

A Rapid and Efficient Protocol for Clonal Propagation of Phenolic-Rich *Lavandula multifida*

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Abstract

Lavandula multifida (fernleaf lavender) grows in confined regions of Portugal. This species is known for its interesting chemical profile, rich in phenolic compounds, and significant antifungal and anti-inflammatory properties, making it an interesting plant for industrial exploitation and justifying alternative propagation methods. Therefore, the present study aims the development of an efficient protocol for large scale propagation of this species, assuring plant uniformity and aiming a sustainable industrial exploitation with preservation of natural resources.

In vitro cultures were established on Murashige and Skoog medium (MS) with different concentrations of benzyladenine (BA) and zeatin (ZEA). Best multiplication rates (6.14 shoots per explant) were achieved using MS supplemented with 1.0 mg/L ZEA and rooting occurred spontaneously on the propagation medium avoiding additional rooting steps. Moreover, plants flowered during the acclimatization phase, confirming a more rapid in vitro development and shorter breeding cycle. This is a very interesting aspect for essential oil extraction. Trichome morphology was examined by scanning electron microscopy and the secretion analyzed through histochemical tests. Trichomes of micropropagated plantlets were identical to those of field-growing plants, and essential oil production was observed at early stages of development.

The present study confirms that micropropagation under controlled conditions could be an interesting alternative for field production of *L. multifida*, since higher amounts of plants can be obtained in less time with guarantee of genetic stability and phytosanitary quality, very important features regarding essential oil production and commercialization.

Keywords: essential oil, *in vitro*, *Lavandula multifida*, micropropagation, trichomes

1. Introduction

The economic exploitation of aromatic and medicinal species has increased due to a renewed interest in the use of natural compounds (Kala, Dhyani, & Sajwan, 2006). These metabolites can be obtained either from wild field-growing or cultivated plants. However, for environmental reasons, the gathering of large amounts of plants from wild species must be avoided since this can threaten the species and reduce biodiversity. Moreover, in aromatic plants, essential oils quality strongly depends upon environmental factors that may interfere in plant yield (Figueiredo, Barroso, Pedro, & Scheffer, 2008). Also chemotypes and ecotypes make it difficult to obtain high quality and uniform essential oils for very competitive markets. In recent years micropropagation techniques have emerged as cloning tools for the multiplication of selected genotypes with interesting chemical profiles (Chaturvedi, Jain, & Kidwai, 2007; Sidhu, 2010; Verma, Mathur, Jain, & Mathur, 2012).

Lavandula spp. essential oils are strictly regulated by ISO standards (e.g. ISO TC 54 - ISO/CD 8902, 2007; ISO TC 54 N- ISO/WD 4719, 2009) that require high-quality of the oils. As in other species, *Lavandula* spp. may be propagated sexually or asexually. However, reproduction through seeds is usually slow and the plants display a

great variation in a number of characteristics such as growth rate and essential oil profile. Therefore, techniques of vegetative propagation are the most effective to produce a large number of individuals displaying a genotype of interest. However, multiplication through cuttings is slow and cumbersome and poor rooting is often observed (Segura & Calvo, 1991; Chawla, 2009; Canhoto, 2010). Moreover, due to repeated cycles of propagation, modifications in morphological and chemical characteristics have been reported among the regenerated plants (Moutet, 1980; Panizza & Tognoni, 1992). Considering all these aspects, micropropagation is assuming an increased importance in plant cloning used for industrial purposes. Micropropagation can assure a large-scale production in controlled conditions, in a short period of time, without negative impacts on habitats (Canhoto, 2010). From the three techniques of micropropagation usually used, proliferation of axillary meristems is the most suitable for plant cloning (Gahan & George, 2008) since propagation by organogenesis or by somatic embryogenesis may induce variability among the regenerated plants (Deverno, 1995; Firoozabady & Mo, 2004; Jin et al., 2008), specially when an intermediate *callus* phase occurs.

Tissue culture techniques have been applied to several *Lavandula* species, as recently reviewed by Gonçalves and Romano (2013). For instance, axillary shoot proliferation was reported for *L. dentata* (Jordan, Calvo, & Segura, 1998; Sudriá et al., 1999, 2001; Echeverrigaray, Basso, & Andrade, 2005; Machado, Silva, & Biasi, 2012), *L. latifolia* (Sánchez-Gras & Calvo, 1996), *L. stoechas* (Nobre, 1996), *L. vera* (Andrade, Echeverrigaray, Fracaro, Pauletti, & Rota, 1999) and *L. viridis* (Dias, Almeida, & Romano, 2002; Nogueira & Romano, 2002). Direct shoot formation from different tissue explants was achieved in *L. latifolia* (Calvo & Segura, 1989a) and shoot formation from *callus* was reported for *L. angustifolia* (Quazi, 1980; Ghiorghita et al., 2009), *L. x intermedia* (Dronne, Jullien, Caissard, & Faure, 1999), *L. latifolia* (Calvo & Segura, 1988, 1989b; Jordan, Calvo, & Segura, 1990), *L. officinalis* x *L. latifolia* (Panizza & Tognoni, 1988) and *L. vera* (Tsuru, Koda, & Inoue, 1999, 2000). A preliminary study on somatic embryogenesis of *L. vera* was carried out by Kintzios et al. (2002).

The health and agricultural potential of the essential oils of *Lavandula* spp. growing wild in Portugal justifies the development of efficient protocols for the rapid and successful *in vitro* propagation of these species. Previously, a reliable protocol for large scale propagation of *L. pedunculata* was established for the first time (Zuzarte, Dinis, Cavaleiro, Salgueiro, & Canhoto, 2010). Now, a protocol for *in vitro* propagation of *L. multifida* is proposed, for the first time. This species is widely used in folk medicine namely in decoctions to treat rheumatism, colds, and also as a digestive (Upson & Andrews, 2004). Its interesting chemical profile, with high amounts of phenolic compounds, validated anti-inflammatory (Sosa et al., 2005) and antifungal properties (Zuzarte et al., 2012) and restricted distribution in Portugal, justify alternative propagation protocols avoiding excessive plant gathering in the wild and assuring plant uniformity. Therefore, the present study aims to develop a rapid and efficient protocol for *in vitro* culture of *L. multifida* for industrial exploitation.

2. Material and Methods

2.1 Plant Material

Aerial parts of *L. multifida* (Figure 1a) were collected at Sesimbra and maintained for a few days in the lab at room temperature with the basal part of the stems dipped in water to promote axillary shoot development and to facilitate the further isolation of axillary buds.

2.2 Establishment of *in vitro* Cultures

The developing axillary buds were dipped in 70% ethanol for 30 s before surface disinfection (5 min) in a 7% (w/v) calcium hypochlorite solution containing a drop of Tween 20. Following rinsing in sterile distilled water, the material was cultured on MS medium (Murashige & Skoog, 1962) supplemented with 3% sucrose. Ascorbic acid (10 mg/L) was added to the culture media to prevent tissue oxidation. The pH of all media was adjusted to 5.75 prior to the addition of 6 g/L agar. Media were sterilized by autoclaving at 121 °C for 20 min at 1.1 atm. Explants were cultured in test tubes (15 mL culture medium per test tube) and maintained in a growth chamber at 25 °C ± 1 °C and a 16 h daily photoperiod. Cultures were transferred to fresh culture medium every four weeks until enough stock material was available to perform further experiments.

2.3 Shoot Multiplication

After three months in culture, nodal segments of the established shoots (≥ 0.5 cm) were subcultured for shoot proliferation on MS medium containing 3% sucrose and different concentrations of benzyladenine (BA: 0.0, 0.1, 0.2, 0.5 and 1.0 mg/L) or zeatin (ZEA: 0.0, 0.1, 0.2, 0.5 and 1.0 mg/L). The media were prepared as described above and the nodal segments were cultured in test tubes and maintained in a growth chamber at 25 °C ± 1 °C and a 16 h daily photoperiod. To evaluate the influence of the cytokinin concentration on shoot multiplication, both the number of explants producing shoots and the number of shoots obtained (≥ 0.5 cm) were recorded.

Results were registered after 30 days of culture. Also, the number of explants developing *callus* was recorded.

2.4 Shoot Elongation

Following the proliferation stage, the shoots (≥ 0.5 cm) were transferred to MS medium with 3% sucrose without plant growth regulators, in order to promote shoot elongation. The media were prepared as described above and the shoots were cultured in test tubes and maintained in a growth chamber at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and a 16 h daily photoperiod. Results were taken after 30 days in culture and both the shoot length and the number of nodes per shoot were registered.

2.5 Shoot Rooting

Shoots from the previous phase were cultured on MS medium with 3% sucrose and supplemented with indol-3-butyric acid (IBA: 0.0; 0.5 and 1.0 mg/L). The media were prepared as described above and the shoots were cultured in test tubes and maintained in a growth chamber at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and a 16 h daily photoperiod. The number of roots per plant as well as the longest and the shortest root in each plant was recorded after 30 days of culture. Then the shoots were placed in MS culture medium without growth regulators and allowed to grow for one month, before acclimatization.

2.6 Acclimatization

Rooted plantlets (15 from each IBA treatment) were placed in plastic pots containing a mixture of garden soil and perlite (1:1) (v/v). The pots were maintained in an acclimatization chamber at $25 \pm 2\text{ }^{\circ}\text{C}$ with 70% of humidity and a 16 h photoperiod for 3 weeks.

2.7 Statistical Analyses

All experiments were conducted under a randomized block design including three replicates with 15 explants per treatment. To compare the effect of different concentrations of cytokinins, one-way ANOVA followed by a Dunnett's multiple comparison test was used. The statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA).

2.8 Light Microscopy and Histochemistry

To assure the uniformity of the *in vitro* propagated plants, the type of glandular trichomes in both the micropropagated plantlets and the respective field-growing parent plant were compared using light microscopy (LM) and histochemical analysis. For LM observations, sections (ca. 50-80 μm) of fresh leaves were obtained using razor blades. The sections were mounted on glass slides using the histomount mounting solution and observed unstained. Observations were made with a Nikon Eclipse E400 light microscope equipped with a Nikon Digital Sight DS-U1 photographic camera, using the Act-2U software.

The histochemical tests were performed in 30-50 μm thick sections of fresh leaves, obtained with a Sorvall® tissue sectioner. Terpenes were identified using NADI reagent (David & Carde, 1964). Observations were made in a Nikon Eclipse E400 light microscope equipped with a Nikon Digital Sight DS-U1 photographic camera, using the Act-2U software.

3. Results and Discussion

3.1 Shoot Establishment

The methodology used for explants disinfection was very efficient, allowing the establishment of more than 80% of aseptic cultures (Table 1). During the establishment phase (Figure 1b) the addition of ascorbic acid to the culture medium was irrelevant since necrotic explants did not occur (Table 1) on media without this antioxidant agent.

Table 1. Influence of ascorbic during the establishment phase of *Lavandula multifida*

Culture Medium	No. inoculated explants	1 week of culture		
		Healthy	Contaminated	Necrotic
MS	60	52 (87%)	8 (13%)	0
MS + 10 mg/L ascorbic acid	60	49 (82%)	11 (18%)	0

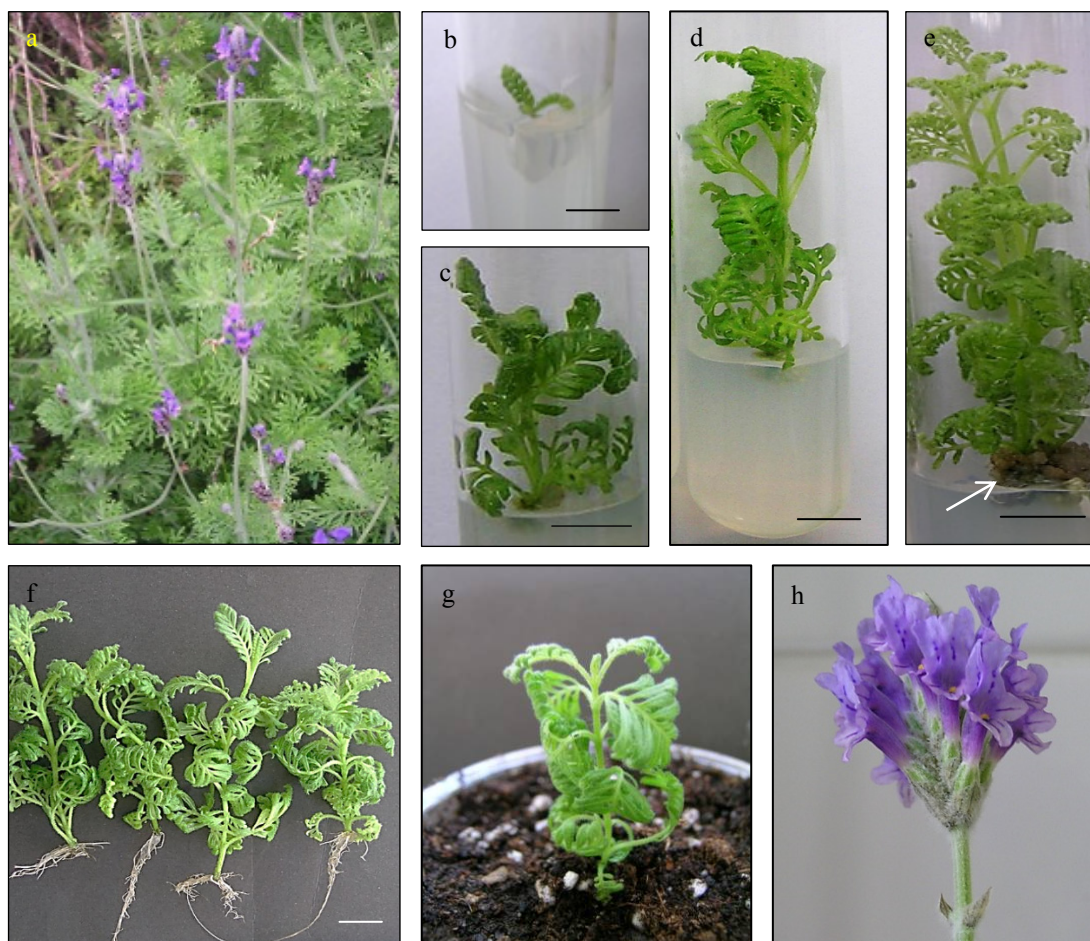


Figure 1. Micropropagation of *Lavandula multifida*

a) Adult plant used for the isolation of axillary buds. b) Establishment phase: inoculation of the axillary buds in test tubes containing culture medium (MS medium without growth regulators). c) Multiplication phase: shoots formed after 30 days of culture in medium with 1.0 mg/L BA. d) Elongation phase: shoots after 30 days of culture in medium without growth regulators. e) Shoot with *callus* (arrow) at the base, after 30 days of culture on MS medium with 1.0 mg/L BA. f) Rooting phase: roots formed spontaneously or due to the addition of IBA to the culture medium. g) Acclimatization phase: transfer of plants to soil. h) Flowering of a plant obtained through axillary shoot proliferation, during the acclimatization phase. Bars = 1 cm.

3.2 Shoot Proliferation

During the proliferation phase (Figure 1c), the different concentrations of BA and ZEA did not significantly interfere with the percentage of explants showing axillary shoot development (Table 2). However, the number of shoots (≥ 0.5 cm) per explant increased with the addition of higher concentrations of ZEA to the culture medium (Table 2). The highest multiplication rate (6.14 shoots per explant) was obtained using MS supplemented with 1.0 mg/L ZEA (Table 2). The efficiency of cytokinins was also shown on shoot multiplication of other lavenders: *L. dentata* (Jordan et al., 1998; Echeverrigaray et al., 2005), *L. officinalis* x *L. latifolia* (Panizza & Tognoni, 1992), *L. pedunculata* (Zuzarte et al., 2010), *L. stoechas* (Mesquita, Guimarães, & Santos Dias, 1990; Nobre, 1996) and *L. vera* (Andrade et al., 1999).

Some shoots displayed hyperhydricity. This is quite common during *in vitro* culture and has also been reported for other *Lavandula* species such as *L. dentata* (Echeverrigaray et al., 2005), *L. stoechas* (Nobre, 1996), *L. pedunculata* (Zuzarte et al., 2010) and *L. vera* (Andrade et al., 1999). Although several factors may induce the appearance of hyperhydricity (Debergh et al., 1992), the excess of cytokinins in the culture medium has been referred as one of the most important in promoting this phenotype (Debergh, Harbaoui, & Lemeur, 1981;

Leshem, Werker, & Shalev, 1988; Kataeva, Alexandrova, Butenko, & Dragavtceva, 1991; Williams & Taji, 1991). In *L. multifida* a positive relationship also seemed to occur between the increase of hyperhydricity and the raise of cytokinins in the culture medium (data not shown). However, this phenotype did not impair plant regeneration in this species since hyperhydricity disappeared following plantlet transfer to MS medium lacking growth regulators.

3.3 Shoot Elongation

Following separation and transfer to MS medium without growth regulators, the shoots from all media elongated (Figure 1d). During the elongation phase, the possible influence of BA and ZEA present in the previous multiplication medium was evaluated with respect to shoot length, and number of nodes *per* shoot, after 30 days of culture in MS medium lacking growth regulators. The longest shoots (1.5 cm) developed in explants from MS medium with 1.0 mg/L BA (Table 2). These results indicate that in *L. multifida* the BA concentration used in the multiplication medium has further influence in the elongation phase, namely on the length of the shoots. This is similar to what has been reported for *L. pedunculata* (Zuzarte et al., 2010) and *L. stoechas* (Nobre, 1996) but contrasts with results obtained with *L. latifolia* (Calvo & Segura, 1989) and *L. dentata* (Jordan et al., 1998).

Once the pre-existing buds started to develop, *callus* proliferated mainly at the cut end of the explants (Figure 1e). Shoot organogenesis from basal *callus* is likely to occur and has been reported in several *Lavandula* species such as *L. angustifolia* (Quazi, 1980), *L. x intermedia* (Dronne et al., 1999), *L. latifolia* (Calvo & Segura, 1988; Jordan et al., 1990), *L. officinalis* x *L. latifolia* (Panizza & Tognoni, 1988) and *L. vera* (Tsuro et al., 2000). Organogenesis may be an alternative to shoot proliferation but a careful analysis of the propagated material must be performed to confirm the uniformity of the regenerated plants.

Table 2. Effect of benzyladenine (BA) and zeatin (ZEA) on shoot proliferation and elongation of *in vitro* cultures of *Lavandula multifida*

	Control	Cytokinins (mg/L)							
		BA				ZEA			
		0.1	0.2	0.5	1.0	0.1	0.2	0.5	1.0
Explants developing axillary shoots (%) ^a	97.44±4.44	97.44 ±4.44	94.87 ±8.88	89.32 ±9.27	92.31 ±7.69	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	97.22 ±4.81
Number of shoots per explant ^a	1.77 ±0.23	1.92 ±0.08	1.80 ±0.23	2.32 ±0.94	2.72 ±0.32	2.11 ±0.27	2.15 ±0.16	4.19 ±1.25 ***	6.14 ±0.78 ***
Shoot length ^b	0.33 ±0.11	0.52 ±0.20	0.8 ±0.26	0.75 ±0.25	1.51 ±0.63 **	0.71 ±0.15	0.79 ±0.32	0.98 ±0.17	0.94 ±0.40
Number of nodes <i>per</i> shoot ^b	1.55 ±0.29	1.87 ±0.40	2.60 ±0.04	3.10 ±0.42	3.73 ±0.52	3.48 ±1.12	3.00 ±0.64	5.10 ±1.80	5.87 ±5.44

Note. ^a After 10 days of culture in MS medium with BA or ZEA; ^b After 30 days of culture in MS medium lacking growth regulators; ** p < 0.01 and *** p < 0.001, in comparison to control.

3.4 Shoot Rooting

During the elongation phase 42% of the shoots rooted spontaneously. Shoots that did not root spontaneously were induced to root using IBA (Figure 1f). The effect of two concentrations of IBA on root formation was evaluated through the number of roots *per* shoot and roots length (the longest and shortest roots *per* shoot, Table 3). Spontaneous rooting is important for *in vitro* propagation as it allows a considerable gain of time in the regeneration process and avoids the use of auxins that are known to induce *callus* and anomalous vascular connections between shoots and adventitious roots (Németh, 1986; Ziv, 1986). In the present study, the two concentrations of IBA used to promote rooting in *L. multifida*, did not show significant differences concerning the number of roots formed but longer roots occurred in the absence of auxins (control) (Table 3).

Table 3. Effect of indol-3-butyric acid (IBA) on rooting of *in vitro* cultures of *Lavandula multifida*

	IBA (mg/L)		
	0.0	0.5	1.0
Number of roots <i>per shoot</i> ^a	4.89±2.01	3.42±1.01	5.78±1.07
Longest root length <i>per shoot</i> ^a	1.83±0.47	0.72±0.22**	1.00±0.08
Shortest root length <i>per shoot</i> ^a	0.54±0.02	0.32±0.07*	0.30±0.16*

Note. ^a After 30 days of culture in MS medium with IBA; * $p < 0.05$ and ** $p < 0.01$, in comparison to control.

3.5 Acclimatization

In vitro rooted plantlets (15 per IBA treatment) were successfully transferred to soil (100% survival rate), exhibiting a normal development with a high degree of homogeneity and no evidences of somaclonal variation (Figure 1g). Also, flowering was observed during the acclimatization phase (Figure 1h), an interesting aspect since early flowering of micropropagated plantlets can shorten the breeding cycle to meet the market demands (Kielkowska & Havey, 2012).

3.6 Microscopic Observations

Microscopic observations showed the presence of the same types of trichomes in *in vitro* plantlets and in field-growing adult plants, including the characteristic peltate trichome of this species (Zuzarte, 2012). Also histochemical tests using Nadi reagent detected essential oils in the trichomes of *in vitro* shoots, at very early stages of plant development (Figure 2). Previous experiments showed that the essential oils of both *in vitro* shoot-cultures and micropropagated plants of *L. viridis* had the same main compounds as the original field-grown parent plant, without remarkable compositional variations (Nogueira & Romano, 2002). Also, the analysis of the volatiles emitted directly (headspace solid phase microextraction) from *in vitro* shoot cultures, and micropropagated plants of *L. viridis* when compared with those from the field-growing parent plant, showed the same major compound, 1,8-cineole (Gonçalves et al., 2008). In *L. pedunculata*, the trichomes and essential oils of *in vitro* plantlets of two selected chemotypes were identical to those occurring in field-growing plants (Zuzarte et al., 2010).

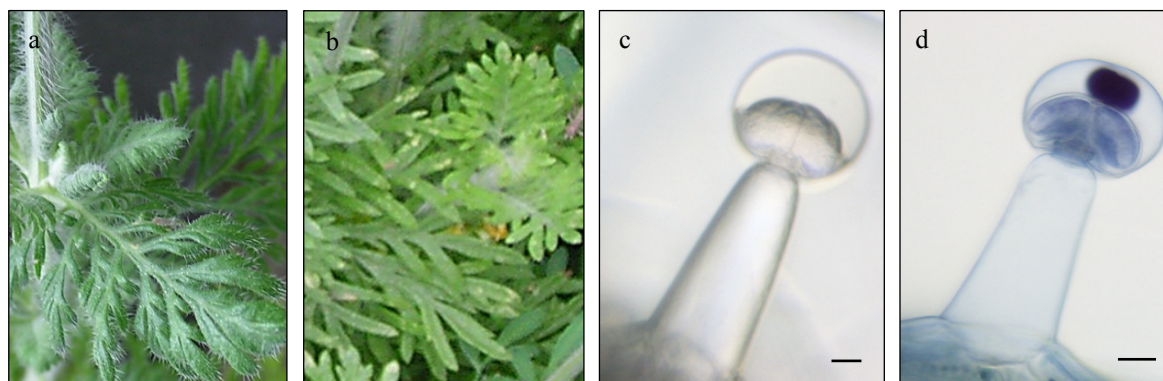


Figure 2. Leaf details of a) parent plant and b) fully acclimatized plant. c) Peltate trichome occurring in the leaves of *in vitro* shoot cultures. d) Detection of essential oils in a peltate trichome in *in vitro* shoot cultures using Nadi reagent. Bars = 10 μ m

4. Conclusions

The present study aimed the development of a rapid and efficient protocol for *in vitro* propagation of *L. multifida*. In fact, the results obtained suggest that micropropagation through axillary shoot proliferation is a reliable method for the rapid multiplication of this species allowing plant conservation without damage of its restricted natural resources in Portugal. Moreover, considering the interesting biological properties of this species, micropropagation under controlled conditions could be an interesting alternative for field production of *L. multifida*, since higher amounts of plants can be obtained in less time. For example, by using only MS with 1.0 mg/L of ZEA as a multiplication medium, it is possible to obtain around 360 acclimatized plantlets, after 7

months of culture.

Further experiments are being considered to compare the essential oil composition of *in vitro* plantlets, acclimatized plants and fully adapted *ex-situ* plants with those from the parent-plants.

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