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ORIGINAL ARTICLE

# A protocol for micropropagation of the medicinal species *Tuberaria lignosa* provides ploidy true-to-type plants with high antioxidant capacity

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## Abstract

*Tuberaria lignosa* (Cistaceae family) has excellent properties as an antioxidant, anti-inflammatory, antimicrobial, and antiproliferative/anti-tumoural. This work aimed to establish a routine micropropagation protocol of *T. lignosa* to supply high-quality plants for the pharmaceutical industry. Apical shoot cuttings (0.5 cm long) were grown on basal medium Murashige and Skoog (MS) and ½MS for 60 days with different growth regulators. ½MS supplemented with 1 mg $L^{-1}$ benzylaminopurine (BAP) provided the best results with shoots showing better growth rates and no vitrification or browning. After 60 days, the best results for shoot proliferation were achieved on ½MS with 1 mg $L^{-1}$ mT, with 100% explants responding and ~ 4 new shoots formed per explant. For elongation and rooting, after 30 days, 1 mg $L^{-1}$ indoleacetic acid (IAA) provided 100% of rooting (~ 18 roots per shoot), and resulting plants were vigorous, also with no signs of vitrification or browning. For acclimatization, the substrate Jiffy Plug provided the best results after 60 days, with a survival rate of 68.42%. In the subsequent 30 days in greenhouse conditions, all plants survived. Acclimatized plants had still less proteins, but high activity of antioxidant enzymes compared with the mother plants, supporting that these cloned plants may be of interest to the pharmaceutical industry. No changes in the DNA-ploidy were detected between micropropagated and the mother plants. The protocol described here provides, after 8 months of explant establishment, a high number of plants/explants, revealing a high potential for future use in this species and in other medicinal and economically relevant species, and in species in need of ex-situ conservation measures.

## Key message

A new ploidy true-to-type micropropagation protocol of the medicinal species *Tuberaria lignose* provided a large number of plants after explant-inoculation on ½MS (with mT for multiplication) and successful acclimatization.

**Keywords** Antioxidant properties · Tuberaria · Medicinal plants · Micropropagation · Perennial rock-rose · Ploidy-fidelity

## Introduction

The family Cistaceae includes a wide number of medicinal species traditionally used in folk medicine, of which the best-known genus is *Cistus* (Papaefthimiou et al. 2014). The genus

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*Tuberaria* is far less known, but several species of this genus, namely *Tuberaria lignosa,* are highly rich in medicinal compounds, presenting a high, yet unexplored, pharmaceutical potential. *T. lignosa* is found in Western–Southern Europe, and in North Africa, particularly in Italy, France, Spain, Portugal, Morocco and Algeria (POWO 2019). In traditional medicine, its infusions or decoctions may be prepared using the whole plant or just leaves or flowers, fresh or dried. Due to its multiple antioxidants, anti-inflammatory, and antimicrobial properties plants/extracts of this species are used in treatments of heartburn, flu, gastrointestinal disorders, or skin infections (e.g., Bedoya et al. 2009, 2010; Pinela et al. 2012). Its richness in bioactive compounds against cancer was also demonstrated (Pereira et al. 2016). Additionally, this species raised interest as a source of ellagitannin (Bedoya et al. 2010), a compound that showed high efficiency against HIV infection (mediated by CD4 downregulation), besides other antiviral bioactivities (Bedoya et al. 2010; Pinela et al. 2016; Salehi et al. 2018). Its extracts, obtained by infusions or decoctions, contain phenolic compounds and ascorbic acid, which are bioactive molecules that can contribute to their antioxidant activity. The chromatographic profiles of the phenolic compounds of this species include mainly ellagitannins, flavonoids (flavones and flavonols), and derivatives of phenolic acids (Bedoya et al. 2010; Huang et al. 2009; Pinela et al. 2012, 2014, 2015).

Among the increasing demand for medicinal products based on natural sources and, in particular, secondary metabolites of medicinal plants (Chen et al. 2016), research on candidate species and industrial propagation for use in the pharmaceutical and nutritional industry is growing worldwide. Most plants of many of these candidate species are not industrially produced, but rather harvested in their natural environments, which poses critical problems related to: (a) the survival of the wild populations and the sustainability of the ecosystems; (b) irregular harvests, mainly dependent on the season; (c) the regular quality of the harvested products is not ensured. On another hand, optimized micropropagation protocols of elite genotypes lead to rapid cycles of production of a large number of plants/materials suitable for industrial transformation and might contribute to overcoming those limitations (Coelho et al. 2020; Chokheli et al. 2020). Micropropagation protocols have already been applied to a large number of industrial and medicinal plants (Alves et al. 2021; Bose et al. 2017; Fernandes et al. 2009; Lodha et al. 2015; Nunes et al. 2018; Pinto et al. 2008; Saha et al. 2020; Salehi et al. 2018). Despite its enormous medicinal and pharmacological importance, as a wild species, no reliable method is available for large scale propagation of *T. lignosa*. In the *Tuberaria* genus, only one work on *T. major* micropropagation with cytokinins [6-benzyladenine (BA), kinetin, and zeatin (ZEA)] for shoot proliferation (Gonçalves et al. 2010), and acclimatized plants showed high plasticity during the acclimation to ex vitro conditions (Osório et al. 2013).

The objective of this work was to establish a robust in vitro culture protocol for the mass micropropagation of genotypes of *T. lignosa*, which may provide plants, in a sustainable manner, to the pharmaceutical industry. The protocol included the steps from its in vitro establishment to the ex vitro acclimatization of the plants resulting from multiplication. Also, considering the interest of this plant as a possible source of natural bioactive compounds, DNA-ploidy level and redox status analyses were made to validate the quality of the propagated plants.

## Materials and methods

### Plant material and in vitro establishment

*Tuberaria lignosa* adult plants were collected in Miranda do Douro, Portugal, in the spring of 2018, and transferred to pots with the same soil. Plants were grown in a greenhouse, water irrigated 2×/week, and treated for 2 weeks with 2 weekly sprays of fungicide Mancozeb (2.0 g $L^{-1}$).

For the in vitro establishment, young branches were first washed in tap water for 30 min and then disinfected in 70% (v/v) ethanol with Tween20® for the 30 s. Then, shoots were disinfected for 20 min in 1.0 g $L^{-1}$ Mancozebe, followed by 10 min in commercial bleach (NaOCl < 5% w/v) with a few drops of Tween20®. Under aseptic conditions, shoots were washed 3 × in sterile distilled water. Apical meristems with leaf primordia were cut and inoculated on 15 mL of culture medium MS or ½MS medium (Murashige and Skoog 1962) supplemented with 1 mg $L^{-1}$ benzylaminopurine (BAP), 30 g $L^{-1}$ of sucrose, and 7 g $L^{-1}$ of agar, and the pH 5.7. The shoot explants were incubated in a growth chamber under a 16/8 h day/night period at 25/22 °C, respectively, with cool-white fluorescent lamps (OSRAM, L58W/840) with a PAR light intensity of ~ 35 µmol $m^{-2}$ $s^{-1}$. After 60 days, the explants were evaluated for survival rates and growth rates. Oxidation/browning, hyperhydricity and vigour were measured using semiquantitative scale: 1 (very low), 2 (low), 3 (moderate), 4 (high), and 5 (very high). In each condition, thirty explants were randomly selected and analysed.

### Shoot multiplication

In vitro shoot apices (0.5–1.0 cm long) were used for the optimization of the best growth regulators combination for shoot multiplication. Shoot apices were inoculated for 60 days on ½ MS basal medium with different cytokinins, namely BAP at 0.5 or 1 mg $L^{-1}$, *meta*-topolin (mT) at 0.5 or 1 mg $L^{-1}$, and zeatin (ZEA) 0.2 mg $L^{-1}$. Two combinations with indole-3-acetic acid (IAA) were also tested: BAP 0.5 mg $L^{-1}$ + IAA 0.25 mg $L^{-1}$, or mT 0.5 mg $L^{-1}$ + IAA

0.25 mg $L^{-1}$. As a reference, another group was grown on ½ MS without growth regulators. For each condition, 30 replicates were made. Incubation conditions were similar to those described above, and two successive multiplication cycles were performed.

### Shoot elongation and rooting

A first approach of combining shoot elongation and rooting was assessed. For that, shoot segments (1 cm, with 2–3 nodes) were separated from the multiplied shoots and used for the elongation and rooting assays. To assess the best growth regulator combination, segments were grown on ½ MS with different growth regulators: indolebutyric acid (IBA) at 0.5, 1 or 2 mg $L^{-1}$, IAA 1 mg $L^{-1}$, gibberellic acid ($GA_3$) 0.2 mg $L^{-1}$ and a combination of 1 mg $L^{-1}$ IAA with 0.2 mg $L^{-1}$ $GA_3$. Growth took place with the same temperature and light intensity as above, for 30 days. For each condition, at least thirty replicates were made.

### Acclimatization

Roots of in vitro plantlets were carefully washed (to remove residues of culture medium) and transplanted to pots with three different substrates: (a) universal (70% brown peat, 25% blonde peat, 5% green compost), (b) Jiffy plugs (40% coconut fiber, 60% peat), and (c) Jiffy mix (60% coconut fibre, 20% coconut chips, 20% peat). The plant growth chamber was maintained at 26 °C (day) and 23 °C (night), and a 16 h photoperiod with a PAR intensity of 60 µmol $m^{-2}$ $s^{-1}$, and a relative humidity initially set to 90% and progressively decreasing to 60% after 6 weeks. The percentage of surviving plants and the number of new leaves and branching were determined 6 weeks after the beginning of the acclimatization. Then the plants were transferred to greenhouse conditions and after 30 days, their survival rate was evaluated. The experiment was replicated with at least 30 explants/treatment.

### Redox status

The redox status of the micropropagated plants was compared with the mother plants by assessing the levels of $H_2O_2$ and of $O_2^{\cdot}$, the antioxidant profile, and the total phenols as described by Mariz-Ponte et al. (2019). For the antioxidant enzymatic pool, soluble proteins were extracted and quantified and superoxide dismutase, catalase, guaiacol peroxidase, and ascorbate peroxidase activities were analyzed as described by Mariz-Ponte et al. (2018). For each assay, three leaves of each micropropagated or mother plant, and at least three plants of each condition were used as replicates.

### DNA-ploidy level analyses

Leaf samples from the mother plants and micropropagated plants were grown in a greenhouse for 30 days. At least three plants of each condition were used as replicates. Estimation of the nuclear DNA content using flow cytometry was based on a methodology already described (Castro et al. 2018). For that, leaves (~ 50 mg) of a reference standard (*Raphanus sativus*) and of sample material were chopped into Woody Plant Buffer (Loureiro et al. 2007), and filtered through a 50 µm nylon filter. To the nuclear suspension, 50 µg $mL^{-1}$ of propidium iodide and RNase were added, and after a 5 min incubation samples were analyzed on a Partec CyFlow® space flow cytometer. To ensure further sample quality, prior to analysis, the instrument stability and linearity were verified using fluorescent beads (Partec GmbH, Görlitz, Germany). The results were acquired using the FloMax software (v. 2.4d) in the form of four graphics: fluorescence pulse integral in linear scale (FL); forward scatter (FS) vs. side scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. To remove debris and improve the quality of the samples, polygon regions were defined in the FL vs. SS scatterplot and further applied to the other graphics. Samples that presented CV values below 6% were accepted. The nuclear DNA content of each sample as a proxy of DNA-ploidy of each sample was calculated for each individual by applying the following formula: (mean FL of the sample nuclei/mean FL of standard nuclei) × 1.11 (nuclear DNA content of *Raphanus sativus*).

### Statistical analysis

The results were analysed using one-way analysis of variance (ANOVA) followed by the Duncan post-test for multiple comparisons between treatments. This analysis was performed using the SPSS Statistics 25.0 program (IBM Corp., Armonk, NY). Some data were also analysed based on the t-student, in which the compared values and significant differences were based on $p < 0.05$, and using the Prism® 8 (GraphPad Software Inc., USA).

## Results

### In vitro establishment

The fungal infection predominated during establishments, with 20.36% ± 1.52 and 18.68% ± 8.16 of the explants showing fungal infection in MS and ½MS (< 7.5% of the explants showed bacterial infection) (Table 1). Among the explants with no infections, there was a clear influence of the medium, specifically on the percentage of the explants' survival, being the ½MS medium the most

appropriate. While in the MS medium the surviving rate was 42.08% ± 14.27, browning followed leading to explant necrosis in 30.25 ± 3.63 of the explants. On the other hand, ½MS provided a survival rate of 72.47% ± 6.06, with necrosis being observed in 2.21% ± 1.55 of explants, only. These data evidence that this last medium, provided significantly better results. Regarding the occurrence of bacterial or fungal contamination, no significant differences were observed between both media. Additionally, it was possible to observe that ½MS medium positively influenced the quality of the explants, in terms of low hyperhydricity or browning (< 3 level) (Table 1).

## Shoot multiplication

In all the treatments in ½MS media, it was possible to observe that the induction of new shoots occurred, particularly on the T4 (with 1 mT mg $L^{-1}$) in which 100% of explants presented new branches (Table 2). This condition demonstrated significant superiority to the others, with an average of 4.11 ± 0.26 shoots per explant and a length of 0.86 ± 0.04 cm in first cycle. In contrast, the lowest rates recorded were observed in T7 (with ZEA), with only 38% of explants showing new shoots, with an average of 1.20 ± 0.35 shoots per explant and 0.54 ± 0.07 cm in length.

Regarding the qualitative parameters in this phase, T3, T4, and T6 induced higher vigour (3–4 levels) and absence of hyperhydricity or oxidation. T0 and T7 induced lowest vigour (1–2) and the highest hyperhydricity and oxidation (4–5). In the second subculture of the multiplication, it was possible to observe that the values of induction, the number of shoots, and their lengths decreased when compared to the first cycle culture (Table 2).

However, in the second cycle, T4 with 1 mT mg $L^{-1}$ showed the highest percentage of shoot induction (75%), the highest average number of shoots per explant (2.50 ± 0.20), and the longest average length of new shoots (0.75 ± 0.05 cm), although they are not significantly different from the values of the other treatments. T7 was the worst in the first cycle, without inducing any response in the second cycle.

## Elongation and rooting

To test the effect of elongation we compared the shoot lengths of different treatment of which, T5, T6, T2, and T1 were the ones that stood out significantly from the others,

**Table 1** Percentage values (%) of the survival rates, infections and explant necrosis of *Tuberaria lignosa* 60 days after being inoculated in vitro on MS or 1/2 M

| Basal medium | Survival (%) | Bacteria infection (%) | Fungi infection (%) | Necrosis (%) |
|---|---|---|---|---|
| MS | 42.08 ± 14.27 | 7.31 ± 2.96 | 20.36 ± 1.52 | 30.25 ± 3.63* |
| ½ MS | 72.47 ± 6.06* | 6.64 ± 4.65 | 18.68 ± 8.16 | 2.21 ± 1.55 |

The other conditions were similar, including both media were supplemented with 1 mg $L^{-1}$ BAP, 30 g $L^{-1}$ of sucrose, and 7 g $L^{-1}$ of agar, and the pH 5.7 (n = 30 for each condition)

*Significant differences ($p < 0.05$)

**Table 2** Effect of growth regulators on shoots multiplication from explants of *Tuberaria lignosa* after 60 days of in vitro culture (first cycle), and on the second cycle of shoots multiplication

| | BAP mT ZEAIAA (mg $L^{-1}$) | | | | 1st cycle | | | 2nd cycle | | | Qualitative traits | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Shoot Induc. (%) | Shoot N°/Exp | Shoot length(cm) | Shoot Induc (%) | Shoot N°/Exp | Shoot Length (cm) | Hyper-hydricity Oxidation | | Vigor |
| T0 | – | – | – | – | 33 | $2.00^{cde} \pm 0.45$ | $0.83^{ab} \pm 0.06$ | 25 | $1.25^{b} \pm 0.35$ | $0.65^{a} \pm 0.09$ | 3–4 | 3–4 | 2 |
| T1 | 0.50 | – | – | – | 67 | $1.50^{de} \pm 0.32$ | $0.71^{b} \pm 0.04$ | 69 | $1.73^{ab} \pm 0.20$ | $0.63^{a} \pm 0.07$ | 3 | 2–3 | 3 |
| T2 | 1 | – | – | – | 78 | $2.21^{bcd} \pm 0.25$ | $0.81^{ab} \pm 0.04$ | 31 | $1.40^{b} \pm 0.30$ | $0.58^{a} \pm 0.08$ | 2–3 | 2–3 | 3 |
| T3 | – | 0.50 | – | – | 94 | $2.88^{b} \pm 0.27$ | $0.84^{ab} \pm 0.04$ | 69 | $2.09^{ab} \pm 0.20$ | $0.66^{a} \pm 0.06$ | 1–2 | 1–2 | 3–4 |
| T4 | – | 1 | – | – | 100 | $4.11^{a} \pm 0.26$ | $0.86^{a} \pm 0.04$ | 75 | $2.50^{a} \pm 0.20$ | $0.75^{a} \pm 0.05$ | 1 | 1 | 4 |
| T5 | 0.50 | – | – | 0.25 | 72 | $2.08^{cde} \pm 0.30$ | $0.72^{ab} \pm 0.04$ | 33 | $1.33^{b} \pm 0.39$ | $0.5^{a} \pm 0.12$ | 1–2 | 1–2 | 3 |
| T6 | – | 0.50 | – | 0.25 | 89 | $3.00^{b} \pm 0.27$ | $0.76^{ab} \pm 0.04$ | 33 | $1.33^{b} \pm 0.39$ | $0.6^{a} \pm 0.12$ | 1 | 1 | 3–4 |
| T7 | – | – | 0.20 | – | 38 | $1.20^{d} \pm 0.35$ | $0.54^{c} \pm 0.07$ | 0 | – | – | 4 | 5 | 1 |

Mean values ± standard error. Columns with different letters are significantly different at p < 0.05 using Duncan's test

with shoot length averages of 1.23 ± 0.19, 1.17 ± 0.30, 1.15 ± 0.27 and 1.14 ± 0.11 cm, respectively (Table 3). Otherwise, the T3 treatment was the one that presented explants with a shorter average shoot length (0.88 ± 0.04 cm).

Regarding the rooting assay, the number of roots formed per explant in T1 showed an average of 17.96 ± 0.76 roots, being significantly higher than the values obtained in the other treatments (Table 3). On the opposite, the absence of growth regulators in the culture medium (T0) resulted in the lowest number of roots formed per shoot, with an average of only 2.25 ± 1.42 roots. Regarding qualitative parameters, we observed that T1, T4, and T5 shoots and roots presented the highest vigour (levels 4–5) and little evidence of hyperhydricity (levels 1–2) or oxidation (levels 1–2). In contrary, the vigour was the lowest (levels 2–3) and hyperhydricity and oxidation were the highest (levels 4–5) (Table 3).

## Acclimatization and redox status

During the pre-acclimatization, plants on Jiffy plugs showed a greater development, with a survival percentage of 68.42%, against 57.89% in the Jiffy mixture and 0% on the universal substrate. The Jiffy plugs also promoted a greater number of new shoots (2.23 ± 0.75 shoots per plant), above the results in the Jiffy mixture (1.60 ± 0.68 shoots per plant) (Table 4).

Regarding the development of new leaves, there were no significant differences between treatments. The analysis of the qualitative parameters revealed that plants of the Jiffy plugs (Fig. 3) presented high vigour and no signs of leaf necrosis, unlike those on the Jiffy mixture. After 30 days in greenhouse conditions, the plants showed a survival rate of 100%, regardless of the substrate used in the pre-acclimatization phase in an acclimatization chamber.

The comparative values of redox parameters are shown in Fig. 1. The levels of $O_2^{\cdot}$- decreased, followed by an increase of $H_2O_2$ and of total phenol antiradical activity, while the levels of soluble proteins were lower in acclimatizing plants (Fig. 1A–D). Interestingly, the activity of antioxidant enzymes per mg proteins was much higher in the acclimatized plants than the values of the mother plants, but the differences were much attenuated when the activities of the antioxidant enzymes were expressed per g FW (Fig. 1E–H).

**Table 4** Effect of substrate on pre-acclimatization of *Tuberaria lignosa* plants after 60 days on growth chamber (n = 30)

| Treatment | Substrate | Survival (%) | Nº shoots/plant | Nº leaves/plant |
|---|---|---|---|---|
| T1 | Universal | 0 | – | – |
| T2 | *Jiffy Plugs* | 68.42 | 2.23 ± 0.75* | 3.85 ± 1.26 |
| T3 | *Jiffy* Mix | 57.89 | 1.60 ± 0.68 | 3.73 ± 1.57 |

Mean values ± standard error

*Significantly different at $p < 0.05$ using t-Student test

## Flow cytometry

The average nuclear DNA content in micropropagated plants (3.83 ± 0.06 pg DNA/2C) was similar to that obtained in field plants (3.82 ± 0.08 pg DNA/2C), with no significant differences ($p > 0.05$; Table 5; Fig. 2). These results demonstrate that both micropropagated and mother plants' populations had similar DNA-ploidy level, and thus ploidy stability was guaranteed in the micropropagated plants.

# Discussion

Micropropagation offers a powerful and sustainable tool for the large-scale production of plants or organs for the pharmaceutical industry. MS is the culture medium mostly used in micropropagation protocols of the Cistaceae family (Airò et al. 2015; Gonçalves et al. 2010; Ruta and Morone-fortunato, 2013). For *T. lignosa*, this medium induced a low response (% of explant establishment) as a consequence of

**Table 3** Effect of growth regulators on elongation and rooting of *T. lignosa* shoots after 30 days of in vitro culture

| | IAA | IBA (mg $L^{-1}$) | $GA_3$ | Shoot elongation (%) | Root induct (%) | Root Nº/explant | Root length (cm) | Qualitative traits | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Hyperhydricity | Oxidation | Vigor |
| T0 | – | – | – | $0.91^{cd} \pm 0.14$ | 27 | $2.25^{c} \pm 1.42$ | $2.70^{a} \pm 0.19$ | 5 | 4 | 2–3 |
| T1 | 1 | – | – | $1.14^{a} \pm 0.11$ | 100 | $17.96^{a} \pm 0.76$ | $1.74^{bc} \pm 0.10$ | 1 | 1–2 | 4–5 |
| T2 | – | – | 0.2 | $1.15^{a} \pm 0.27$ | 60 | $6.45^{b} \pm 0.95$ | $1.72^{bc} \pm 0.13$ | 3 | 2 | 3 |
| T3 | 1 | – | 0.2 | $0.88^{d} \pm 0.21$ | 67 | $4.75^{bc} \pm 1.00$ | $2.07^{b} \pm 0.12$ | 3 | 3 | 3 |
| T4 | – | 0.5 | – | $1.01^{bc} \pm 0.20$ | 73 | $6.82^{b} \pm 0.86$ | $1.41^{c} \pm 0.14$ | 1 | 1 | 4–5 |
| T5 | – | 1 | – | $1.23^{a} \pm 0.19$ | 100 | $4.82^{bc} \pm 0.76$ | $1.67^{c} \pm 0.10$ | 1 | 2 | 4–5 |
| T6 | – | 2 | – | $1.17^{a} \pm 0.30$ | 80 | $5.71^{b} \pm 0.82$ | $1.70^{bc} \pm 0.11$ | 2 | 2 | 3 |

Mean values ± standard error. Columns with different letters are significantly different at $p < 0.05$ after a Duncan's test

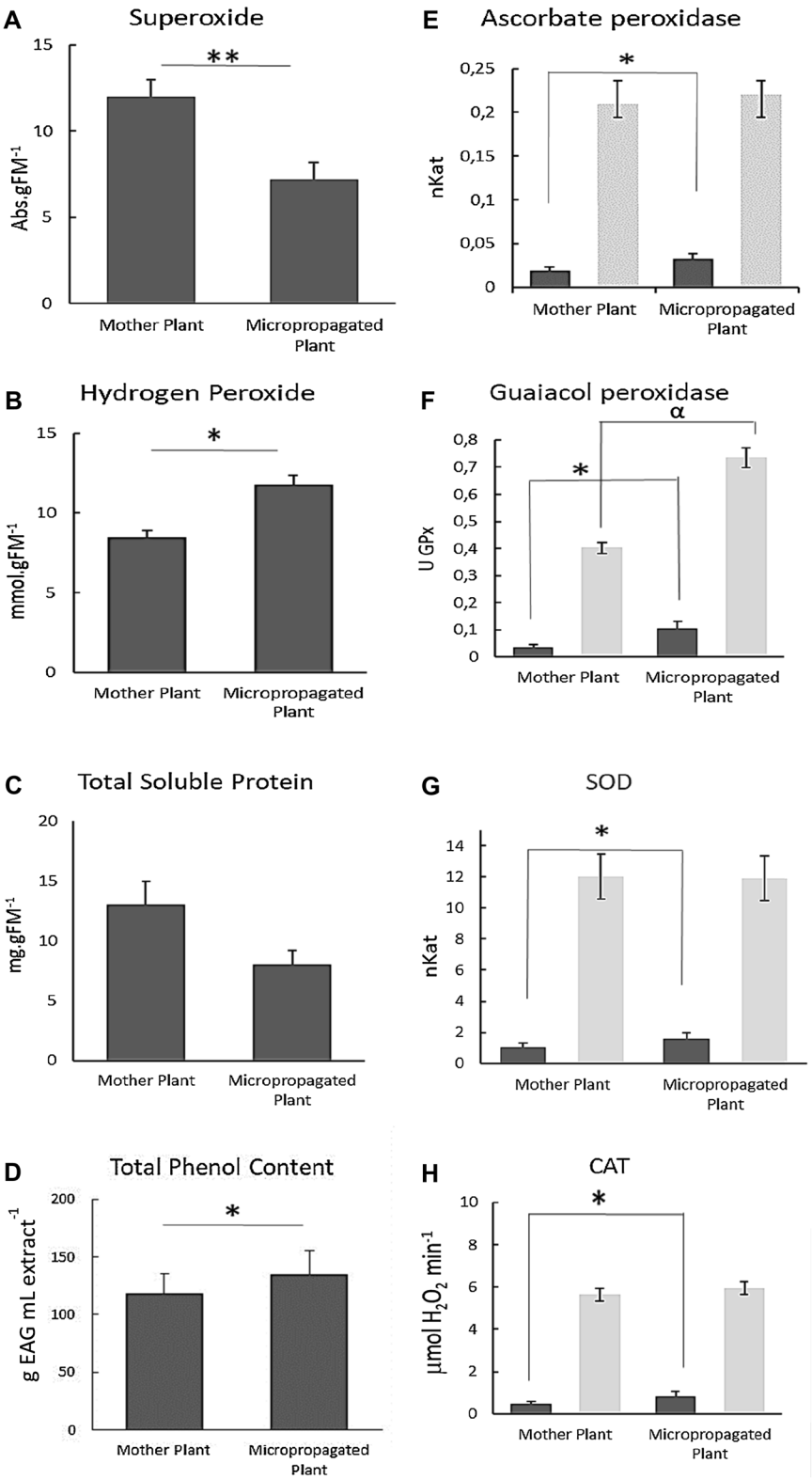


**Fig. 1** Comparative data between mother and micropropagated plants. **A** Superoxide activity; **B** Hydrogen peroxide; **C** total soluble proteins; **D** total phenol content; **E**–**H** Enzymatic activity expressed as function of mg of TSP (dark column) or as function of gFW (light column). **E** Ascorbate peroxidase (APx); **F** guaiacol peroxidase (GPx) **G** superoxide dismutase (SOD); **H** catalase (CAT). FM (Fresh Mass) and TSP (Total Soluble Protein). The bars represent means ± standard deviation. Significant differences between values expressed as function of mg of TSP are indicated as *, and between values expressed as gFW are indicated as α mean ($p < 0.05$)

high mortality rates, and, in the surviving explants, a high incidence of the physiological malformations associated with hyperhydricity (with excessive hydration, low lignification, and stomata malfunction) followed by leaf necrosis was observed. On the contrary, ½MS medium, provided significantly higher rates of established explants, lower mortality, and, also, the established explants showed no signs of hyperhydricity. Most of the studies that include hyperhydricity

**Table 5** Nuclear DNA content in leaves of *Tuberaria lignosa* mother plants and micropropagated plants

| | DNA content (pg DNA) | CV (%) |
|---|---|---|
| Mother plant | 3.82 ± 0.08 | 4.84 ± 0.10 |
| Micropropagated plant | 3.83 ± 0.06 | 5.58 ± 1.37 |

were related to the use of the MS culture medium, with a high content of ammonium nitrate (e.g., Cui et al. 2019), as observed in this study, supporting the preferable use of ½MS in certain species, like *T. lignosa*. The fact that this species is usually found in poor soils with few nutrients, supports its best development in ½MS.

After the optimization of the establishment phase, the best growth regulators combination was tested for the multiplication and the proliferation of axillary shoots, including the interaction of auxins and cytokinins. The best condition for both the 1st and 2nd cycles of multiplication was ½MS supplemented with 1 mg $L^{-1}$ mT. To our knowledge, this is the first time that mT is used with success in the micropropagation of Cistaceae species. BAP is by far one of the most widely used cytokinins in micropropagation and was reported to have success in the shoot multiplication of *Cistus clusii* (Ruta and Morone-fortunato 2013). mT is a recently

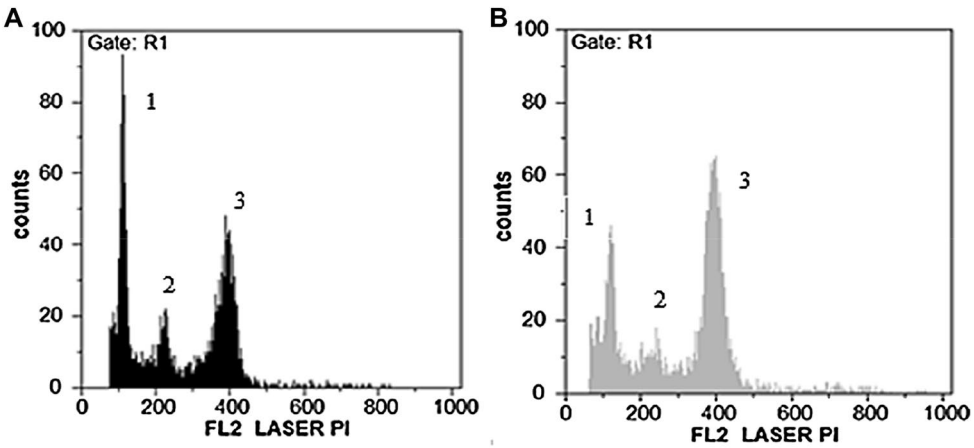


**Fig. 2** Histograms of relative fluorescence intensity (PI) obtained for *Tuberaria lignosa* nuclei, **A** mother plant **B** micropropagated plant. Peak 1—$G_0/G_1$ of *Raphanus sativus*; peak 2—$G_2$ of *Raphanus sativus*; peak 3—$G_0/G_1$ of *Tuberaria lignose*

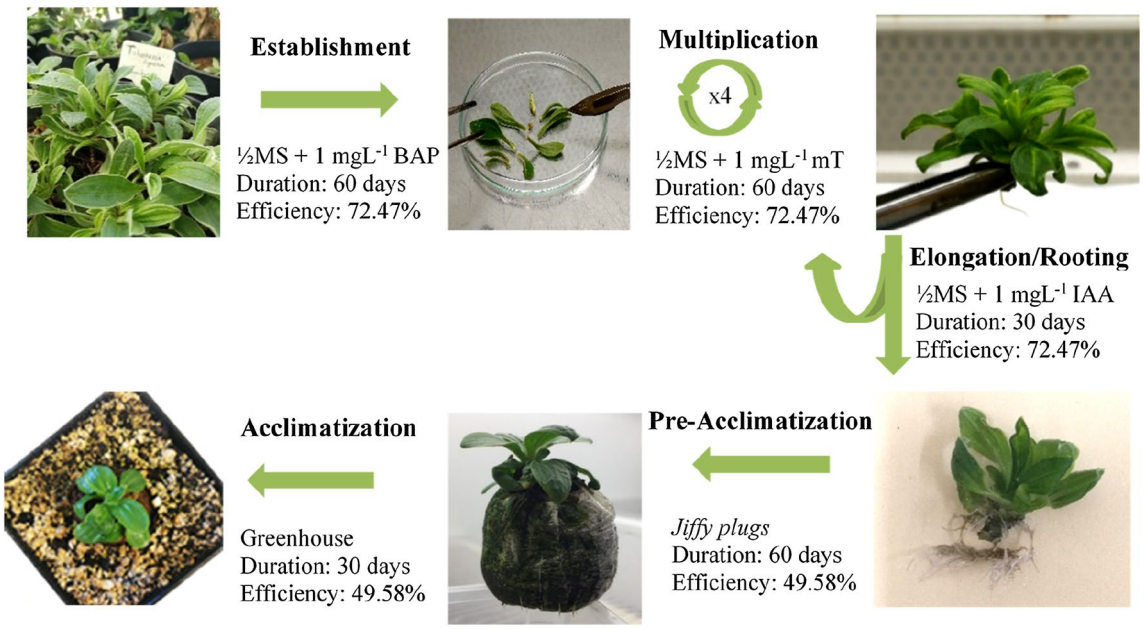


**Fig. 3** An efficient protocol for the micropropagation of *Tuberaria lignosa* with genetic stability. In this new protocol, the establishment have a success of 72.47%, multiplication has a success of 100% with a multiplication factor of 4×, elongation /rooting has a success of 100 with ~ 17 roots per plant and the process of acclimatization have a rate of 68.42% Following this new protocol, it will be possible, to obtain a total of efficiency of 49.58%. For example, with 250 explants it's possible to obtain a total of 495 new plants starting, after 8 months

used growth regulator that has already been used in several species in which, compared to other cytokinins including BAP, it has shown greater potential, inducing a higher number of shoots (Amoo and Staden 2013; Bairu et al. 2007). A study carried out in *Prunus* spp. revealed that mT positively influenced the growth and quality of the shoots, reducing the occurrence of hyperhydricity of the explants (Bairu et al. 2007), as we observed in our work. Concerning the qualitative parameters, plants resulting from consecutive multiplication cycles in the medium supplemented with BAP presented a lower vigour associated with higher hyperhydricity, when compared with those obtained in the culture medium supplemented with mT. Likewise, BAP compromised the quality of the shoots of *T. major* (Gonçalves et al. 2010) and other Cistaceae species (Lopez and González Valdés 2006), supporting the lower effects of this cytokinin in comparison with mT.

Before rooting, most species need a shoot elongation phase due to the continuous exposure of explants to cytokinins in the multiplication phase, they can remain at a reduced size. Once during the multiplication stage in the best condition, the shoots still showed a short length (~0.8 cm), it can impair the success of the protocol. Thus, different combinations of auxins and $GA_3$ were compared to promote shoot elongation. In general, the elongation was significantly increased by the addition of IAA, IBA, and $GA_3$. The culture medium was maintained ½MS, as at this stage the concentration of salts of the basal media like MS is often reduced to half to facilitate rooting (Gonçalves et al. 2010). This was observed in *T. major*, where ½MS provided better results than MS (Gonçalves et al. 2010), and in other Cistaceae species (Morte and Honrubia 1992). The shoots resulting from multiplication presented good rooting capacity, as we observed a high rooting frequency in all treatments (60–100%), the control being an exception. These results can be explained by the high endogenous levels of growth regulators, as observed in the rooting of *T. major* (Gonçalves et al. 2010). The medium supplemented with 1 mg $L^{-1}$ of IAA was the best treatment in which all plants developed, on average, 17.96 ± 0.76 roots. Ruta and Morone-Fortunato (2013) tested growth hormones such as IAA, IBA, and NAA in Cistaceae species, and obtained the best result with IAA (8.3 roots per plant). Using 0.5 mg $L^{-1}$ IBA on *T. major* rooting, Gonçalves and collaborators (2010) presented similar results to those of *T. lignosa* with ~6–7 roots formed per shoot. However, while best rooting results for *T. major* were found with 0.5 mg $L^{-1}$ NAA (9.13 ± 0.8 roots per shoot) (Gonçalves et al. 2010), in *T. lignosa* the best results, with much higher rates of vigorous roots, were achieved with 1 mg $L^{-1}$ IAA, being the best rates reported so far for the genus *Tuberaria*. Further analysis of the results, showed that with this protocol, the supplementation of the medium with 1 mg $L^{-1}$ of IAA is the best condition for the elongation and rooting stage, thus allowing to merge the two stages, reducing the time of micropropagation of this species.

The acclimatization process is by far the most stressing stage of the micropropagated plants, due to the need to reduce the nutritional supplementation, and at the same time stimulating photosynthesis (promoting autotrophism) and avoiding dehydration. This complex balance implies, for example, regulating cuticle formation and stomata function, while ensuring the adequate functionality of roots (often dysfunctional) or promoting the formation of new roots. All these complex events must occur simultaneously, while avoiding the infestation by opportunistic pests and pathogens. In *T. lignosa* acclimatization, the used commercial substrate is extremely rich in organic and mineral elements which was hypothesised to decrease the stress of micropropagated plants by decreasing nutritional sources. However, this substrate led to zero survival rates and the necrosis of all micropropagated plants in our work. *Tuberaria lignosa* has a preference for nutrient-poor media, as was initially observed in the in vitro establishment phase. Between the two other substrates we compared, mixing Jiffy and Jiffy plugs, it was observed that the latter, in addition to a higher percentage of survival rate, also showed a greater number of shoots. In this case, the optimized protocol for micropropagation *T. lignosa* includes Jiffy plugs during the acclimatization. This is the first report of its use in the genus *Tuberaria*, but it has already proved to be an excellent substrate in acclimatizing other plants, including *Anthurium* (Bahavana et al, 2018), *Corema album* (Alves et al. 2021) and chestnut (Cuenca et al. 2017).

The high survival rates presented by the plants after the transfer to greenhouse conditions support the use of this protocol for large scale production, by ensuring gradual acclimatization of the micropropagated plants to ex vitro conditions as occurred with *T. major* (Osorio et al. 2013). In particular, there was an evident antioxidant activation (e.g., phenols levels and peroxidases activity) during acclimatization, supporting that these may be interesting sources in the pharmaceutical industry. Stress induced during acclimatization is well characterized in other species like *Cassia alata* L. (Ahmed and Anis 2014), *Ulmus minor* Mill (Dias et al. 2011), and *Rauvolfia tetraphylla* L. (Faisal and Anis 2009). For example, during the acclimatization of micropropagated *T. major*, changes in the $H_2O_2$ levels were observed, but the levels of malondialdehyde content (a result of lipid peroxidation) and membrane integrity remained constant (Osorio et al. 2013), supporting the increase of ROS and associated antioxidant defense systems. In the current study, when expressed per g FW, only the peroxidases (significant for guaiacol peroxidase) increased during acclimatization, remaining the other enzymes close to the values of the mother plants. However, when expressed as mg TSP, all enzymes showed increased activity in acclimatized plants, which is explained by the lower amounts of the proteins. The

lower amounts of TSPs in the acclimatizing leaves may be, at least in part, due to lower amounts of ribulose bisphosphate carboxylase oxygenase, an abundant leaf protein. On other hand the significant increase in the activity of GPx (a representative of the Class III peroxidases) in these leaves indicates that these peroxidases are necessary at this stage of development/acclimatization. This class of peroxidases is involved in a number of physiological processes in plants such as auxin metabolism, cell wall modification (e.g. lignification, suberization), cross-linking of cell wall components, and synthesis of phytoalexins, besides fighting the raise of ROS levels. Triggering an antioxidant battery like the increase of phenols or antioxidant enzymes during acclimatization, might thus be relevant for industrial purposes.

Together with the establishment of an efficient micropropagation protocol and the subsequent acclimatization of the micropropagated plants, it is also important to evaluate the genetic fidelity of the micropropagated plants by comparing their nuclear DNA content with that of the mother plants. Ensuring genetic fidelity allows to certify that the protocol provides plants with similar characteristics to those identified in the field. Such genetic fidelity may be evaluated by multiple molecular tools (e.g. single nucleotide polymorphism, simple sequence repeats, among others). However, flow cytometry is a fast and highly efficient approach to assess ploidy fidelity (e.g., Brito et al. 2010; Nunes et al. 2018), as a major source of genetic abnormalities in vitro is the occurrence of aneuploidy or polyploidy. We have used FCM to ensure the DNA-ploidy stability of micropropagated plants in multiple species like *Pinus elliottii* hybrid (Nunes et al. 2018) or wild olive (Brito et al. 2010). Regarding *T. lignosa,* results showed high ploidy stability between micropropagated and field mother plants, ensuring that no major gross mutations occurred during the micropropagation process, thus supporting the use of this protocol. These results also confirm that plants regenerated from well-developed meristematic tissues have a low probability of ploidy variations.

## Conclusions

Overall, a pioneering step was taken in the in vitro culture of the medicinal species *T. lignosa* by having established a robust true to type micropropagation protocol with high rates of success between the different stages of explant establishment to plant acclimatization. In general, with this protocol, it was possible to obtain micropropagated plants after 5 months of the establishment of meristematic apexes. This process started with the establishment stage with a success rate of 72.47%, followed by a multiplication in which the best medium was ½MS with mT 1 mg $L^{-1}$ with a multiplication factor of 4, and by elongation/rooting in all plants and with, an average of 17 roots per plant in IAA 1 mg $L^{-1}$. Acclimatization lasted a total of 3 months and the substrate Jiffy plugs, was the best, presenting a success rate of 68.42% (Fig. 3). In short, following this new protocol, it will be possible, after 8 months, to obtain a total of 495 new plants starting from 250 explants.

The whole approach of this work, from the micropropagation methodology to the described acclimatization protocol, proved to be a promising method to introduce a reduction in the dependence on natural plants used for medicinal purposes, being at the same time an important resource towards the conservation of these species. Finally, these methods can be applied not only for other medicinal plants, but also for economically relevant species.

**Author contributions** DR performed in vitro cultures assisted by JD and some biochemical assays; NMP performed and supervised biochemical assays; SC and JL performed FCM assays; CD, CS and JCG designed and supervised the work and analyzed data. CS, DR and NMP elaborated the manuscript supported by all authors.

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**Data availability** Original data will be made available to those who require their access to authors.

## Declarations

**Conflict of interest** Authors declare no conflict of interest.

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