Received: 2020-07-18

Accepted: 2020-12-08

Available online: 2020-12-31

DOI: 10.2478/hepo-2020-0019

EXPERIMENTAL PAPER

In vitro clonal propagation and efficient acclimatization with use of hydrogel of intensively sweet medicinal plant *Lippia dulcis* Trev.

MAGDALENA TOMASZEWSKA-SOWA

University of Science and Technology
Department of Agricultural Biotechnology
Bernardyńska 6
85-029 Bydgoszcz, Poland,

phone: + 48 52 374 95 21; e-mail: magda@utp.edu.pl

Summary

Introduction: The leaves of *Lippia dulcis* contain high amounts of hernandulcin. It is one thousand fold sweeter than sucrose, however, it hardly contains any calories.

Objective: The aim of this research was to optimisation of micropropagation and acclimatization of *L. dulcis*.

Methods: The nodal explants were inoculated on phytohormone-free MS medium. After 6 weeks the explants were inoculated onto the MS medium with different plant growth regulators. Well-developed rooted plantlets were adapted to *ex vitro* conditions using hydrogel.

Results: On the medium with BAP and NAA the highest number of shoots were produced. The highest average shoot length, number of the leaves and the leaf area were recorded on the medium with GA₃. Adding IBA increased the number of roots. The addition of hydrogel enhanced the acclimatization efficiency.

Conclusions: There was observed a positive, stimulating influence of growth regulators on mass propagation and increase in the number of leaves and the leaf area and influence of hydrogel on the development of plantlets during acclimatization.

Key words: *phytohormones, hydrogel, Lippia dulcis, micropropagation, rooting*

Słowa kluczowe: *fitohormony, hydrożel, Lippia dulcis, mikrorozmnażanie, ukorzenianie*

INTRODUCTION

An intensive development of civilization as well as an easy and quick access to synthetically produced drugs and healthy substances have outclassed the benefits of natural products. The active compounds in plants are often equal to their chemical equivalents in terms of potency. Furthermore, their proper application ensures a reaction effectiveness with no negative side effects. *Lippia dulcis* Trev. (*Phyla scaberrima*, *Lippia scaberrima*, *Zapania scaberrima*) is one of such traditional natural medicinal plants of *Verbenaceae* family. It is up to 40 cm tall and its white flowers produce an intensive aroma. Long plant shoots end with leaflets dark green in colour, sometimes changing into violet. They creep spectacularly on the ground. *Lippia* comes from the tropical sites and is popular in Asia and South America. The first reports on *L. dulcis* come from 1552 when its sweet leaf taste was discovered. It is due to the fact that the leaves contain high amounts of edible sweetening substance, hernandulcin, an organic chemical compound of the terpenoids group, analog of bisabolol. It is one thousand fold sweeter than sucrose, however, it hardly contains any calories [1-3]. In some Asian countries, *L. dulcis* has been added to desserts, used for sweetening tea or to provide flavour of water. It can be used as a sweetener, although it is sensitive to high temperatures. From the leaves of *Lippia dulcis* also verbascoide (kusagin, acteoside) was extracted, a phenolic compound and a very strong antioxidant, showing anti-inflammatory and pain-relieving, immunosuppressive, immune modeling, anticancer and hepatoprotective properties [4] (fig. 1). In folk medicine, *L. dulcis* has been used in the treatment of bronchitis, cough, indigestion, liver disorders, hypertension, and dysentery. *L. dulcis* shows a specific aroma as a result of camphor oil found in its tissues. For that reason people, especially children and pregnant women, should not consume high amounts of fresh leaves. Due to numerous active substances in tissues, *L. dulcis*, as demonstrated by the pharmacological and biochemical studies so far, shows antioxidative, anti-inflammatory and antibacterial properties towards *Salmonella typhi* and *Shigella flexneri*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Vibrio cholerae* [1, 2, 5]. In addition, Abe *et al.* [6] found that the *L. dulcis* extracts had significant antiproliferative effects on several lines of human cancer cultures. Despite its health-boosting properties the plant is not popular a lot.

Figure 1.

Hernandulcin (a), verbascoide (b)

In vitro methods of propagation and callus formation of plants of the *Verbenaceae* family have been described quite extensively. However, there are very few articles about *L. dulcis* *in vitro* propagation. Urrea *et al.* 2009 [7] indicate the possibility of using shoot tips and nodal segments for micropropagation and induction of callus formation. The shoot cultures and hairy root cultures of *L. dulcis* have been used as a source of hernandulcin, which is sweeter than sucrose and can be found in human diet [8, 9]. In the present study we evaluate the effect of different plant growth regulators on shoot and leaf induction, regeneration and rooting of *L. dulcis* and propose an optimal system of acclimatization of this species to *ex vitro* conditions.

MATERIAL AND METHODS

The initiation of culture and disinfection of plant material.

Explants of *L. dulcis* were collected from *ex vitro* plants growing in greenhouse conditions (fig. 2, 3A). Nodal fragments without leaves, 1.5–2.0 cm in length, were chemically disinfected. At the first stage, the vegetable parts, rinsed with running tap water, were immersed for 1 minute into a 70% EtOH solution for degreasing, degassing, and surface disinfection. Then the explants were treated with 10% calcium hypochlorite ($\text{Ca}(\text{OCl}_2)$) solution with 3 drops of Tween 20 for 15 minutes. Finally shoot parts were rinsed three times with sterile distilled water. The sterilized nodal explants were inoculated on phytohormone-free Murashige and Skoog medium (MS) [10] supplemented with 3% sucrose and solidified with 0.8% agar (fig. 3B). The medium was adjusted to pH 5.7 and autoclaved at 0.5 MPa at 121°C for 25 minutes. The cultures were carried out in a plant growth chamber under a 16-hour photoperiod with the light intensity of $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and the temperature of $25\pm 2^\circ\text{C}$. The number of shoots initiated per explant were recorded.

Figure 2.

Ex vitro plants of *Lippia dulcis* growing under greenhouse conditions

Figure 3.

Different stages in the micropropagation of *Lippia dulcis*: fragment of *Lippia dulcis ex vitro* shoot (A), initiation of shoot formation on the MS medium (B), secondary explants (C), root induction (D)

Shoot proliferation process

After 6 weeks of shoot culture, individual plants were selected and cut into nodal fragments with one bud of maximum 2 cm in length. The explants were inoculated onto MS medium with different concentrations and combinations of BAP (6-benzylaminopurine, Sigma - Aldrich, Germany) and NAA (naphthaleneacetic acid, Sigma - Aldrich, Germany) and GA₃ (gibberellic acid, Sigma - Aldrich, Germany) (Table 1) and incubated under such growth conditions (Fig. 3C). For each replication of the experiment 50 explants were used and the experiment was repeated three times. After 8 weeks the newly-formed shoots were counted and the mean shoot length was recorded. The results were subjected to statistical analysis and the proliferation rate was determined.

Rooting of elongated shoots

After 8 weeks, the amplified axillary shoots which regenerated on the medium with the addition of 1.0 mg dm⁻³ BAP and 1.0 mg dm⁻³ NAA were transferred onto the rooting MS medium (fig. 3D). As a rooting growth medium a solid MS medium without any growth regulators as well as an MS medium supplemented with 0.5 mg dm⁻³ IBA (indole-3-butyric acid, Sigma - Aldrich, Germany) or 0.5 mg dm⁻³ IAA (indole-3-acetic acid, Sigma - Aldrich, Germany) were used. After the successive 8 weeks the following parameters were evaluated: the percentage roots, the root number and the length of roots (fig. 4).

Figure 4.

Effect of IBA (A) and MS free medium (B) on *in vitro* rooting of shoots in *Lippia dulcis*

Table 1.

Effect of plant growth regulators on shoot and leaf induction and regeneration

Plant growth regulator (mg dm ⁻³)			Average number of shoots/explant (mean ±SD)	Average shoot length (cm) (mean ±SD)	Average no. of leaves per shoot (mean ±SD)	Average leaf area (cm ²) (mean ±SD)
BAP	NAA	GA ₃				
–	–	–	2.02±0.80c	3.06±1.23c	3.96±0.8b	0.86±0.13 ns
0.5	–	–	2.14±0.85bc	3.94±1.58bc	3.44±1.23b	0.81±0.21 ns
1.0	–	–	2.26±0.94bc	3.80±1.56bc	3.54±1.09b	0.89±0.20 ns
0.5	1.0	–	4.08±1.19ab	4.42±1.67b	3.74±0.95b	1.0±0.31 ns
1.0	1.0	–	5.14±0.99a	4.07±1.76b	4.04±1.04b	0.95±0.25 ns
0.5	–	1.0	3.42±1.31b	5.34±1.82a	4.40±1.64ab	0.92±0.26 ns
1.0	–	1.0	3.72±1.32b	5.02±1.73ab	4.60±1.57a	0.90±0.17 ns
0.5	1.0	1.0	4.80±0.95a	5.66±1.82a	4.70±1.63a	0.98±0.11 ns
1.0	1.0	1.0	4.34±1.09a	6.26±1.35a	5.40±1.17a	1.02±0.11 ns

Results are mean ±SD (standard deviation); means followed by the same letter do not differ significantly at $\alpha=0.05$

Acclimatization of plantlets

Well-developed rooted plantlets were adapted to *ex vitro* conditions. The plants were removed from the jars and thoroughly washed with water and transferred to plastic pots. The pots were filled with sterilized substrate in two variants: a mixture of sand, soil and vermiculite (2:1:1) and a mixture of sand, soil, vermiculite and hydrogel (1:1:1:1), with pH ranging from 5.5 to 6.5. They were placed in the growth chamber with the light intensity of 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and the temperature of 25±2°C under a 16-hour photoperiod. After 28 days of adaptation to *ex vitro* conditions, the plants were transferred to greenhouse conditions.

Statistical analyses

The results on the number of shoots per explant, leaf number and leaf area as well as on the root length and number were subjected to statistical analyses. The statistical analyses comprised the analysis of variance and the significance of differences between means, verified with the Tukey test at significance level of $\alpha=0.05$. The analyses of variance were performed using the Statistica software.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

As for bacterial and fungal control, the combination of alcohol and calcium hypochlorite yielded 78% of

axenic cultures. Three hundred sterilized nodal segments produced 234 sterile shoots. Stems emerging from lateral buds demonstrated a normal plant habit: they were green in colour, with no signs of destructive impact of the active substance used in the process of sterilization. After 6 weeks of nodal fragments culture, along with at least one lateral bud derived from *in vitro*, the cultures were used as secondary explants for shoot proliferation. Urrea *et al.* 2009 [7] also used the shoot tips and nodal segments of *L. dulcis* for micropropagation and callus formation. This kind of explant was offered as the best explant for *in vitro* proliferation and reported by Boustani *et al.* [11], Juliani *et al.* [12], and Castellanos-Hernandez *et al.* [13]. As it was noted by Servin *et al.* [14], the nodal explants on the half MS medium with no growth regulator has been the best in forming sprouts and roots from buds. Similarly, Urrea *et al.* 2009 [7] considered the MS medium without growth regulators to be the most effective for induction and multiplication of *L. dulcis* shoots from shoot tips and nodal segments. In another study of *in vitro* propagation in plant species of the *Verbenaceae* family it was detected that nodal segments on the medium supplemented with BAP and NAA induced multiple shoots [15]. In present experiment, the explants were inoculated on the modified MS medium to proliferate and to grow shoots. Over successive 10 weeks, the number and length of shoots were estimated, the number of leaves was determined and the width of the leaf blade – measured. Among the plants originating from different variants of the experiment, the medium supplemented with 1.0 mg dm⁻³ BAP and 1.0 mg dm⁻³ NAA produced the highest number of

shoots from a single explant (approximately 5.14). This combination of phytohormones also triggered the highest multiple shoots response in *Lippia filifolia* [15]. In the micropropagation of *Lippia junelliana*, an increase in the number of shoots was observed with an increase in BA concentration. The best results were achieved by combining BA ($4.4 \mu\text{M}$) and IBA ($0.04 \mu\text{M}$) [12], whereas other researchers reported the highest number of shoots per explant of *Lippia graveolens* from the medium with BAP only. The best results were obtained in the medium containing 2 mg dm^{-3} BA, less shoots and roots were formed when there were lower concentrations of BA or BA in combination with auxin in the medium [13]. In the presence of another phytohormone from the auxin group - 2,4-D at a concentration of 0.1 mg dm^{-3} Urrea *et al.* 2009 [7] obtained intensive callus formation on explants isolated from *L. dulcis* plants. In our experiment, short internodes of *L. dulcis* plants growing on culture media, containing BAP or NAA were observed. To produce longer internodes, GA_3 was applied following [16]. The presence of GA_3 in the medium enhanced the length of shoots and the number of leaves per shoot after 10 weeks of cultivation. The longest shoots (6.26 cm) were produced on the medium supplemented with 1.0 mg dm^{-3} BAP, 1.0 mg dm^{-3} NAA and 1.0 mg dm^{-3} GA_3 , while only cytokinins and auxins in the same concentrations without GA_3 resulted in shorter shoots (4.07 cm). The effect of gibberellic acid on shoot elongation was also reported by Rani and Nair [17]. In *in vitro* micropropagation of *Vitex negundo*, the elongation of shoots was observed on the MS medium containing 2.4 mM GA_3 [17]. The number of the leaves formed and the leaf area also depended on the phytohormonal composition of the medium. With GA_3 in the medium, the highest number of leaves per shoot and the largest leaf area were noted. The highest number of leaves (5.4) per shoot was observed on the medium with BAP, NAA and GA_3 (1.0 mg dm^{-3}). In that case the biggest leaf area (1.02 cm^2) it was also reported (tab. 1).

After four weeks of culture, the regenerated shoots were transferred to the rooting media. Root initiation was observed within three weeks of culture. The highest rooting rate and root length were produced on the medium supplemented with 0.5 mg dm^{-3} IBA. For that species, the addition of IBA to induce rhizogenesis increased the number of roots per explant significantly (by about 50%), as well as they were about 53% longer (tab. 2). Adding IBA significantly increased the number and the length of the roots described also in the studies of Castellanos-Hernandez *et al.* [13]. According to them, best results were obtained in medium containing 1.5 mg dm^{-3} IBA. In the presence of this phytohormone, an average of 15 roots per shoot with a length of up to 10 cm was obtained. Similarly, Boustani *et al.* [11] reported root formation when exposed shoots to auxin in the medium. When medium were supplemented just with cytokinins without combination of auxin regenerated shoots were not rooted. The authors described only the formation of shoots and leaves from the callus. Much research on the *Verbenaceae* family reports on the root development in plants of that family to be very easy, however, some report observed the highest rooting percentage on the MS free medium. Moreover, a treatment without growth regulators produces a large and vigorous root system [12].

The effectiveness of water use in the acclimatization process is important for the improvement of the survival and performance of plant plantlets. Water absorbed by a hydrogel (superabsorbent polymer) has the potential to reduce drought stress after planting and it can extend the survival time of plantlets by approximately 50%, as compared with the plantlets without hydrogel in soil [18]. A total of 200 plantlets were placed in controlled conditions. To improve the process of acclimatization, two variants of substrate were analysed. As the results show, the mixture of sand, soil, vermiculite and hydrogel (1:1:1:1) increased the acclimatization efficiency, a 43.3% increase as compared with the mixture of sand, soil and vermiculite (2:1:1) (fig.5). A positive influence

Table 2.

Effect of IBA and IAA on *in vitro* root induction in *Lippia dulcis*

Plant growth regulator [mg dm^{-3}]	Root induction [%]	Average number of roots/ shoot [mean $\pm$ SD]	Average root length [cm] [mean $\pm$ SD]
0	100.0 $\pm$ 0.0 a	8.40 $\pm$ 1.73c	2.90 $\pm$ 0.41b
0.5 IBA	100.0 $\pm$ 0.0 a	12.65 $\pm$ 1.87a	4.45 $\pm$ 0.96a
0.5 IAA	97.0 $\pm$ 0.4 b	10.20 $\pm$ 1.51b	2.85 $\pm$ 0.73b

Results are mean $\pm$ SD (standard deviation); means followed by the same letter do not differ significantly at $\alpha=0.05$

of hydrogel on plant development of plantlets during acclimatization was also observed by El-Asmar *et al.* [19]. The hydrogel improves fresh and dry above-ground biomass in corn by 25% and it prolongs the survival time of pine plantlets by 90%. According to Wang and Boogher [20], hydrogels absorb various amounts of water during expansion and have been found to increase water retention in potting media, thus reducing the irrigation frequency and delaying the onset of wilting. Adding hydrogel to soil demonstrated a positive impact on the development of argan seedling parameters [21], it significantly enhanced growth, root collar diameter and the slenderness index of plants. The biodegradable superabsorbent cellulose-based hydrogel used by Montesano *et al.* [22] for sweet basil plants grown on perlite with hydrogel added showed an increase in fresh biomass.

Figure 5.

Effectiveness of acclimatization of plants depending on the type of substrate used

CONCLUSIONS

Our study demonstrates that the MS medium supplemented with BAP and NAA is useful for mass propagation of *L. dulcis*. GA_3 application to the medium in *in vitro* culture stimulates plant growth and triggers an increase in the number of leaves and the leaf area. The process of rooting was stimulated by the presence of IBA in the medium. A positive influence of hydrogel on the development of plantlets during acclimatization was observed. The mixture of sand, soil, vermiculite and hydrogel increased the acclimatization efficiency. The method can be used for the production of plants on a commercial scale. Plants from *in vitro* cultures can be a source of plantlets for planting organic crops and for home gardens.

Conflict of interest: Authors declare no conflict of interest.

REFERENCES

1. Compadre CM, Hussain RA, Compadre RLL, Pezzuto JM, Kinghorn AD. The intensely sweet sesquiterpene hernandulcin: isolation, synthesis, characterization, and preliminary safety evaluation. *J Agric Food Chem* 1987; 35:273-279.
2. Souto-Bachiller FA, de Jesus-Echevarria M, Cárdenas-González OE, Acuna-Rodríguez MF, Meléndez PA, Romero-Ramsey L. Terpenoid composition of *Lippia dulcis*. *Phytochemistry* 1997; 44(6):1077-1086.
3. de Oliveira PF, Machado RAF, Bolzan A, Barth D. Supercritical fluid extraction of hernandulcin from *Lippia dulcis* Trev. *J Supercrit Fluids* 2012; 63:161-168. doi: <http://dx.doi.org/10.1016/j.supflu.2011.12.003>
4. Wilczańska-Barska A, Chmura B, Krauze-Baranowska M. Akteozyd – fenylopropanoid o cennych właściwościach farmakologicznych [Acteoside – phenylpropanoid with valuable pharmacological activities]. *Post Fitoter* 2010; 3:157-161 [in Polish].
5. Kaneda N, Lee IS, Gupta MP, Soejarto DD, Kinghorn AD. (+)-4-hydroxyhernandulcin, a new sweet sesquiterpene from the leaves and flowers of *Lippia dulcis*. *J Nat Prod* 1992; 55:1136-1141.
6. Abe F, et al. Trypanocidal constituents in plants 1. Evaluation of some Mexican plants for their trypanocidal activity and active constituents in guaco, root of *Aristolochia taliscana*. *Biol Pharm Bull* 2002; 25:1188-1191.
7. Urrea A, Castrillon P, Monsalve Z. *In vitro* propagation and tisular differentiation in *Lippia dulcis*. *Actual Biol* 2009; 31(9):21-29.
8. Sauerwein M, Flores H.E, Yamazaki T, Shimomura K. *Lippia dulcis* shoot cultures as a source of the sweet sesquiterpene hernandulcin. *Plant Cell Rep* 1991; 9:663-666.
9. Sauerwein M, Yamazaki T, Shimomura K. Hernandulcin in hairy root cultures of *Lippia dulcis*. *Plant Cell Rep* 1991a; 9:579-581.
10. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 1962; 15:473-497.

11. Boustani A, Omid M, Torabi S, Zarekarizi AR. Callus induction and plant regeneration in Lemon verbena (*Lippia citrodora* L.), an important medicinal plant. *Trakia J Sci* 2016; 1:30-38. doi: <http://dx.doi.org/10.15547/tjs.2016.01.004>
12. Juliani HR (Jr.), Koroch AR, Juliani HR, Trippi VS. Micropropagation of *Lippia junelliana* (Mold.) Tronc. *Plant Cell Tissue Organ Cult* 1999; 59:175-179. doi: <http://dx.doi.org/10.1023/A:1006396531647>
13. Castellanos-Hernandez OA, et al. In vitro clonal propagation and regeneration of the commercially important plant Mexican oregano (*Lippia graveolens*). *In Vitro Cell Dev Biol – Plant* 2013; 49(5):620-625. doi: <http://dx.doi.org/10.1007/s11627-013-95384>
14. Servin C, Bruzzese D, Sapio OD, Gattuso M, Gattuso S. Evaluation of in vitro behavior of *Aloysia citriodora* Palau: Histological and chemical study. *Mol Med Chem* 2006; 11:19-20.
15. Peixoto PH, et al. In vitro propagation of endangered *Lippia filifolia* Mart. and Schauer ex Schauer. *In Vitro Cell Dev Biol – Plant* 2006; 42(6):558-561. doi: <http://dx.doi.org/10.1079/IVP2006814>
16. Giridhar P, Sowmya KS, Ramakrishna A, Ravishankar GA. Rapid clonal propagation and stevioside profiles of *Stevia rebaudiana* Bertoni. *Int J Plant Dev Biol International* 2010; 4(1):47-52.
17. Rani DN, Nair GM. Effects of plant growth regulators on high frequency shoot multiplication and callus regeneration of an important indian medicinal plant, Nirgundi (*Vitex negundo* L.). *In Vitro Cell Dev Biol – Plant* 2006; 42:69-73. doi: <http://dx.doi.org/10.1079/IVP2005727>
18. Bartnik C. Wpływ hydrożelu na przeżywalność siewek i sadzonek sosny pospolitej w warunkach suszy [The influence of hydrogel on the survival rate of plantlets and the plants of *Pinus sylvestris* L. during drought]. *Studia i materiały centrum edukacji przyrodniczo-leśnej* 2008; 10/2(18):329-338 [in Polish].
19. El-Asmar J, Jaafar H, Bashour I, Farran MT, Saoud IP. Hydrogel banding improves plant growth, survival, and water use efficiency in two calcareous soils. *Clean – Soil, Air, Water* 2017; 45(7):1-9. doi: <http://dx.doi.org/10.1002/clen.20170>
20. Wang YT, Boogher CA. Effect of a medium-incorporated hydrogel on plant growth and water use of two foliage species. *J Environ Hortic* 1987; 5(3): 125-127.
21. Defaa C, Achour A, Mousadi AE, Msanda F. Effets de l'hydrogel sur la survie et la croissance des plantules d'arganier sur une parcelle de régénération en climat aride. *JABS* 2015; 92: 8586-8594. [in French] doi: <http://dx.doi.org/10.4314/jab.v92i1.3>
22. Montesano FF, Parente A, Santamaria P, Sannino A, Serio F. Biodegradable superabsorbent hydrogel increases water retention properties of growing media and plant growth. *Agric Agric Sci Proc* 2015; 4:451-458. doi: <http://dx.doi.org/10.1016/j.aaspro.2015.03.052>