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Establishment and clonal propagation of *Lippia dulcis* Trevir. through *in vitro* single node cultures

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ABSTRACT

Lippia dulcis Trevir. (Verbenaceae) is a species with medicinal potential that is used as a tranquilizer and to control diabetes in the western region of Pará, Brazil. The objective of this study was to determine the establishment and clonal propagation of *Lippia dulcis* through single nodal segments grown in different concentrations of salts in a MS (Murashige and Skoog) medium (MS, MS/2, MS/4) and determine the best period for subculturing the species. Nodal segments cultivated in the MS medium showed a higher survival percentage and better development than those grown in more dilute media (MS/2, MS/4). The concentration of the salts affected the dry weight gain of some plant parts. Plantlets cultivated in the MS medium obtained better leaf dry weight and total leaf area than plantlets cultivated in half-strength MS. The half-strength MS medium proved to be most effective for root length growth and root dry weight gain*in vitro*. The growth curve showed that day 30 was the time for subculturing the species. Starting with only one nodal segment, 3125 plantlets were obtained after sixth months of cultivation at a multiplication rate of five. The plantlets of *Lippia dulcis* were removed from the flasks and acclimatized with success in a greenhouse.

Index terms: Growth analysis; micropropagation; shoot proliferation; nodal segment.

INTRODUCTION

The Verbenaceae family comprises species with potential for industrial and therapeutic uses due to their aromatic compositions (Castilho et al., 2019). *Lippia dulcis* (Trevir.), popularly known as sweetgrass, is an aromatic species whose leaves are used in infusions to control diabetes and as a tranquilizer (Castilho et al., 2019). Additionally, it is employed as a decoction for the treatment of coughs, colds, bronchitis, asthma, and colic (Moreno-Murillo et al., 2010). Osuji et al. (2015) and Kinghorn et al. (2011) reported that *Lippia dulcis* is approximately a thousand times sweeter than sucrose because it contains hernundulcin sesquiterpene. This plant has shown potential for use as a natural low-calorie sweetener in the dietary management of diabetes/obesity (Kinghorn et al., 2011).

Micropropagation is a technique used to improve the quality of agricultural products, including medicinal plants. From this perspective, nutrients and their compositions are determining factors for the conversion of explants into plantlets and then into scions (Carvalho et al., 2018; Reed et al., 2013), with the definition of the culture medium being the basis for the establishment of the *in vitro* cultivation protocols (Araruna et al., 2017).

The culture medium is the source of the substances essential for the growth and development of the explant (Pinhal et al., 2017), varying according to the plant species and the cultivation process (Costa et al., 2007; Greenway et al., 2012; Martins et al., 2015). Inorganic salts provide macronutrients (calcium, magnesium, sulfur, potassium, phosphorus and nitrogen) and micronutrients (zinc, iron, copper, manganese, chlorine, molybdenum and boron), and these elements are considered essential for plant

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development (Carvalho et al., 2018; Reed et al., 2013). Although there is no standard formulation, several species, from arboreal to medicinal, have shown good results when grown in a MS medium (Murashige; Skoog, 1962), and modifications and dilutions to the medium may be needed according to the nutritional needs of each species (Carvalho et al., 2018; Monfort et al., 2015).

The use of medicinal plants for pharmaceutical purposes can be impaired by many factors, such as the heterogeneity of individuals due to genetic and biochemical variability in addition to difficulties with propagation; therefore, it is important to carry out studies that make cultivation on scale possible while promoting species conservation (Ghorbanpour et al., 2017; Morais et al., 2012). *Lippia dulcis* is a species that can be propagated sexually (seeds) and asexually (cuttings) (Adams; Oliveira, 2016; Dwivedi, 2022; Ribeiro et al., 2022). Due to micropropagation is a method of multiplying genetically identical individuals, more quickly, all year round, protected from pests and pathogens, it can be a tool used to improve cultivation and increase the production of secondary metabolites in medicinal plants (Chandana et al., 2018). Much work has been conducted on the active principles of medicinal plants; however, there is still a lack of studies on the cultivation, propagation and *in vitro* conservation of these medicinal plant species. In the literature, very few studies were found on the micropropagation of *L. dulcis*. Some studies have already demonstrated that different *in vitro* conditions can affect the growth of *Lippia* species. Rocha et al. (2022) reported that light intensity and light quality influenced plantlets propagation, providing better conditions for the acclimatization phase. Lazzarini et al. (2019a) observed the influence of the explant type and natural ventilation system and concluded that explants with a pair of leaves and a ventilation system with four membranes promoted the greatest growth, leaf area, and carvacrol and thymol contents in *L. gracilis*. Hsie et al (2019) reported that sucrose concentration in the culture medium with porous membrane led to higher in vitro growth rates in *Lippia rotundifolia*. Urrea et al. (2009) reported that more effective for induction and multiplication of *Lippia dulcis* from shoot tips or nodal segments was MS medium free of plant growth regulators.

In view of this context, the objective was to determine the establishment and clonal propagation of *Lippia dulcis* through single nodal segments grown in different concentrations of salts from the MS medium and determine the best period for subculturing the species.

MATERIAL AND METHODS

Experiment, location, and plant material identity

The experiments were conducted at the Laboratory of Tissues Culture and Medicinal Plants at the Department of Agriculture of the Federal University of Lavras, Brazil. The existence of a voucher specimen deposited at UFLA's herbarium in the Biology Department was confirmed – registration n° ESAL 30314 and in the PAMG (Herbarium of the Agricultural Research Corporation of Minas Gerais - EPAMIG, Belo Horizonte, Minas Gerais, Brazil) – registration n° PAMG 57966.

Establishment of the nodal segments

The *Lippia dulcis* Trevir. is a species with opposite phyllotaxy (two axillary buds per nodal segment). Therefore, the first and second nodal segments (± 1.5 cm) were removed from one-year-old mother plants with good phytosanitary state grown in a greenhouse (spring-summer). These explants were washed in a neutral soap (a liquid detergent) for 5 min followed by washing with running tap water for 15 min. Further, they were surface-sterilized for 15 minutes with a 50% bleach (1.25% active chlorine - NaOCl) solution containing 2-3 drops of Tween 20® and washed thoroughly in sterile distilled water. The base of the cuttings was removed, and the explants were incubated in 25x150 mm glass test tubes with 15 mL mediaat three salt concentrations: full strength MS basal medium (Murashige and Skoog, 1962), half-strength MS medium (MS/2) and quarter-strength MS medium (MS/4). The media were supplemented with 30 g L^{-1} sucrose and 0.6 g L^{-1} agar (Himedia®, type I) at pH 5.7 ± 0.1 and autoclaved (121 °C and 1.05 kg cm^2 per 20 minutes). The growth chamber temperature was set to 25±1 °C, while the photoperiod, provided by an Osram 36 W cool-white fluorescent light, was fixed to 16/8 hours with a light intensity of 42 µmol m^{-2} s^{-1}. After a period of 15 days of the nodal segments were established in the MS, MS/2 and MS/4 media, the shoot length and the percentage of survival were evaluated without

the plantlets being removed from the test tube. The experimental design was completely randomized, composed of 3 concentrations of salts: MS, MS/2 and MS/4 and 7 repetitions (with 5 tubes), with 1 explant per tube, totaling 105 plantlets.

Growth analysis

Since the plantlets had better growth when they were inoculated in the MS and MS/2 media, they were subcultured on the same respective media. After 45 days, their growth parameters were evaluated: shoot length (SL) and root length (RL); leaf dry weight (LDW), stem (SDW), root (RDW) and total (TDW); and total leaf area (TLA). Total leaf area was measured using a WinFOLIA™ software and an EPSON PERFECTION V700 PHOTO scanner. Various parameters were used to evaluate plantlet growth *in vitro* and to infer plant behavior. Five representative plantlets from each treatment were chosen, and from these, the leaf areas of all the leaves were evaluated. The leaf area ratio (LAR = TLA/TDW), specific leaf area (SLA = TLA/LDW), specific leaf weight (SLW = LDW/TLA), which is the inverse of the SLA and gives an estimate of leaf thickness, and leaf weight ratio (LWR = LDW/LDW+SDW) were also evaluated. These parameters were calculated according to Benincasa (2003). The experimental design was completely randomized, composed of 2 concentrations of salts: MS and MS/2 and 10 repetitions (with 5 tubes) with 1 explant per tube, totaling 100 plantlets.

Growth curve

The apical and nodal segments of the plantlets grown in the MS medium were used to build the growth curve. A total of 140 plantlets from each segment (totaling 280) were inoculated in test tubes with 15 mL of MS culture medium. The *in vitro* cultivation conditions were the same as for the pre-established plantlets. Apical and nodal segment growth was evaluated every five days, starting on day zero (day of inoculation) until completion after 30 days, totaling seven evaluations. At each evaluation, the fresh and dry weight values used to build the growth curve were measured.

Multiplication rate

The multiplication rate *in vitro* was evaluated from the multiplication of apical and nodal segments of pre-established plantlets. Five subcultures were carried out with intervals of 30 days between each subculture. After 30 days, the resulting number of nodal segments produced per plantlet was determined, and a new segment was used for subculturing in the same respective media. This cycle was repeated a total of 5 times. Segment data for the 5 cycles were pooled to determine the average segment rates to obtain an estimate of culture responses over time.

Acclimatization

For acclimatization, 10 plantlets (± 8-10 cm) with roots from each treatment (MS and MS/2) were used, which were transferred to polystyrene trays and commercial substrate and kept in a greenhouse for 40 days with shading nets (60%) with automated irrigation, in season spring. Finally, the percentage of survival (%) and the length of stem (LS) and length of root (LR) were calculated.

Statistical analysis

The growth parameters were subjected to analysis of variance and, when significant, were compared using the Scott–Knott test at 5% probability level. Statistical analyses of the observed data were performed in R software.

RESULTS AND DISCUSSION

Nodal segment establishment

The concentration of salts in the culture medium can affect the growth of the explant. At 15 days, nodal segments grown in the full MS medium showed greater growth (2.09 cm) and survival (68.57%) than segments maintained in the MS/2 and MS/4 media (Table 1 and Figure 1). The different concentrations of salts did not inhibit the induction and growth of roots, with the percentage of rooting of the plantlets equal to 100%. However, in Figure 1, it is possible to observe that the concentrations of media affect the number of roots, the shoots and the roots length (data not shown). The MS/4 medium did not provide sufficient nutrients for the growth of cultivated explants. According to Taiz et al. (2017), the suppression of plant growth and reproduction caused by nutritional stress is related to the essential function of mineral nutrients, which are components of enzymes and the structural constituents of cells. In addition, several researches

demonstrate that the concentration of nutrients in the culture medium can affect growth, number of shoots, root induction, biomass production, cell wall composition and the content of volatile compounds (Carvalho et al., 2018; Lawson; Bridges; Adelberg, 2023; Magangana; Stander; Makunga, 2018; Munthali et al., 2022; Sim et al., 2020; Zarei et al., 2022).

Table 1: Shoot length (SL - cm) and survival (%) of *Lippia dulcis* plantlets, grown in different concentrations of MS basic salts, at 15 days of *in vitro* culture.

Treatments	Shoot lenght (cm)	Survival (%)
MS	2.09 ± 0.88 a	68.57 ± 15.7 a
MS/2	1.22 ± 0.23 b	54.28 ± 9.8 b
MS/4	1.03 ± 0.39 b	54.28 ± 9.8 b

The means followed by the same letters in the column, do not differ by the Scott-Knott Test ($p < 0.05$).

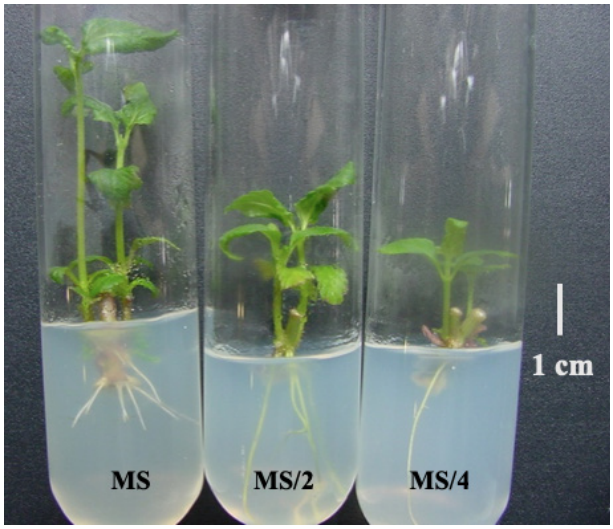

Figure 1: Nodal segment establishment of *Lippia dulcis* plantlets grown in different concentrations of MS medium salts, at 15 days of *in vitro* cultivation.

Growth analysis

After the explant was established, the single nodal segments were cultured in the full-strength MS medium and MS/2 to evaluate the growth of the plantlet after 45 days. The concentration of salts affected the dry weight gain of some of the parameters. Leaf dry weight (LDW) and total leaf area (TLA) varied depending on the concentration of salts. Plantlets cultivated in the MS medium obtained a gain of 18.42% in LDW and 60.81% in TLA compared to plantlets cultivated in MS/2 (Table 2). The results showed that there was no statistically significant difference between treatments for the parameters shoot length (SL), stem (SDW) and total dry weight (TDW) (Table 2).

Nodal segments of *Lippia dulcis* cultivated in culture media with lower concentrations of salts had greater development of the root system. Plantlets grown in the MS/2 medium had a root length of 5.26 cm, and those grown in the MS medium had a root length of 3.92 cm, which corresponds to an increase of 34% when cultivated with a lower concentration of salts. Similarly, the root dry weight gain was 48% higher in the MS/2 medium than in the MS culture medium (Table 2 and Figure 2). Half-strength MS medium proved to be the most effective for root dry weight gain *in vitro*.

The difference in the concentration of salts in the culture media is an important factor in the induction of roots (Monfort et al., 2018; Monfort et al., 2015). Good nutrition for *in vitro* plantlets depends on the equilibrium of salt concentrations in the culture medium. In general, high concentrations lead to inhibition, and lower salt concentrations stimulate rooting in the species (Golle et al., 2012; Monfort et al., 2018; Shekhawat; Kannan; Manokari, 2015). According to Lucho et al. (2019) different concentrations of salts in a culture medium can affect the growth of shoots, as they influence the availability of water, absorption and assimilation of nutrients. In this present study it was demonstrated that for *Lippia dulcis* the complete MS medium provided better growth compared to MS/2 (Figure 2 and Table 2).

The ratio of root to aerial parts (RDW/LDW+SDW) was higher in the medium with a lower concentration of salts, showing a greater allocation of photoassimilates for the root than for the aerial part (Table 2). Plantlets grown in the MS medium distributed 13% of the dry weight for roots, 51% for leaves and 36% for stems, and in MS/2 medium, it was 21% for roots, 43% for leaves and 36% for stems.

The medium MS formulations contained salts of approximately 4.63 g L^{-1} and a nitrogen supplement of 60.01 mM. Salt concentration can affect the growth and rooting of the species, and it (Martins et al., 2015; Monfort et al., 2018; Shekhawat; Kannan; Manokari, 2015; Singh et

al., 2015). In addition, salt concentrations in MS affected the plantlet growth of *Ocimum basilicum,* and explants grown in the MS and MS/2 media showed the best growth in comparison to MS/4 and 2MS (Monfort et al., 2018). Nutrients play an important role in the regulation of both plant morphogenesis and growth (Carvalho et al., 2018; Wada et al., 2013).

Table 2: Growth parameters of *Lippia dulcis* plantlets grown in MS and MS/2, after 45 days of *in vitro* culture.

Growth parameter	Medium	
	MS	MS/2
Shoot length - SL (cm)	7.20±1.01 a	6.46±0.82 a
Root length - RL (cm)	3.92±0.47 b	5.26±1.36 a
Leaf dry weight - LDW (mg)	61.14±7.21 a	51.63±7.14 b
Shoot dry weight - SDW (mg)	43.24±5.72 a	43.15±4.08 a
Root dry weight - RDW (mg)	16.07±3.45 b	23.92±3.35 a
Total dry weight - TDW (mg)	120.45±15.32 a	118.70±12.80 a
RDW/LDW+SDW	0.15±0.02 b	0.25±0.03 a
Total leaf area - TLA (cm^2)	1.71±0.33 a	1.064±0.41 b
Leaf area ratio - LAR ($cm^2\ mg^{-1}$)	0.014±0.004 a	0.009±0.004 b
Specific leaf area - SLA ($cm^2\ mg^{-1}$)	0.029±0.008 a	0.021±0.008 a
Leaf weight ratio - LWR ($mgmg^{-1}$)	0.586±0.013 a	0.543±0.028 b
Specific leaf weight – SLW ($mg\ cm^{-2}$)	35.73±8.24 b	48.52±11.79 a

The means followed by the same letters in the line, do not differ by the Scott-Knott Test ($p < 0.05$).

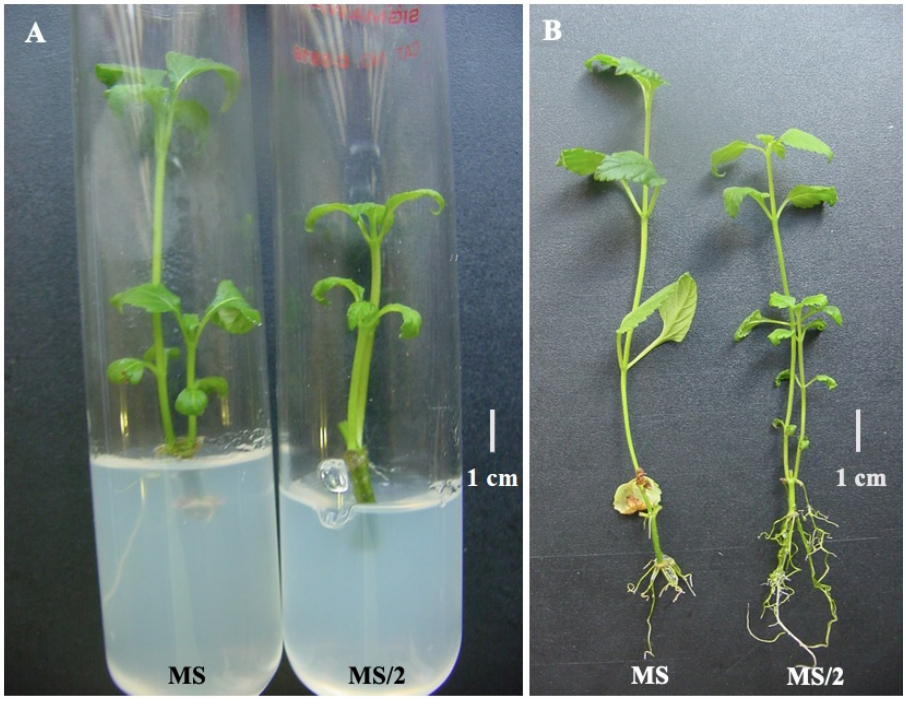

Figure 2: Growth of *Lippia dulcis* plantlets cultivated in MS and MS/2 medium. A) After 15 days of *in vitro* culture; B) After 45 days of *in vitro* culture.

According Araújo et al. (2021), the LAR expresses the leaf area corresponding to 1 g of dry weight. The results obtained for LAR (0.014 cm^2 mg^{-1}) suggest that *Lippia dulcis* plantlets grown in MS had a larger leaf area that was useful for carrying out photosynthesis, which contributed to gains in LDW shown in Table 2. Rocha et al. (2022) found that the highest LAR contributed to higher dry weight averages in *Lippia dulcis* under in vitro conditions. According to Benincasa (2003), the leaves are the center for the production of photosynthates (dry weight), and the rest of the plant depends on the export of this material through the leaves. LWR represents the fraction of dry weight that is not exported from the leaves to other plant parts. The results found for the leaf weight ratio (LWR - 0.586 mg mg^{-1}) indicate that *Lippia dulcis* plantlets cultivated in the MS medium exported fewer photoassimilates to other organs. This is evident from the observation that the plantlet cultivated in the MS medium retained 51% of its dry weight in the leaf part, while in the MS/2 medium, it retained 43%. Rocha et al. (2022) observed that under *in vitro* conditions (natural ventilation system) *Lippia dulcis* also showed a direct correlation between LAR and biomass gain, as the highest average LAR provided higher LDW.

The specific leaf area (SLA) is an important factor from a physiological point of view as it describes the allocation of leaf biomass per unit area (Bastos et al., 2019). Plantlets cultivated in the MS and MS/2 media obtained the same SLA value statistically (0.029 and 0.021 cm^2 mg^{-1}, respectively), showing equal leaf areas to accumulate dry weight. According to Barreiro et al*.* (2006), decreases in SLA indicate an increase in leaf thickness resulting from the increase in the number and size of plant cells. SLW is also indicative of leaf thickness (Benincasa, 2003). The *Lippia dulcis* plantlets cultivated in the MS/2 medium had a higher SLW (48.52 mg cm^{-2}), which indicates that they had greater leaf thicknesses, but the total leaf areas were smaller (Table 2). The lowest mean SLW (35.73 mg cm^{-2}) of plantlets cultivated in the full MS may be related to the highest TLA (1.71 cm^2), because with an increase in leaf area to maximize light capture, plants decrease in leaf specific weight. Rocha et al. (2022) observed the same correlation between SLW and TLA in *L. dulcis* cultivated in vitro, as the lower the SLW, the higher the TLA.

Growth curve

A growth curve was constructed from the fresh and dry weights of the plantlets over 30 days (Figure 3). On the curve, it can distinguish an initial phase of slow growth (lag) up to 10 days, then an exponential phase up to 20 days and then a phase of linear growth up to 30 days, where the largest increase in the rate of dry weight gain occurred. After 10 days of *in vitro* cultivation, the development of the root system and the expansion of the leaves increased, and the plantlet removed water and nutrients from the culture medium in which it was developing and initiated anabolic processes dependent on photosynthesis. During this period, the highest plantlet growth occurred (Figure 3).

After the 30-day period, it can infer that the plant will undergo slow growth, with the eventual cessation of the process; thus, the plantlets had not yet reached their maximum growth. However, it has been observed in practice that the period employed in this study is better for carrying out the subculturing of plantlets for new growth. After this period, the plantlet enters the senescence phase, resulting in a decrease in the accumulation of dry weight, its translocation to other organs, and the consequent degeneration of the photosynthetic system.

Growth curve revealed that specie displayed significant plasticity in growth rates across of the 30 days. The allocation of biomass to different plant organs depends on species, ontogeny, medium concentration and on the environment where of the explant growth (Mendonça et al., 2012; Chen, 2015). The effects of days were greater suggesting that just in the case of days, biomass allocation is an important factor in plant response to growth. Nodal and apical segments growth curve studies are significant for identifying the appropriate time for the passage (subculture). Thus, it facilitates the monitoring of culture, particularly biomass accumulation and explant growth maintenance.

Multiplication rate

In vitro apical and nodal segments of *Lippia dulcis* were repeatedly subcultured for 5 subcultures in the Murashige and Skoog medium without plant growth regulators. A multiplication rate of 4-5 nodal segments $plantlet^{-1}$ was observed after the fifth subculture. *Lippia dulcis* is a species that produces

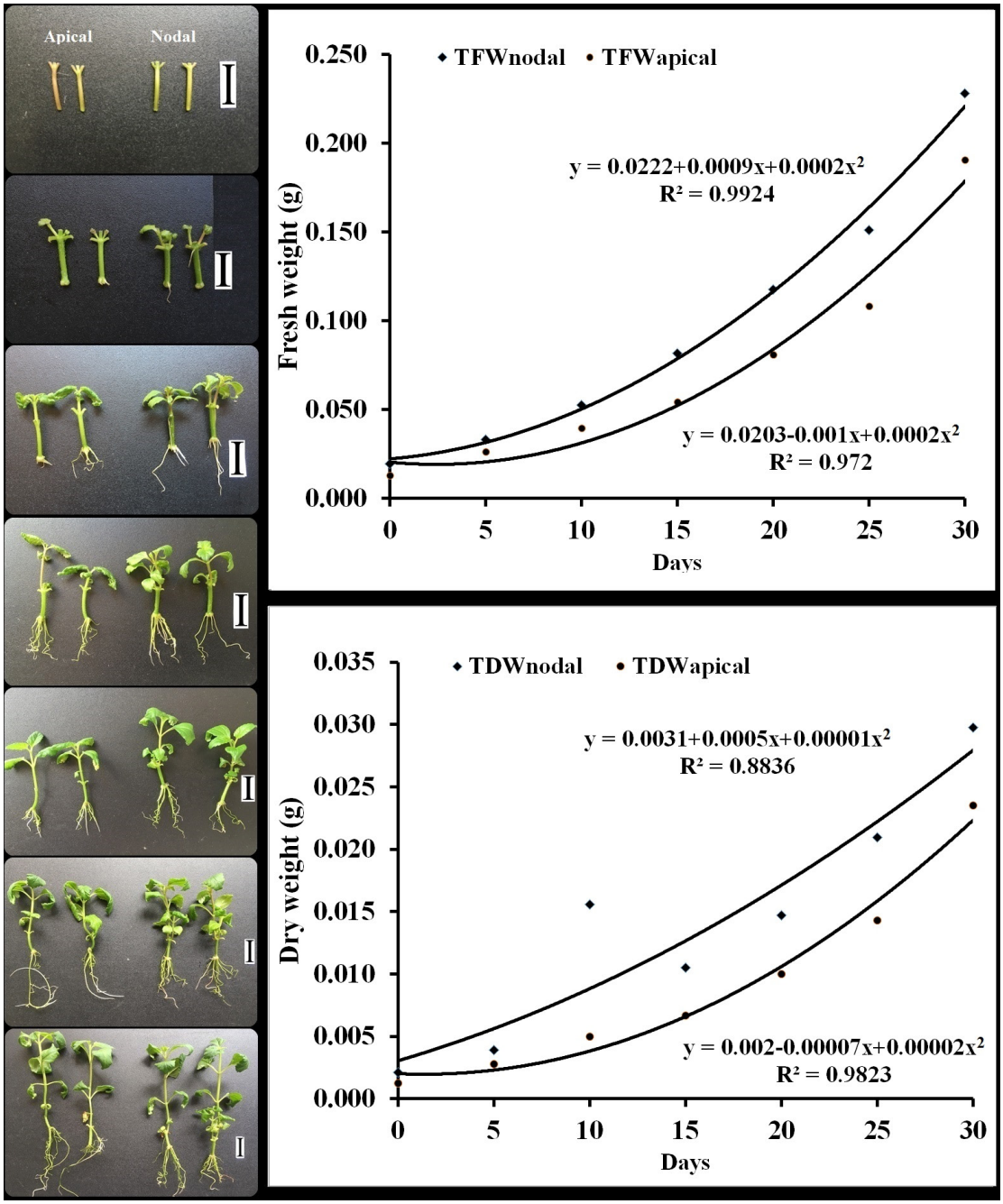

Figure 3: Growth curve of *Lippia dulcis* plantlets grown *in vitro* for 30 days. TFW – Total fresh weight; TDW - total dry weight. Scale = 1cm.

elongated shoots in *in vitro* cultures. Plantlets can be cut into single nodes that are subcultured, and the leaves are trimmed. Starting with only one nodal segment, 3125 plantlets were obtained after sixth months of cultivation, with a multiplication rate of five (Figure 4). If the establishment starts with 50 explants,156,250 plantlets can be obtained within 180 days. The subculture effect on the multiplication rate of *in vitro* cultures varies from one species to another. Node culturing has become

a safer technique for clonal stability. The segment formation capacity remained stable from the first to fifth subcultures. The production of plants from single- or multiple-node cultures has proven to be the most applicable and reliable method of true-to-type *in vitro* propagation without the use of plant growth regulators (George et al., 2008). Apical and nodal segment micropropagation showed good visual quality and vigor. It was not necessary to add plant growth regulators to the medium; normally, high doses of plant growth regulators can cause genetic variation and hyperhydration in the explant. The single node culture used in this study could be an important step toward helping to establish a program for the reintroduction of *Lippia dulcis* into its natural habitats. No visible morphological variations or aberrations of the plantlets were found in successive subcultures.

The multiplication phase aims to produce a larger number of plantlets in a short amount of time. It is important to take into account other factors; that is, the ideal is a combination of a satisfactory multiplication rate with minimum variation between the explants. The culture medium in the micropropagation process can be supplemented or not supplemented with plant growth regulators. Cytokinin are normally incorporated into the culture medium for the proliferation of axillary

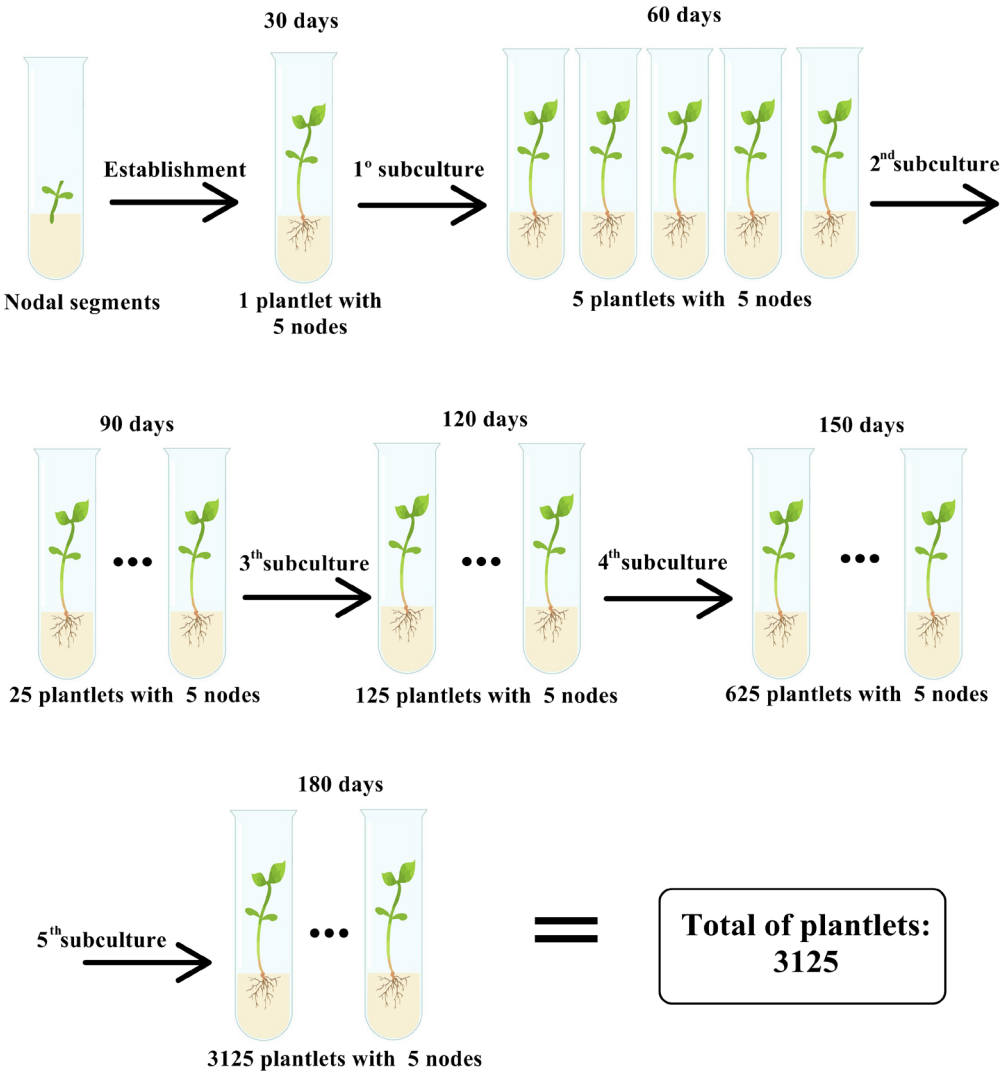

Figure 4: Number of plantlets obtained after fifth subculture through the node culture of *Lippia dulcis*.

or adventitious shoots (Hsie et al., 2019; Lazzarini et al., 2019b). Importantly, to obtain a satisfactory multiplication rate with minimum variation between explants, the culture medium was one of the variables that could be used for this study. However, an excessive salt concentration in the medium can be toxic and compromise the development of plantlets (Carvalho et al., 2018). Therefore, a node culture was used without plant growth regulator supplementation for the multiplication of *L. dulcis*. The optimum levels of cytokinins required for shoot regeneration vary according to genotype. In addition, benzylaminopurine has several side effects, such as difficulties in rooting, toxicity, hyperhydricity, stunted shoots, and callus formation in apple micropropagation (Magyar-Tábori et al., 2010).

The multiplication rate of *Melissa officinalis* was approximately 2.1 times higher for plantlets subcultured in a MS medium with node culture than for those subcultured in a MS medium in the presence of BA (Reis et al., 2008). Shoot formation capacity can also decreased over repeated subculturing in genotypes of cherry, plum and pear (Vujović; Ružić; Cerović, 2012).

Acclimatization

The plantlets of *Lippia dulcis* were removed from flasks and acclimatized with success in the greenhouse (Figure 5). In all treatments, 100% survival of the plantlets was observed for both the MS and MS/2 media. The stem length (8.36 cm MS and 7.55 cm MS/2) and the root length (17.25 cm MS and 14.35 cm MS/2) were also not influenced (Figure 5). However, the plantlets from MS presented more flowers than those from MS/2 (Figure 5, white circles). The high levels of nitrogen present in the

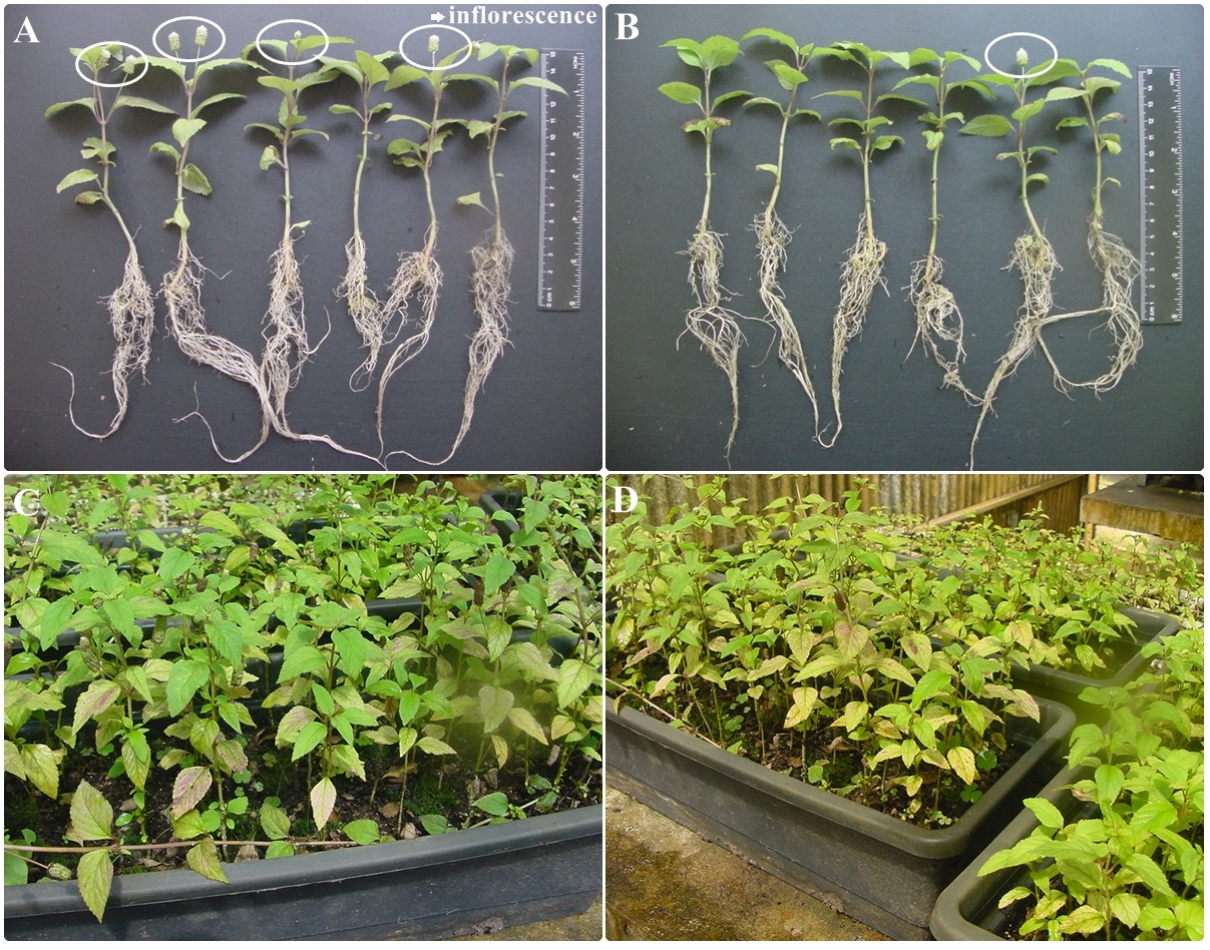

Figure 5: Acclimatization in greenhouse of *Lippia dulcis* plants: A) MS - complete culture medium after 40 days, B) MS/2 - half the concentration of MS salts after 40 days, C and D) after 90 days.

complete MS medium may have provided a faster growth rate compared to plantlets grown in MS/2 (Petersen et al., 2021; Phillips; Garda, 2019). In this way, the plantlets had a smaller LAG phase, thus reaching the early reproductive stage.

CONCLUSIONS

The complete MS medium is the recommended concentration of salts for the establishment and maintenance of *Lippia dulcis* explants *in vitro*. Node cultures without the supplementation of plant growth regulators proved to be efficient and safe for the multiplication of the species. Plantlets grew and rooted normally without the use of plant growth regulators. The growth curve showed that day 30 was the time for subculture of the species. A multiplication rate between 4 and 5 through node cultures was more suitable for obtaining *in vitro* plantlets of *L. dulcis*.

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REFERENCES

ADAMS, R. P.; OLIVEIRA, P. F. Comparison of intensely sweet volatile leaf oils of *Lippia dulcis* (Verbenaceae) with low and high camphor from Brazil and Mexico. **Phytologia**, 98:207-214, 2016.

ARARUNA, E. D. C. et al. Salt concentrations in culture media for the development of *Dipteryx alata in vitro*. **Pesquisa Agropecuária Brasileira**, 52:1295-1300, 2017.

ARAÚJO, D. X. et al. Photon flux density and wavelength influence on growth, photosynthetic pigments and volatile organic compound accumulation in *Aeollanthus suaveolens* (Catinga-de-mulata) under *in vitro* conditions. **Industrial Crops and Products**, 168:113597, 2021.

BARREIRO, A. P. et al. Growth analysis of basil plants submitted to plant growth regulators. **Bragantia**, 65:563-567, 2006.

BASTOS, K. V. L. D. S. et al. Determination of the specific leaf area of different species of three physiognomies of the South Pantanal. **Ambiência**, 15:695-712, 2019.

BENINCASA, M. M. P. **Plant growth analysis: basics notions**. Jaboticabal: Funep 2003. 42 p.

CARVALHO, A. A. D. et al. Mesos components ($CaCl_2$, $MgSO_4$, KH_2PO_4) induced changes in growth and ascaridole content of *Dysphania ambrosioide*s L. *in vitro*. **Industrial Crops and Products**, 122:28-36, 2018.

CASTILHO, C. V. V. et al. *In vitro* propagation of a carvacrol-producing type of *Lippia origanoides* Kunth: A promising oregano-like herb. **Industrial Crops and Products**, 130:491-498, 2019.

CHANDANA, B. C. et al. Role of plant tissue culture in micropropagation, secondary metabolites production and conservation of some endangered medicinal crops. **Journal of Pharmacognosy and Phytochemistry**, 7: 246-251, 2018.

CHEN, C. Application of growth models to evaluate the microenvironmental conditions using tissue culture plantlets of Phalaenopsis Sogo Yukidian 'V3'. **Scientia Horticulturae**, 191: 25-30, 2015.

COSTA, A. S. et al. *In vitro* establishment of *Lippia sidoides* Cham. **Horticultura Brasileira**, 25:68-72, 2007.

DWIVEDI, R. S. Hernandulcin (Sesquiterpene). In*:* DWIVEDI, R. S. (Ed.) **Alternative sweet and supersweet principles**: Natural sweeteners and plants, Singapore: Springer Nature Singapore, p. 389-403, 2022.

GEORGE, E. F. et al. **Plant propagation by tissue culture** 3rd Edition. The Netherland, The Back Ground Springer, 2008. 29-64p.

GHORBANPOUR, M. et al. **Importance of medicinal and aromatic plants in human life**. Switzerland Springer, 2017. 23p.

GOLLE, D. P. et al. *In vitro* establishment and development of *Eugenia involucrata* DC.: Influence of explant source and nutritional medium. **Ciência Florestal**, 22:207-214, 2012.

GREENWAY, M. B. et al. A nutrient medium for diverse applications and tissue growth of plant species *in vitro*. **In Vitro Cellular & Developmental Biology - Plant**, 48:403-410, 2012.

HSIE, B. S. et al. Growth regulators induced shoot regeneration and volatile compound production in *Lippia rotundifolia* Cham., a threatened medicinal plant. **Industrial Crops and Products**, 137:401-409, 2019.

KINGHORN, A. D. et al. The relevance of higher plants in lead compound discovery programs. **Journal of Natural Products**, 74:1539-1555, 2011.

LAWSON, J. D.; BRIDGES, W. C.; ADELBERG, J. W. IBA delivery technique and media salts affected *in vitro* rooting and acclimatization of eight *Prunus* genotypes. **Plants**, 12:289, 2023.

LAZZARINI, L. E. S. et al. Explant type and natural ventilation systems influence growth and content of carvacrol and thymol of *Lippia gracilis* Schauer. **Plant Cell, Tissue and Organ Culture** 137:33-43, 2019a.

LAZZARINI, L. E. S. et al. Growth regulators affect the dry weight production, carvacrol and thymol content of *Lippia gracilis* Schauer. **Industrial Crops and Products**, 129:35-44, 2019b.

LUCHO, S. R. et al. Salt stress-induced changes in *in vitro* cultured *Stevia rebaudiana* Bertoni: Effect on metabolite contents, antioxidant capacity and expression of steviol glycosides-related biosynthetic genes. **Journal of Plant Growth Regulation**, 38:1341-1353, 2019.

MAGANGANA, T. P.; STANDER, M. A.; MAKUNGA, N. P. Effect of nitrogen and phosphate on *in vitro* growth and metabolite profiles of *Stevia rebaudiana* Bertoni (Asteraceae). **Plant Cell, Tissue and Organ Culture**, 134:141-151, 2018.

MAGYAR-TÁBORI, K. et al. The role of cytokinins in shoot organogenesis in apple. **Plant Cell, Tissue and Organ Culture**, 101:251-267, 2010.

MARTINS, J. P. R. et al. Effects of salts and sucrose concentrations on *in vitro* propagation of *Billbergia zebrina* (Herbert) Lindley (Bromeliaceae). **Australian Journal of Crop Science**, 9:85-91, 2015.

MENDONÇA, E. G. et al. Growth curve and development of the internal calli structure of *Eucalyptus camaldulensis* Dehn. **Brazilian Archives of Biology and Technology**, 55: 887-896, 2012.

MONFORT, L. E. F. et al. Effects of plant growth regulators, different culture media and strength MS on production of volatile fraction composition in shoot cultures of *Ocimum basilicum*. **Industrial Crops and Products**, 116:231-239, 2018.

MONFORT, L. E. F. et al. Micropropagation and *in vitro* seed germination of atroveran. **Revista Ceres**, 62:215-223, 2015.

MORAIS, T. et al. Applications of tissue culture in medicinal plants. **Revista Brasileira de Plantas Medicinais**, 14:110-121, 2012.

MORENO-MURILLO, B. et al. Essential oil from leaves of *Lippia dulcis* grown in Colombia. **Natural Product Communications**, 5(4):613-614, 2010.

MUNTHALI, C. et al. A model nutrition control system in potato tissue culture and its influence on plant elemental composition. **Plants**, 11:2718, 2022.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. **Physiologia Plantarum**, 15:473-497, 1962.

OSUJI, G. O. et al. Molecular regulation of the metabolic pathways of the medicinal plants: *Phyla dulcis*. **American Journal of Plant Sciences**, 6:1717, 2015.

PETERSEN, F. et al. Influence of the nitrate-N to ammonium-N ratio on relative growth rate and crude protein content in the duckweeds *Lemna minor* and *Wolffiella hyalina*. **Plants**, 10:1741, 2021.

PHILLIPS, G. C.; GARDA, M. Plant tissue culture media and practices: An overview. **In Vitro Cellular & Developmental Biology-Plant**, 55:242-257, 2019.

PINHAL, H. F. et al. Concentration of MS medium and cutting of seeds on *in vitro* establishment of baruzeiro (*Dipteryx alata* Vog.). **Bioscience Journal**, 33, 2017.

REED, B. M. et al. Mineral nutrition influences physiological responses of pear *in vitro*. **In Vitro Cellular & Developmental Biology - Plant**, 49:699-709, 2013.

REIS, É. S. et al. Influence of culture medium on *in vitro* seed germination and multiplication rate of *Melissa officinalis* L. **Revista Ceres**, 55:160-167, 2008.

RIBEIRO, F. N. S. et al. Phenology and vegetative propagation of *Lippia dulcis* Trevir (Verbenaceae). **Research, Society and Development**, 11:e298111638261, 2022.

ROCHA, T. T. et al. Morphoanatomy and changes in antioxidant defense associated with the natural ventilation system of micropropagated *Lippia dulcis* plantlets. **Plant Cell, Tissue and Organ Culture** 151:467-481, 2022.

SHEKHAWAT, M. S.; KANNAN, N.; MANOKARI, M. *In vitro* propagation of traditional medicinal and dye yielding plant *Morinda coreia* Buch.–Ham. **South African Journal of Botany**, 100:43-50, 2015.

SIM, S. et al. Influence of inorganic salts on biomass production, biochemical composition, and bioethanol production of *Populus alba*. **iForest - Biogeosciences and Forestry**, 13:566-574, 2020.

SINGH, A. et al. Effect of $MgCl_2$ and double concentration of Murashige and Skoog medium on *in vitro* plantlet and root cultures generation in halophytic grasswort *Salicornia brachiata*. **Plant Cell, Tissue and Organ Culture**, 120:563-570, 2015.

TAIZ, L. et al. **Physiology and plant development**. Porto Alegre:Artmed Ed., 2017. 888 p.

URREA, A. I. et al. Propagación *in vitro* y desdiferenciación tisular en *Lippia dulcis*. **Actualidades Biológicas**, 31: 21-29, 2009

VUJOVIĆ, T.; RUŽIĆ, D.; CEROVIĆ, R. *In vitro* shoot multiplication as influenced by repeated subculturing of shoots of contemporary fruit rootstocks. **Horticultural Science**, 39:101-107, 2012.

WADA, S. et al. Mesos components ($CaCl_2$, $MgSO_4$, KH_2PO_4) are critical for improving pear micropropagation. **In Vitro Cellular & Developmental Biology - Plant**, 49:356-365, 2013.

ZAREI, A. et al. Improvement of mineral nutrition and rooting efficiency of *Cannabis sativa* L. for *in vitro* large-scale propagation. **In Vitro Cellular & Developmental Biology-Plant**, 1-11, 2022.