O$	1.74 ± 0	1572	2.22 ± 0	1574
Spathulenol	$C_{15}H_{24}O$	6.21 ± 0.4	1597	11.22 ± 1.2	1598
Caryophyllene oxide	$C_{15}H_{24}O$	2.43 ± 0	1594	2.11 ± 0.06	1599

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Table 1- Continued

Compounds	Chemical formula	Composition in micro-propagated plantlet (%)	Micro-propagated plantlet (KI)	Composition in stem cutting plantlet (%)	Cutting plantlet (KI)
Bisabolol (Dihydro 10,11-ar-alpha)	$C_{15}H_{26}O$	0.29 ± 0.08	1604	1.19 ± 0.03	1601
α-Muurolol	$C_{15}H_{26}O$	0.60 ± 0	1654	1.09 ± 0.01	1656
(*E*)-*β*-Ocimene	$C_{10}H_{16}$	1.86 ± 0	1051	-	-
Citronella	$C_{10}H_{18}O$	0.51 ± 0.1	1160	-	-
α-Humulene	$C_{15}H_{24}$	0.18 ± 08	1461	-	-
Carvotan acetone	$C_{10}H_{14}O$	-	-	0.52	1260
Globulol	$C_{15}H_{26}O$	0.89 ± 0.2	1597	-	-
Percent of essential oil	-	0.4 ± 0.08	-	0.6 ± 0.1	-
The number of known compounds	-	35	-	34	-
Percent of known compounds	-	95	-	91.73	-

Table 2- Compound groups and their percentages of the essential oils *L. citriodora* in micro-propagated and stem cutting plantlets

Compounds group	Percentage (%) in micro-propagated plantlet	Percentage (%) in stem cutting plantlet
Monoterpene hydrocarbons	14.8	12.4
Oxygen-containing monoterpenes	40.24	36.52
Sesquiterpene hydrocarbons	16.6	14.91
Oxygen-containing sesquiterpenes	12.89	17.83
Others	13.72	9.56

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stem cutting plantlets, oxygenated monoterpenes were the prevailing group (40.24 and 36.52%, respectively) as compared to monoterpene hydrocarbons (14.8 and 12.4%, respectively). The oxygenated monoterpenes fraction was the main group of the compounds in both plantlets studied, while sesquiterpene hydrocarbons constituted only 16.6 and 14.91% of the oil in micro-propagated and stem cutting plantlets, respectively. In micro-propagated plantlets as compared to stem cuttings, the fraction of oxygenated monoterpene was enriched, mainly due to an increase in geranial and neral's percentage. Comparison of the main components profiles for essential oils of *L. citriodora* in micro-propagated and stem cutting plantlets were showed in Figure 1.

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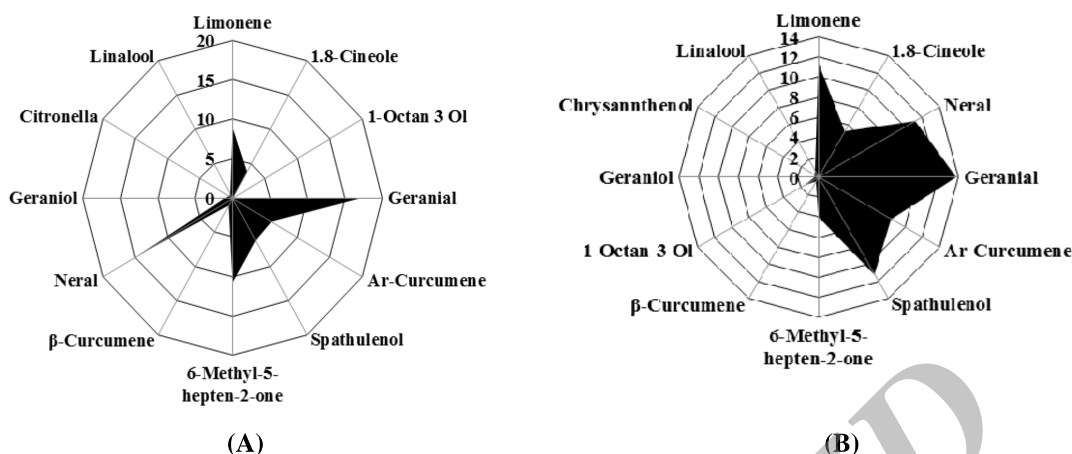

Figure 1- Comparison profiles of the main components for essential oils *L. citriodora* in micro-propagated plantlet (A) and stem cutting (B)

The major monoterpene hydrocarbons were represented by (*E*)-*β*-ocimene (1.86%), limonene (8.93%), sabinene (0.84%) and *α*-pinene (0.23%) in micro-propagated plantlets, and limonene (11.41%), sabinene (0.55%), and *α*-pinene (0.44%) in stem cutting plantlets. The main oxygenated monoterpenes were demonstrated by geranial (17.26%), neral (14.48%), 1.8-cineole (3.82%), *α*-terpineol (1.07%), geraniol (0.99%), citronella (0.51%), linalool (0.63%), *Cis*-sabinene hydrate (0.38%), and rosefuran epoxide (0.24%) in micro-propagated plantlets, and geranial (13.81%), neral (11.21%), 1.8-cineole (5.29%), rosefuran epoxide (1.64%), *α*-terpineol (1.63%), linalool (0.68%), *Cis*-sabinene hydrate (0.60%), chrysannthenol (0.46%), geraniol (0.37%), and *trans*-limonene oxide (0.31%) in stem cutting plantlets.

The major sesquiterpene hydrocarbons were displayed by *ar*-curcumene (6.02%), (*E*)-caryophyllene (3.74%), bicyclogermacrene (2.47%), *β*-curcumene (1.09%), *α*-zingiberen (1.02%), *β*-sesquiphellandrene (0.65%), *α*-copaene (0.49%), aromadendrene (0.41%), *β*-cedrene (0.22%) and *α*-humulene (0.18%) in micro-propagated plantlets, and *ar*-curcumene (8.41%), (*E*)-caryophyllene (1.62%), bicyclogermacrene (0.98%), *α*-copaene (0.93%), *β*-sesquiphellandrene (0.81%), aromadendrene (0.91%), *β*-curcumene (0.48%), *β*-cedrene (0.39%), and *α*-zingiberen (0.38%) in stem cutting plantlets. The main oxygenated sesquiterpene were represented by spathulenol (6.21%), caryophyllene oxide (2.43%), (*E*)-nerolidol (2.08%), globulol (0.89%), *α*-Muurolol (0.6%), (*Z*)-nerolidol (0.39%), and bisabolol (0.29%) in micro-propagated plantlets, and spathulenol (11.22%), (*E*)-nerolidol (2.22%), caryophyllene oxide (2.11%), bisabolol (1.19%), *α*-murolol (1.09%), and (*Z*)-nerolidol (0.51%) in stem cutting plantlets.

As Table 2, it indicated that the essential oil was characterized by the presence of terpenoids (monoterpenes and sesquiterpenes), except for 6-methyl-5-hepten-2-one, geranyl acetate, geranyl propionate, 1-octan-3-ol whose contribution was very low. The oil was devoid of compounds from the phenylpropanoid pathway.

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Discussion

The comparison of these two plantlets (micro-propagated and stem cutting plantlets) showed that the essential oil content in the stem cutting plantlets was more than in the *in vitro* condition. The results of present research are in accordance with findings of Juliani et al. (1999) for *Lippia junelliana* Mold, who reported that the shoot cultures show a lower essential oil accumulation in comparison with parent plants. Some of the most significant factors in increasing production of secondary metabolites in culture are medium components, explants, and physical conditions [6]. The literature emphasizes that a variety of media and environmental factors can lead to qualitative and quantitative differences in the essential oil produced. It has been found that stem cutting plantlets has much more essential oil rather than micro-propagated plantlets grown in the greenhouse. At the same time, a number of other factors such as the growing conditions can influence in the essential oil composition. Considering that the component of essential oil biosynthesis is generally more related with the primary photosynthetic process [12], the composition of the essential oil is also affected by the production method [13]. Micro-propagated and stem cutting plantlets display a range of phytochemical productions, which have been described as responses to various chemical and physical factors of the early culture environment. The difference in the amount and components of essential oil in plantlets is due to medium composition, type and composition of growth regulators, mineral salts, carbon source, and environmental factors of pre-acclimation in the early growth of micro-propagated plantlets [14, 15].

We observed a pattern of metabolites in the *L. citriodora* plantlets which it disclosed an even part of the biosynthetic pathway with a narrow range of metabolites, a very low amount of monoterpene and sesquiterpene hydrocarbons and a high content of the oxygenated monoterpenes. Oxygenated monoterpenes were the prevailing group as compared to sesquiterpenes. Interestingly, a recent report has showed that in snapdragon flowers the plastid-localized methylerythritol phosphate (MEP) pathway provided isopentenyl diphosphate (IPP) precursors for both plastidial monoterpene and cytosolic sesquiterpene biosynthesis [16]. Although the exact intermediates were not identified, the authors provided evidence that under certain conditions, exchange of metabolites of the cytosolic and the plastidial isoprenoid pathways across the plastid envelope occurred and the transport might be bi-directional [17]. The main compounds were geranial and neral in the micro-propagated plantlets, and geranial and limonene in the stem cutting plantlets. Whereas, the amount of geranial were the main component in both plantlets, but it was less in the stem cutting plantlets. It is recognized that a high biosynthesis of geranial proceed subsequently in the metabolic pathway and their accumulation in the *in vitro* plantlets of *L. citriodora* that they are preferentially formed at this stage of development. Due to the growing commercial importance of *L. citriodora* secondary metabolites, there is great interest in enhancing their production through biotechnology. This study has proved that tissue culture of *L. citriodora* is able to biosynthesize geranial and neral in quantities. The molecular structures of geranial, neral, 6-methyl-5-hepten-2-one and limonene were presented in Figure 2. Chrysannthenol, carvaton acetone, and *trans*-limonene oxide component were

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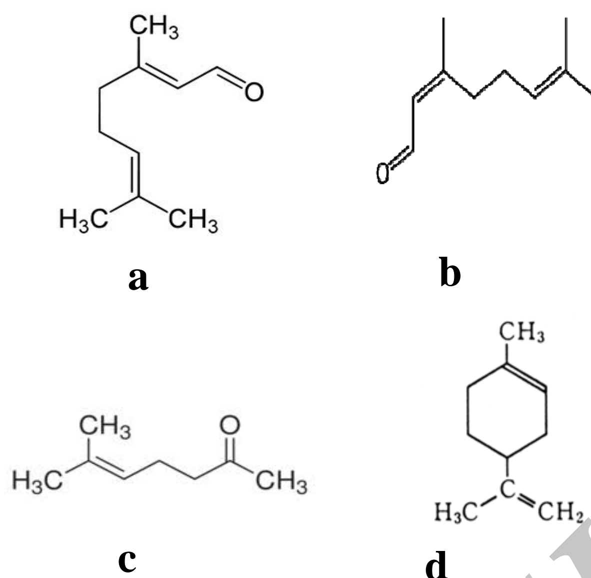

Figure 2- Molecular structures of geranial (a), neral (b), 6-methyl-5-hepten-2-one (c), limonene (d)

found only in the stem cutting plantlets, and also, nerol, globulol, citronella, *α*-humulene and (*E*)-*β*-ocimene were detected only in the micro-propagated plantlets.

In the present research, *L. citriodora* has been studied for the biosynthesis and accumulation of phytochemicals in the micro-propagated and stem cutting plantlets. The manipulation of plant terpene metabolism has long been a focus of interest in plant biotechnology and culture [18]. Recent advances in the biotechnology of plant cultures suggest that these systems will become a viable source of important secondary metabolites. A growing interest in the development of efficient protocols to micro-propagate certain species of *Salvia* has also been evident in more recent years, some times correlated with the production of terpenes and phenolic constituents [19]. The rate of change and stability in the composition of essential oils was showed suitable adaptability to the *in vitro* conditions for some secondary metabolites. *In vitro* propagation of medicinal plants with enriched bioactive principles and culture methodologies for selective metabolite production is found to be highly useful for commercial production of important medicinally compounds. The increased use of plant culture systems in recent years is perhaps due to an improved understanding of the secondary metabolite pathway in important economically plants [20]. The increasing demand for useful secondary metabolites has intensified the application of proliferation methods to reproduce under controlled growing conditions and to obtain homogenous productions.

Conclusion

L. citriodora H.B.K. can be cultured in the *in vitro* condition due to reproduce quickly and easily of this plant in medium as an alternative method to proliferation and increase in secondary metabolites such as geranial and neral. Micro-propagation for the production of valuable secondary metabolites is beneficial independent of the control medium change. In general, the essential oil content in micro-propagated plantlets was less than stem cutting

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plantlets while the number of compounds and geranial and neral content was increased.

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