

# Micropropagation and molecular characterization of *Hypericum perforatum* L. subsp. *veronense* (Schrank) H. Lindb., a valuable medicinal plant with ornamental value

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## ABSTRACT

St. John's wort (*Hypericum perforatum*) is a perennial species with a worldwide interest due to its pharmaceutical characteristics which is also of ornamental value for gardening with native elements. Given that *in vitro* culture allows mass multiplication of selected genotypes, we investigated the micropropagation of the *ex situ* cultivated Greek native *H. perforatum* subsp. *veronense* using novel substances and its genetic fingerprinting. Apart from common auxins and cytokinins, a culture medium (BB) was used for the first time for its micropropagation and the effect of betaine, PVP, SNP and triacontanol were explored for the first time. Betaine addition of 200 mg/L produced proliferated shoots and roots without the need of plant growth regulators although a 0.5 mg L<sup>-1</sup> BA was the best treatment in proliferation experiments. Shoot number was highest in 0.5 BA + 0.1 IBA (mg L<sup>-1</sup>), in 1 µM sodium nitroprusside (SNP) + 1 mg L<sup>-1</sup> IAA + 200 mg L<sup>-1</sup> betaine and in PVP (0.5-1.5 mg L<sup>-1</sup>) + 1 mg L<sup>-1</sup> IBA + 200 mg L<sup>-1</sup>. Greater SNP concentration had either no differences or negative effect, while the greatest root number were recorded in Control. All three treatments of triacontanol (1-10 µg L<sup>-1</sup>) + 0.5 mg L<sup>-1</sup> BA + 200 mg L<sup>-1</sup> betaine resulted in higher shoot number. However, the statistically greatest root number were recorded in low concentration of SNP, or in absence of PVP and triacontanol. The micro-plants of *H. perforatum* subsp. *veronense* obtained from the SunCap™ sealed vessels and transplanted into the soil substrates presented 90.2% success; they were then transplanted to 1 L pots and were successfully established in the field for cultivation. The molecular characterization of *H. perforatum* subsp. *veronense* was achieved with DNA barcoding fingerprints deposited in the GenBank regarding four molecular markers of cpDNA (*petB/petD*, *rbcl*, *trnL/trnF* and *rpoC1*).

**Keywords:** St John's wort, *in vitro* clonal propagation, SNP, PVP, triacontanol, acclimatization, phylogeny, DNA Barcoding

## INTRODUCTION

St. John's wort (*Hypericum perforatum* L.) is a perennial species which is native in paleotemperate regions and introduced in other continents. Since ancient times, this

species has been used for its medicinal properties as a diuretic, emmenagogue, hemostatic and wound healing agent (Istikoglou *et al.*, 2010; Saddiqe *et al.*, 2010). Nowadays, it is on focus of research with particularly encouraging results in the treatment of depression and other mental and neurological disorders (Santarém and Astarita, 2003; Mohammed *et al.* 2019; Tavakoli *et al.* 2020; Isenberg, 1998). Due to its melatonin content, *H. perforatum* is used as an anticonvulsant and sleep quality enhancer (Murch and Saxena, 2006). Recently, St. John's wort was shown to have potential as a novel anticancer drug (Schempp *et al.*, 2002). It also contains glycosides, hypericin and pseudo-hypericin, flavonoids (16% in leaves), xanthenes, phenolic acids and essential oils (0.13% in whole plant) (Southwell and Bourke, 2001; Council of Europe, 2000, EMA 2022). The chemical compounds for which the plant has gained popularity as a medicinal agent are the naphthodianthrone (hypericin and pseudohypericin) and the acylphloroglucinol derivative (hyperforin and adhyperforin) (Fornasiero *et al.*, 1998; Kakouri *et al.*, 2023). *H. perforatum* is a traditional herbal medicinal product with the following indications approved by EMA: (i) relief of temporary mental exhaustion, (ii) symptomatic treatment of minor skin inflammations (such as sunburn) and as an aid in healing of minor wounds, (iii) symptomatic relief of mild gastrointestinal discomfort, and (iv) supportive treatment of nervous restlessness and associated with difficulties in falling asleep (EMA 2023).

The cultivation of St. John's wort can offer high-profit margins due to its well-known pharmaceutical qualities compared to other local crops due to valuable components used in cosmetology and pharmacology (Murch *et al.*, 2000a). As a wild-growing species of temperate regions, it is associated with broad ecological adaptation, natural resistance to pests and diseases. In addition, its desirable ornamental characteristics combined with low demands, inputs needed and reduced installation expenses when cultivated make it a promising species for landscaping or gardening with native plants as well as a valuable phytogenetic resource for breeding and genetic improvement purposes (Touchell *et al.*, 2008). To date, commercial St. John's wort production has been mainly based on field-grown plant material, though the quality of these products may be affected by different environmental conditions altering the concentration of medicinal metabolites (Murch *et al.*, 2000a). According to previous research, the concentration of hypericin and pseudohypericin in the leaves of St. John's wort may vary considerably between different seasons, while their accumulation is strongly affected by the presence of heavy metals (Prasad *et al.*, 1999). However, *in vitro* technologies may solve many cultivation and propagation problems in medicinal plants related to quality assurance by supplying plant material raised in a controlled environment.

*In vitro* culture techniques offer a viable system for rapid, mass multiplication of selected genotypes of valuable germplasm (Mallón *et al.*, 2010). In addition to its applications in basic propagation, tissue culture can be a useful tool in improving the medicinal features of *H. perforatum* such as hypericin and secondary metabolites concentration (Santarém and Astarita, 2003; Gadzovska *et al.*, 2005; Savio *et al.*, 2011). *In vitro* regeneration culture of *H. perforatum* is feasible from plant tissues like nodal segments (Ravindran *et al.*, 2022), flower stamens (Kirakosyan *et al.*, 2000; Goel *et al.*, 2009), hypocotyl tissue (Banerjee *et al.*, 2012; Murch *et al.*, 2000b) and leaves (Pretto and Santarém 2000; McCoy and Camper 2002), employing MS medium under the influence of various types and concentrations of cytokinin and auxin (Mikhovich *et al.* 2021; Ravindran *et al.*, 2022). More specifically, research on *H. frondosum* Minchx. shows that high levels of benzylamino purine (BA) facilitate optimum *in vitro* regeneration from leaves (Touchell *et al.*, 2008). BA is the most used cytokinin for *in vitro* regeneration (Mikhovich *et al.*, 2021; Ravindran *et al.*, 2022), while the reaction of explants to cytokinin depends on its concentration in the nutrient substrate as well as its absorption, metabolism, transport in the explant (Auer *et al.*, 1999),

and culture conditions (Feito *et al.*, 1994). Many studies have been published on the appropriate type and optimum concentration of cytokinin regarding different species (e.g., Hutchinson and Zimmerman, 1987). Several auxins have been also used for the regeneration of *Hypericum* spp., including indole-3-butyric acid (IBA) and  $\alpha$ -naphthaleneacetic acid (NAA), with the number of formed roots increasing proportionally with the concentration of administered auxin (Mikhovich *et al.*, 2021; Ravindran *et al.*, 2022; Moura, 1998). However, the selection of the appropriate auxin type and its concentration largely depends on species genotype (George, 1993), while the *in vitro* combination of auxins and cytokinins often results in blastogenesis. Recently, other substances such as betaine, sodium nitroprusside (SNP), polyvinylpyrrolidone (PVP) and trioctanol (TRIA) have been employed in the tissue culture of other medicinal-aromatic plants with impressive results (Akula *et al.*, 2000; Sarropoulou and Maloupa, 2019).

*Hypericum* spp. have been genetically studied both due to their phytochemical properties and the taxonomic implications involved. Several studies have examined and correlated the phytochemical profiles of various *Hypericum* spp. to their molecular profiles by AFLPs (Aziz *et al.*, 2007), RAPDs and SSRs (Smelcerovic *et al.*, 2006). Population genetics have also been studied broadly for members of this genus (Jayanthi *et al.*, 2004; Barcaccia *et al.*, 2006; Piperić *et al.*, 2008; Béjaoui *et al.*, 2010, 2011; Rezaei *et al.*, 2012; Morshedloo *et al.*, 2014; Rahnvard 2017; Houda *et al.*, 2021), whereas other studies adopted an ecological approach (Gaudel *et al.*, 2006; Dlugosch *et al.*, 2007; Molins *et al.*, 2014). Several phylogenetic studies have also been reported for members of genus *Hypericum* L. (Crockett *et al.*, 2004; Park *et al.*, 2004; Percifield *et al.*, 2007; Howard *et al.*, 2009; He *et al.*, 2013; Meseguer *et al.*, 2013, 2014, 2015; Nürk *et al.*, 2013, 2015; Robson *et al.*, 2016; Bi *et al.*, 2021; Cao *et al.*, 2021) indicating their significance. To our knowledge, *Hypericum* taxa from Greece have only been studied in the frame of a phytochemical-molecular profile correlation (Zeliou *et al.*, 2020), including phylogenetic analysis based on the *matK* and *trnH-psbA* plastid regions for five *Hypericum* taxa and the discrimination of different Greek *H. perforatum* subsp. *perforatum* populations (Pyrka *et al.*, 2021). No further study has ever been reported assessing the phylogenetic relationship of *H. perforatum* subsp. *veronense* from Greece, using a combination of molecular markers to indicate the position of this subspecies in phylogenetic terms.

In this context, the present investigation aimed to explore for the first time new substances and novel media with possible promoting action in the micropropagation of *H. perforatum*. In terms of germplasm selection, we focused our research on a well-documented Greek native genotype of *Hypericum perforatum* L. subsp. *veronense* (Schrank) H.Lindb. originating directly from wild-growing populations of Lefkada Island (Ionian Islands, Greece). This study also aimed to provide sequence data for four molecular regions regarding *H. perforatum* subsp. *veronense* (i.e., *petB/petD*, *rbcL*, *trnL/trnF* and *rpoC1*), defining the identity and allowing the phylogenetic characterization of the taxon. *Hypericum perforatum* as well as other specific *Hypericum* genotypes are commonly harvested across Greece directly from the wild to prepare the traditional reddish ointment as infused in extra virgin olive oil. Furthermore, the increasing local, regional, and global demand for natural plant products directly harvested from wild-growing populations combined with the ongoing habitat degradation, man-induced land reclamation and over-grazing may limit the natural range and/or the reproductive potential of many widely used medicinal-aromatic plants such as *H. perforatum* subsp. *veronense* (Grigoriadou *et al.*, 2020). Therefore, the *ex situ* conservation of different genotypes of *H. perforatum* subsp. *veronense* should also be enabled with the aim to facilitate the sustainable utilization of such valuable germplasm.

## MATERIALS AND METHODS

### Plant material

The initial wild-growing plant material of *H. perforatum* subsp. *veronense* (Hypericaceae) was originally collected on 26/10/2006 at St. Maura Castle of Lefkada Island (Ionian Islands, Greece) located at sea level (N38° 50' 70", E20° 43' 14"). This material was then transferred to the nursery of the Balkan Botanic Garden of Kroussia, Institute of Plant Breeding and Genetic Resources (IPBGR), Hellenic Agricultural Organization Demeter, where it was taxonomically identified and was assigned to the IPEN (International Plant Exchange Network) accession number GR-1-BBGK-06,3755. The ex situ cultivated mother plants of *H. perforatum* were maintained in 3 L pots inside a non-heated greenhouse from October to March 2016 and plant nutrition was applied on a monthly basis using a water-soluble fertilizer 20-20-20 (N-P-K) + micronutrients (Poly-Feed® GG; Haifa Chemicals Ltd, Haifa, Israel). To obtain clean explant material for the establishment of tissue cultures, the stock mother plants of *H. perforatum* subsp. *veronense* were treated periodically with the following fungicide formulations: 0.6 g L<sup>-1</sup> Octave® 46WP (BASF, Ludwigshafen, Germany), 1.5 g L<sup>-1</sup> Morgan 83WP (Ellagret S.A., Mandra, Greece), and 1 g L<sup>-1</sup> Switch 25/37.5WG (Syngenta, Basel, Switzerland). The plants produced fast growing shoot tips during spring and were maintained in the greenhouse until the completion of the plant tissue culture experiments.

### *In vitro* establishment and culture conditions

Shoot tips taken from *H. perforatum* subsp. *veronense* potted plants were initially surface disinfected using 70% (v/v) ethanol (Disolol 97%; Chem-Lab, Zedelgem, Belgium) for 3 to 4 min and then with 2% (v/v) sodium hypochlorite solution [12 to 13% (v/v) NaOCl; Chem-Lab] with 160 µL of Tween® 20 per 100 mL solution shaken on a rotary shaker for 25 min, and were rinsed three times with sterile distilled water.

All shoot tips were transferred in 30 x 200 mm test tubes containing 10 mL of MS (Murashige and Skoog 1962) medium supplemented with 25 g L<sup>-1</sup> sucrose (Duchefa, Haarlem, The Netherlands) and solidified with the medium was free of plant growth regulators. The pH was adjusted to 5.8 using 1N HCl or 1N KOH (Chem-Lab) prior the addition of 3 g L<sup>-1</sup> Phytagel™ (Sigma-Aldrich®, Saint Louis, MO) and autoclaved at 121 °C for 20 min. Cultures were maintained at 22 ± 2°C and 16-h photoperiod under cool white, fluorescent light (40 µmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux) (TL-D 58W/54-765, Philips, Carquefou, France).

The explants that proliferated clusters of shoots were dissected into single shoots and were transferred into Magenta® B-cap-sealed, 62.4 mm × 95.8 mm, 200 mL glass (baby food jars) (Sigma-Aldrich®), containing 30 mL of the above prepared medium. The procedure was repeated every four weeks, until adequate plant material yielded for further experimentation. In addition, the BB basal culture medium (Tsoktouridis *et al.*, 2019) was tested for experimentation.

In the preliminary experiments, 2- to 3-weeks-old *in vitro*-formed proliferated shoot tips were used as initial explants. They were cultured on a BB medium supplemented with Betaine (0 - 50 - 100 - 200 - 400 mg L<sup>-1</sup>). The experiment for shoot proliferation was based on a completely randomized design and consisted of five treatments with three replications per treatment, averaging over three explants (3 baby food jars x 3 explants per jar). The explants were transferred into baby food jars, each containing 35 mL of BB culture medium. Afterwards, shoot tips were cultured on a BB medium supplemented with 0.5 mg L<sup>-1</sup> of 6-benzyladenine (BA) combined with 200 mg L<sup>-1</sup> Betaine.

In the main experiment, the BB basal medium was used, supplemented with 200 mg L<sup>-1</sup> Betaine, 25 g L<sup>-1</sup> sucrose and five different combinations of BA and auxins (0 - 0.5 BA - 0.5 BA+0.1 IAA - 0.5 BA+0.1 NAA - 0.5 BA+0.1 IBA mg L<sup>-1</sup>). Shoot tips (meristem tips) and in some cases shoot internodes (both with four leaves) were used as explants. The

experiment was based on a completely randomized design and consisted of five treatments with six replications per treatment, averaging over three explants (6 baby food jars x 3 explants per jar). The results were evaluated eight weeks after the beginning of the experiment, and measurements concerning the number of shoots, shoot length, and shoot fresh weight per explant and treatment were performed.

*In vitro* maintained shoot tips namely meristems with four leaves were used as explants for three individual experiments using separately SNP, polyvinylpyrrolidone (PVP), and triacontanol. The explants were cultured on BB basal medium supplemented with 30 g L<sup>-1</sup> sucrose, and 3 g L<sup>-1</sup> Phytagel™ and: (a) 0, 1, 2, 5, 10, 20, 50, 100 or 200 μM of SNP plus 1 mg L<sup>-1</sup> IAA in the first experiment; (b) 0, 0.5, 1 or 2 mg L<sup>-1</sup> PVP plus 1 mg L<sup>-1</sup> IBA in the second one ; and (c) 0, 1, 5 or 10 μg L<sup>-1</sup> Triacontanol plus 0.5 mg L<sup>-1</sup> BA in the third one . The experiments were based on a completely randomized design and consisted of nine (1<sup>st</sup>), four (2<sup>nd</sup>) and four (3<sup>rd</sup>) treatments with five replications per treatment, averaging over four explants (5 baby food jars x 4 explants per jar). The number of roots and root length produced from the base of micro-plants per explant and treatment and the number and length of proliferated shoots were measured after eight weeks.

### **Acclimatization and plant establishment *ex vitro***

The *in vitro*-rooted plants were transferred to baby food jars containing hormone-free BB medium and each vessel was supplemented with 10 mL of BB liquid nutrient solution. The Magenta® B-cap was replaced by a transparent sterile membrane over which a sterilized special SunCap™ filter (Sigma-Aldrich®) was applied for sealing with a 5 cm diameter x 6 mm width rubber band. The cultures were maintained at 22 ± 2°C and 16-h photoperiod under cool white, fluorescent light (40 μmol m<sup>-2</sup> s<sup>-1</sup>) for two weeks before acclimatization in greenhouse conditions. The same procedure was followed for the *in vitro* acclimatization with the filter paper (Whatman® qualitative filter paper, Grade 1, Sigma-Aldrich®) used to replace the SunCap™ filters.

The *in vitro*-rooted acclimatized micro-plants in vessels with (a) SunCap™ filters; (b) filter papers; and (c) control material (*in vitro*-rooted micro-plants in baby food jars only capped with Magenta® B-cap) were all carefully rinsed with tap water to remove the adhering medium, and then they were transferred into 30 cm x 50 cm plastic seedling trays (TRAY 50 X 30CM 60 HOLE INSERT, Alegre grow shops, Athens, Greece) filled with a substrate mixture consisted of peat moss (Klasmann, TS2), vermiculite and perlite (1:1:1 v/v). The seedling trays were then placed in a nylon tabletop tunnel with adjustable relative humidity (RH), ranging from 75-82% RH in the first week to 55-62% RH during the second week. After two weeks, the trays of *H. perforatum* subsp. *veronense* were transferred onto a greenhouse bench (25 ± 4°C temperature, 50 ± 5% RH) for two more weeks, where they were irrigated by sprinkling. The plants were transplanted into 1 L pots containing the same substrate and were transferred outdoors the completion of the acclimatization of the plants. The number and percentage (%) of successfully acclimatized plants were recorded two months later.

After the successful mass propagation of approximately 600 acclimatized and well-rooted *H. perforatum* subsp. *veronense* plants, the new plants were transferred to five different fields for private commercial cultivation at geographically distant areas in different altitudes in Greece, i.e., in Sifnos island at 300 m above sea level (asl), in Syros island at 100 m (asl), in Amorgos island (asl), in Kefalonia island at 50 m (asl), and in Kipourgio of Grevena at 830 m (asl).

### **Statistical analysis**

Data resulting from the two experiments of shoot proliferation and rooting of *H. perforatum* subsp. *veronense* were analyzed according to the Completely Randomized Design (CRD) with the analysis of variance (ANOVA) method. Separation of means was accomplished with the Tukey or Dunnett's T3 multiple comparisons procedure at

significance level  $\alpha = 0.05$  ( $P \leq 0.05$ ). All statistical analyses were performed with the SPSS® v.20.0 statistical software (IBM, Armonk, NY).

### Molecular analyses

Fresh young leaves of *H. perforatum* subsp. *veronense* GR-1-BBGK-06,3755 were used for the molecular analyses of this study. DNA extraction, amplification and DNA sequence analysis were performed according to Tsoktouridis *et al.* (2019). The oligonucleotide primers (InVitrogen Inc., Paisley, Scotland, UK) used for PCR amplification targeted the *petB/petD*, *rbcL*, *trnL/trnF* and *rpoC1* regions of cpDNA. DNA sequences of the above-mentioned molecular markers for other *Hypericum* taxa were retrieved from GenBank and aligned for each molecular marker using the Mega11 software (Tamura *et al.*, 2021). A unified phylogenetic tree for all the molecular markers was constructed using the Neighbor-Joining statistical method and Maximum Composite Likelihood substitution model. All new-generated sequences were submitted to Genbank.

## RESULTS

### Disinfection and establishment of *in vitro* cultures of *H. perforatum* subsp. *veronense*

Due to the sensitivity of *H. perforatum* subsp. *veronense* plant tissue to the high concentrations of NaOCl, the most appropriate concentration of NaOCl for the establishment of *in vitro* cultures from explant material was 2% (v/v). Fungicides were applied for one month in the stock mother plants within the greenhouse to reduce the amounts of fungal and microbial contaminants. The plants were also followed a standard fertilization scheme in order to produce microbe-free multiple fast-growing apical shoots. This procedure was very successful in obtaining many shoots used as initial explant material for the *in vitro* establishment. The disinfected apical shoots were placed in MS medium without any growth regulators for three weeks to ensure sterility, until the initiation of the next experiments on shoot proliferation (Figure 1a).

### Shoot proliferation of *H. perforatum* subsp. *veronense*

*In vitro*-maintained shoots of *H. perforatum* subsp. *veronense* were used for a pre-experiment, employing the BB basal medium supplemented with Betaine (0, 50, 100, 200 or 400 mg L<sup>-1</sup>). The results of this pre-experiment showed that the addition of 200 mg/L betaine in the BB basal medium produced an average of 30.8 shoots and 8 roots per explant without the need of plant growth regulators (Table 1). The addition of betaine resulted also a positive effect in the general appearance of the proliferated shoots (more vigorous, with larger leaves and better color, Figure 1b). For this reason, 200 mg L<sup>-1</sup> betaine was added in all of the following experiments. An additional experiment was set up to investigate the best BA concentration for shoot proliferation of *H. perforatum* subsp. *veronense*.

A shoot proliferation pre-experiment of *H. perforatum* subsp. *veronense* shoot tips (1.5-2 cm long) was also took place in a BB basal medium supplemented with BA at 0, 0.5, 1, 1.5, 2 mg L<sup>-1</sup> and 200 mg L<sup>-1</sup> betaine. The results of this pre-experiment showed shoot and callus proliferation for all treatments with BA; however, the control produced the longest shoots (5 cm) and roots (2.4 cm). The increased concentration of BA, especially in 1.5 and 2 mg L<sup>-1</sup> treatments, caused pale-green leaf-color in the proliferated shoots, leaf, and meristem necrosis and therefore, the 0.5 mg L<sup>-1</sup> BA treatment was used in the next proliferation experiments (Table 2). After BA proliferation experiment, *H. perforatum* subsp. *veronense* shoots were placed in a BB regulator-free medium for four weeks (Figure 1c).

The combination of BA with auxins caused significant differences in *H. perforatum* subsp. *veronense* proliferation. Control resulted the lowest shoot number (2.4) but the highest shoot length (5 and 2.3 cm). In addition, root formation (root number, root length) was recorded only for the Control (100% of the explants), while callus formation was observed

when treated with BA. The highest shoot number (6.3) was recorded in 0.5 BA+0.1 IBA (mg L<sup>-1</sup>) treatment but no statistical differences resulted in fresh weight (Table 3, Figure 1d). The combination of SNP and IAA in the BB culture medium supplemented with 200 mg L<sup>-1</sup> betaine was significantly affected the number of *H. perforatum* subsp. *veronense* proliferated shoots. The highest shoot number (4.4) was recorded with 1 µM SNP. However, in the other treatments, shoot number, root number and length of the proliferated *H. perforatum* subsp. *veronense* explants were either not statistically significant or presented negative effect; in the 100 µM SNP treatment, small pale-green color leaves were produced, and in the 200 µM SNP treatment, necrosis of leaves and explants were recorded. In 200 µM SNP treatment, the lowest shoot length, root number and root length were observed (Table 4, Figure 2).

The combination of PVP and IBA in the BB culture medium supplemented with 200 mg L<sup>-1</sup> betaine was significantly affected the number of *H. perforatum* subsp. *veronense* proliferated shoots in all three treatments compared to the control. However, no statistical differences among these three treatments was resulted. On the contrary, the statistically longest shoot length, root number and length of the proliferated explants were recorded for the Control (Table 5).

The combination of triacontanol and BA in BB culture medium supplemented with 200 mg L<sup>-1</sup> betaine, was used as rooting agent for *H. perforatum* subsp. *veronense* explants. However, the statistically significant highest shoot length and root number of the proliferated explants were recorded in control. The three different concentrations of triacontanol in combination with IBA resulted in statistically higher shoot number compared to the control, but no significant differences were detected among them. The formed roots were short, and for the treatment of 5 µg L<sup>-1</sup> triacontanol, the roots were shorter than the control (Table 6, Figure 3).

### Acclimatization of *H. perforatum* micro-plants

All vigorously rooted microplants were acclimatized first *in vitro* (Figure 4a) and then *ex vitro* (Figure 4b) under greenhouse conditions. The *H. perforatum* subsp. *veronense* *in vitro* plants (SunCap™ sealed vessels) were transplanted in a substrate inside a non-heated greenhouse and presented a 90.2% success. The micro-plants obtained from the filter paper sealed vessels presented low survival rate (29.8%), and thus this method was not recommended for further use. The micro-plants obtained from vessels using Magenta® B-cap and transplanted into the same substrate presented 83.2% survival rate. During the third hardening phase (outdoors), all plants that were transplanted to 1 L pots had a 100% success (Figure 4c). Mass propagation was succeeded during the next year and the plants of *H. perforatum* subsp. *veronense* were successfully established in the field for cultivation at five different geographically remote areas with altitude ranging from sea level to 830 m asl.

**Table 1.** Effect of betaine in growth regulator-free BB medium on *Hypericum perforatum* subsp. *veronense* shoot proliferation. The number, length and fresh weight of proliferated shoots and roots were counted for every treatment after eight weeks of culture.

| Betaine treatment (mg L <sup>-1</sup> ) | Shoot      |                 |                       | Root       |                 |                       |
|-----------------------------------------|------------|-----------------|-----------------------|------------|-----------------|-----------------------|
|                                         | Number (1) | Length (cm) (1) | Fresh weight (mg) (1) | Number (2) | Length (cm) (2) | Fresh weight (mg) (1) |
| 0                                       | 4.0 a      | 6.2 ab          | 144 a                 | 6.1 a      | 1.7 a           | 48 a                  |
| 50                                      | 6.4 a      | 5.5 ab          | 110 a                 | 7.3 ab     | 2.8 b           | 67 ab                 |
| 100                                     | 16.2 b     | 6.8 b           | 147 a                 | 8.3 b      | 2.4 b           | 69 ab                 |
| 200                                     | 30.8 c     | 5.4 ab          | 169 a                 | 8.0 b      | 2.8 b           | 128 b                 |
| 400                                     | 20.5 b     | 5.3 a           | 152 a                 | 7.2 ab     | 2.6 b           | 112 b                 |

\*Mean values in the same column followed by different letter(s) are statistically different at significance level  $p < 0.05$  according to the results of the Tukey (1) or Dunnett's T3 (2) multiple range test. Every treatment was carried out with 6 jars and 3 explants per jar

**Table 2.** Effect of BA (0, 0.5, 1, 1.5 or 2 mg L<sup>-1</sup>) and 200 mg L<sup>-1</sup> betaine in BB medium on *Hypericum perforatum* subsp. *veronense* shoot proliferation. The callus formation, number and length of proliferated shoots and roots were counted for every treatment after eight weeks of culture.

| BA treatment (mg L <sup>-1</sup> ) | Shoot  |             | Root   |             | Callus diameter (cm) |
|------------------------------------|--------|-------------|--------|-------------|----------------------|
|                                    | Number | Length (cm) | Number | Length (cm) |                      |
| 0                                  | 1.6 a  | 5.0 b       | 7.3    | 2.4         | -                    |
| 0.5                                | 3.5 b  | 1.7 a       | -      | -           | 0.4 a                |
| 1.0                                | 4.0 b  | 1.5 a       | -      | -           | 0.4 a                |
| 1.5                                | 3.3 b  | 1.4 a       | -      | -           | 0.5 a                |
| 2.0                                | 3.5 b  | 1.4 a       | -      | -           | 0.5 a                |

\* Mean values in the same column followed by different letter(s) are statistically different at significant level  $p < 0.05$ , according to the results of Dunnett's T3 multiple range test. Every treatment was carried out with 6 jars and 3 explants per jar.

**Table 3.** Effect of 0.1 mg L<sup>-1</sup> IAA, NAA or IBA and 0.5 mg L<sup>-1</sup> BA in BB medium supplemented with 200 mg L<sup>-1</sup> Betaine on *Hypericum perforatum* shoot proliferation. The callus formation, number and length of proliferated shoots and roots, and explant fresh weight were counted for every treatment after eight weeks of culture.

| Treatment (mg L <sup>-1</sup> ) | Shoot number (1) | Shoot length (cm) (1) | Root number | Root length (cm) (2) | Callus diameter (cm) (2) | Explant fresh weight (mg) (1) |
|---------------------------------|------------------|-----------------------|-------------|----------------------|--------------------------|-------------------------------|
| 0                               | 2.4 a            | 6.5 c                 | 5.0         | 2.3                  | -                        | 99 a                          |
| 0.5BA                           | 5.7 b            | 1.2 b                 | -           | -                    | 0.5 a                    | 73 a                          |
| 0.5BA+0.1IAA                    | 6.2 b            | 0.8 a                 | -           | -                    | 0.7 a                    | 153 a                         |
| 0.5BA+0.1NAA                    | 6.3 b            | 1.2 b                 | -           | -                    | 0.6 a                    | 135 a                         |
| 0.5BA+0.1IBA                    | 11.8 c           | 0.7 a                 | -           | -                    | 0.4 a                    | 143 a                         |

\*Mean values in the same column of each plant followed by different letter(s) are statistically different at significant level  $p < 0.05$  according to the results of the Tukey (1) or Dunnett's T3 (2) multiple range test. Every treatment was carried out with 6 jars and 3 explants per jar.

**Table 4.** Effect of SNP and 1 mg L<sup>-1</sup> IAA in BB medium supplemented with 200 mg L<sup>-1</sup> betaine on *Hypericum perforatum* subsp. *veronense* shoot proliferation. Number and length of proliferated shoots and roots were counted for every treatment after eight weeks of culture.

| SNP (μM) | Shoot number (1) | Shoot length (cm) (1) | Root number (1) | Root length (cm) (2) |
|----------|------------------|-----------------------|-----------------|----------------------|
| 0        | 1.4 ab           | 6.5 b                 | 5.0 c           | 2.3 e                |
| 1        | 4.4 c            | 3.5 a                 | 4.1 c           | 1.9 de               |
| 2        | 2.8 abc          | 5.0 ab                | 3.7 bc          | 1.3 bcde             |
| 5        | 1.3 ab           | 5.1 ab                | 1.4 ab          | 0.9 abc              |
| 10       | 2.2 abc          | 5.4 ab                | 2.6 abc         | 1.2 bcd              |
| 20       | 1.0 ab           | 6.4 b                 | 3.2bc           | 1.1 bcd              |
| 50       | 3.0 bc           | 4.0 ab                | 4.8 c           | 2.1 cde              |
| 100      | 1.0 ab           | 4.6 ab                | 0.5 a           | 1.9 ab               |
| 200      | 0.6 a            | 2.8 a                 | 0.0 a           | 0.0 a                |

\*Mean values in the same column followed by different letter(s) are statistically different at significant level  $p < 0.05$  according to the results of the Tukey (1) or Dunnett's T3 (2) multiple range test. Every treatment was carried out with 6 jars and 3 explants per jar.

**Table 5.** Effect of PVP and 1 mg L<sup>-1</sup> IBA in BB medium supplemented with 200 mg L<sup>-1</sup> betaine on *Hypericum perforatum* subsp. *veronense* shoot proliferation. Number and length of proliferated shoots and roots were counted for every treatment after eight weeks of culture.

| PVP treatments (mg L <sup>-1</sup> ) | Shoot number (1) | Shoot length (cm) (2) | Root number (1) | Root length (cm) (2) |
|--------------------------------------|------------------|-----------------------|-----------------|----------------------|
| 0                                    | 1.2 a            | 6.2 b                 | 6.1 b           | 1.7 b                |
| 0.5                                  | 2.5 b            | 3.2 a                 | 1.6 a           | 1.3 a                |
| 1.0                                  | 2.2 b            | 2.9 a                 | 2.6 a           | 1.1 a                |
| 1.5                                  | 2.3 b            | 2.4 a                 | 1.9 a           | 0.7 a                |

\*Mean values in the same column followed by different letter(s) are statistically different at significant level  $p < 0.05$  according to the results of the Tukey (1) or Dunnett's T3 (2) multiple range test. Every treatment was carried out with 6 jars and 3 explants per jar.

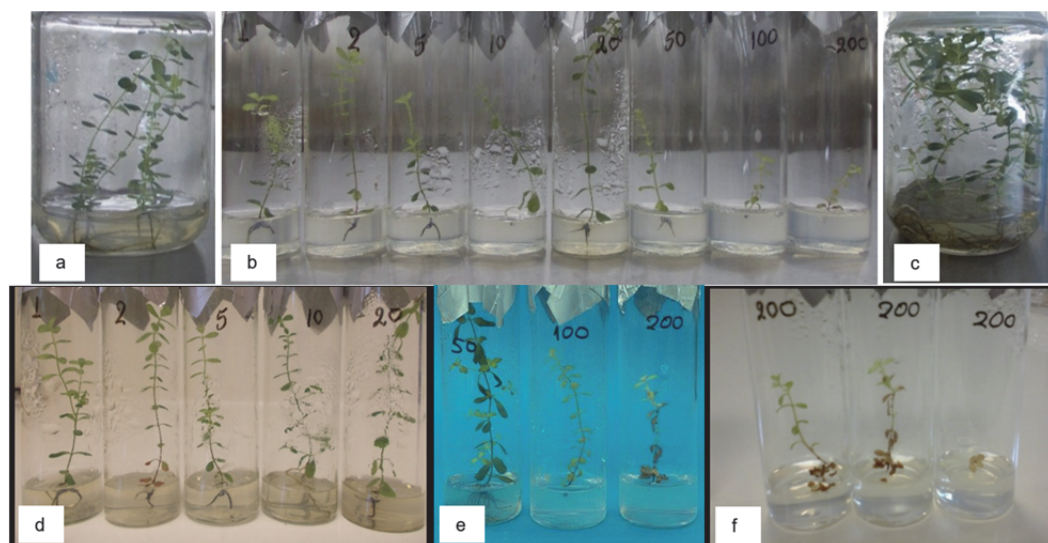
**Table 6.** Effect of triacontanol and 0.5 mg L<sup>-1</sup> BA in BB medium supplemented with 200 mg L<sup>-1</sup> Betaine on *Hypericum perforatum* subsp. *veronense* shoot proliferation. Number and length of proliferated shoots and roots were counted for every treatment after eight weeks of culture.

| Triacontanol Treatments (μg L <sup>-1</sup> ) | Shoot number (1) | Shoot length (cm) (2) | Root number (2) | Root length (cm) (1) |
|-----------------------------------------------|------------------|-----------------------|-----------------|----------------------|
| 0                                             | 1.1 a            | 6.2 b                 | 6.1 c           | 1.7 b                |
| 1                                             | 1.9 b            | 3.3 a                 | 1.9 ab          | 1.8 ab               |
| 5                                             | 2.1 b            | 2.5 a                 | 1.0 a           | 0.9 a                |
| 10                                            | 2.6 b            | 3.2 a                 | 2.7 b           | 1.5 b                |

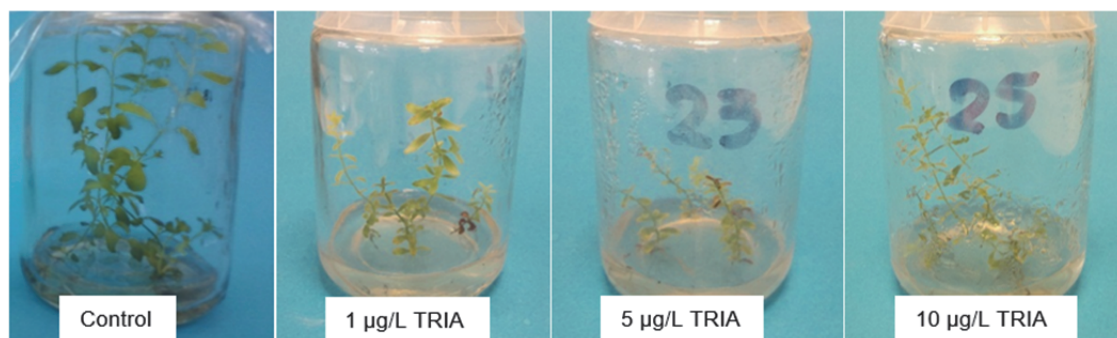
\*Mean values in the same column followed by different letter(s) are statistically different at significant level  $p < 0.05$  according to the results of the Tukey (1) or Dunnett's T3 (2) multiple range test. Every treatment was carried out with 6 jars and 3 explants per jar.



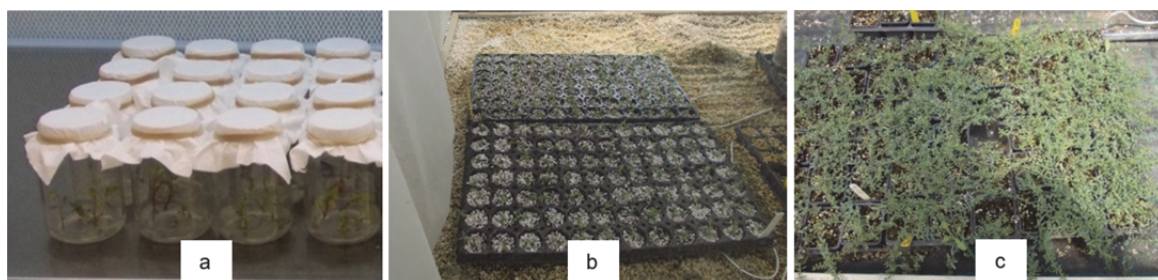
**Figure 1.** (a) Sterilized *Hypericum perforatum* subsp. *veronense* apical shoot explants in MS medium without any growth regulators; (b) Effect of betaine in growth regulator-free BB medium on *H. perforatum* shoot proliferation (from left to the right: 0-50-100-200-400 mg L<sup>-1</sup>) (see Table 1); (c) Proliferated shoots in BB growth regulator-free medium after four weeks; (d) Effect of auxins and BA in BB medium supplemented with 200 mg L<sup>-1</sup> betaine on *H. perforatum* subsp. *veronense* shoot proliferation at the end of the experiment (from left to the right: Control, 0.5 BA, 0.5 BA+0.1 IAA, 0.5 BA+0.1 NAA, 0.5 BA+0.1 IBA mg L<sup>-1</sup>). All growth regulators' treatments were statistically significant compared to the control (see Table 3).



**Figure 2.** Effect of SNP and 1 mg L<sup>-1</sup> IAA in BB medium supplemented with 200 mg L<sup>-1</sup> betaine on *Hypericum perforatum* subsp. *veronense* shoot proliferation (see Table 4); **(a)** Control after four weeks; **(b)** Different concentrations (from left to right: 1-2-5-10-20-50-100-200 μM) of SNP after four weeks; **(c)** Control after eight weeks; **(d)** Different concentrations (from left to right: 1-2-5-10-20 μM) of SNP after eight weeks; **(e)** Different concentrations (from left to right: 50-100-200 μM) of SNP after eight weeks; **(f)** Toxic effect of 200 μM SNP in explants after eight weeks.



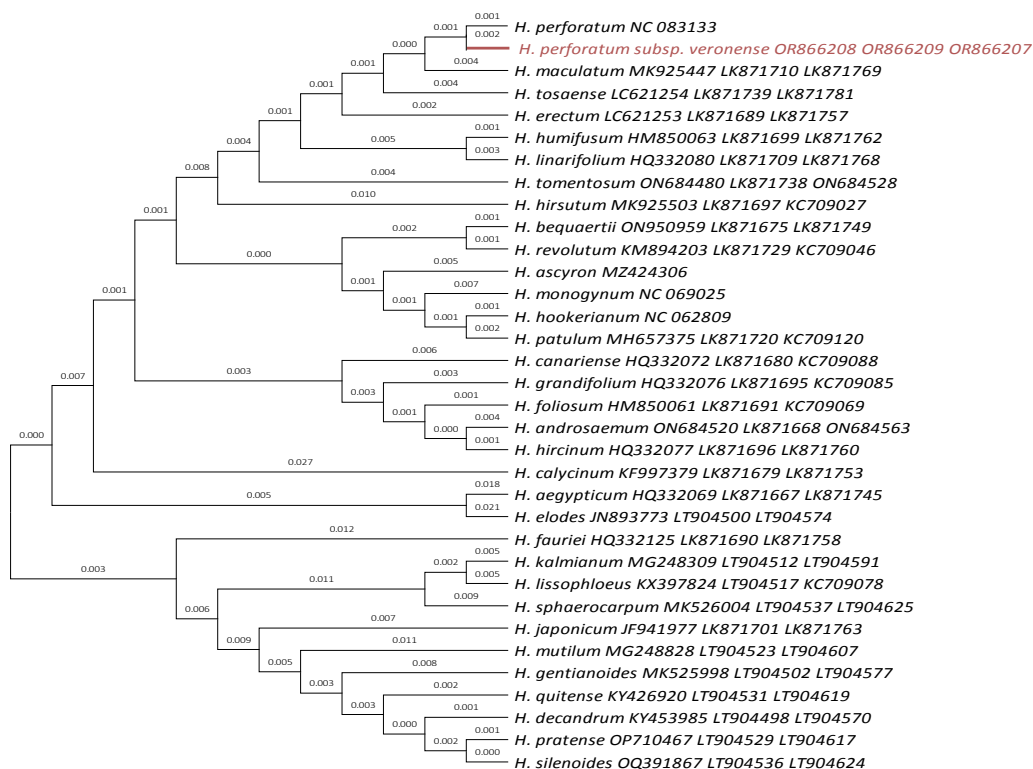
**Figure 3.** Effect of triacontanol (TRIA) concentrations and 0.5 mg L<sup>-1</sup> BA in BB medium supplemented with 200 mg L<sup>-1</sup> betaine on *Hypericum perforatum* subsp. *veronense* shoot explants at the end of the experiment (see Table 6).



**Figure 4.** Acclimatization of *Hypericum perforatum* subsp. *veronense* micro-plants **(a)** *in vitro* and **(b)** in the greenhouse three days after transplanting, and acclimatized plants in 1 L pots **(c)** two months after transplanting in a soil substrate with peat moss, vermiculite, and soil (1:1:1 v/v).

# Molecular analyses

The sequences generated in this study for the molecular markers *trnL/trnF* (461 bp), *rbcl* (604 bp), *petB/petD* (1108 bp) and *rpoC1* (514 bp) were deposited in the Genbank obtaining the accession numbers OR866207 - OR866210. A unified phylogenetic tree for the above molecular markers was constructed indicating the phylogenetic relationships of *H. perforatum* subsp. *veronense* with other species of the genus *Hypericum* (Figure 5), which represented 33 *Hypericum* taxa retrieved from the GenBank. Due to inadequate information on GenBank for *rpoC1*, this molecular marker was excluded from the phylogenetic analysis. Using nBLAST, each sequence produced in this study was aligned to identify the species with the highest sequence similarity to *H. perforatum* subsp. *veronense* GR-1-BBGK-06,3755. First of all, it is worth mentioning the complete chloroplast genome of *Hypericum perforatum* (NC083133), a specimen of the same species from China, that was compared to the Greek specimen from Lefkada, Greece studied herein. The total length of the new DNA sequences deposited to the GenBank for the latter was 2687 bp, thus developing a reference genetic dataset for *H. perforatum* subsp. *veronense*. The *petB/petD* molecular marker exhibited a 99.78% similarity (899 out of 901 bp identical nucleotides) with *H. perforatum* subsp. *veronense* (LK871724) and the *rbcl* region was similar (99.83%; 602/603 bp identities) to *H. perforatum* subsp. *perforatum* (ON684488). Finally, the sequence generated for the *trnL* region in this study matched with the Japanese endemic *H. tosaense* Makino and *H. perforatum* subsp. *veronense* by 99.08% (539/544 bp identities), whereas the *rpoC1* sequence matched to *H. perforatum* from China (NC\_083133, JX664944, GQ436208, FJ395794) by 97.86%, with 502 identical nucleotides out of 513 (material probably referring to cultivated subsp. *perforatum*).



**Figure 5.** Phylogenetic tree illustrating the relationships among *Hypericum* species based on the molecular plastid markers *petB/petD*, *rbcl* and *trnL/trnF*. The tree was constructed using the Neighbor-Joining method and Maximum Composite Likelihood substitution model. Accession numbers for sequences obtained in this study from Genbank are indicated for each molecular marker, respectively, next to taxon names. The clade marked in red represents *H. perforatum* subsp. *veronense* GR-1-BBGK-06,3755 studied herein.

## DISCUSSION

Previous multiplication protocols for *H. perforatum* have mainly employed the MS medium and the use of common plant growth regulators (Mikhovich *et al.*, 2021; Ravindran *et al.*, 2022). However, compounds like betaine (Saini *et al.*, 2012), triacontanol (Tantos *et al.*, 2001), sodium nitroprusside (Ötvös *et al.*, 2005) and polyvinylpyrrolidone (Krishna *et al.*, 2008) or modified culture media (Tsoktouridis *et al.*, 2019; Tzatzani *et al.*, 2023) have never been employed in plant tissue culture of *H. perforatum* to date and, therefore, they are reported for the first time in the micropropagation of *H. perforatum* subsp. *veronense* as referred to herein. The glycine betaine (GB) is a vital compatible osmolyte that has received considerable attention due to its multiple roles in plant growth, metabolism and abiotic stress resistance. Various studies have reported enhanced GB accumulation in plants under heat stress, salinity, UV radiation and heavy metals (Ashraf and Foolad, 2007; Annunziata *et al.*, 2019; Alhaithloul *et al.*, 2020). The effectiveness and benefits of BG in plant tissue culture has been not widely studied, despite of the positive results on plant growth and robustness (Chen and Xu, 2015). For the micropropagation of *H. perforatum* subsp. *veronense*, the addition of 200 mg/L betaine to the BB culture basal medium produced proliferating shoots and roots without the need for plant growth regulators and improved the general appearance of the proliferated shoots (more vigorous, with larger leaves of darker color). The BB culture media also proved to be effective (explants at satisfactory levels) as a standard basal medium.

When cultures are exposed to excessive BA, the shoots fail to elongate or to root (Shiji and Siril, 2018). In accordance with the latter, our findings indicated that 1.5 and 2 mg L<sup>-1</sup> BA caused pale-green leaf-color in the proliferated shoots, and leaf and meristem necrosis, while rooting was only observed in BA-free culture medium. Among the existing auxins, indolebutyric acid (IBA) and naphthaleneacetic acid (NAA) are widely used in many different plant species (Carimi and Pasquale, 2003). However, the *in vitro* combination of auxins with cytokinins provides results of shoot induction (Kwiecien *et al.*, 2023; Tzatzani *et al.*, 2023).

In most plant species, the application of auxins achieves the formation of adventitious roots and the number of roots in every shoot increases proportionally with the concentration of the administered auxin as an optimal value (George, 1993). In accordance with the latter, we observed root induction when 1 mg L<sup>-1</sup> IAA was only added in the culture medium (control treatment).

According to Ötvös *et al.* (2005), SNP in combination with auxins may activate cell division and formation of embryogenic cells in *Medicago sativa* L, showing that in the absence of exogenous auxins and with a concentration of 10 µM SNP, cell division remains unaffected. Conversely, when combined with 0.22 or 1 µM 2,4-D, SNP at the same concentration remarkably increases the frequency of cell division. Recent research has evaluated the impact of SNP (10 µM) on shoot multiplication and plant regeneration in *Vanilla planifolia* Andrews (Tan *et al.*, 2013). Other studies have illustrated that nitric oxide from SNP contributes to root formation in *Cucumis sativus* L. using a concentration of 10 µM and in *Solanum lycopersicum* L. with an optimal concentration of 200 µM (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2006). Moreover, findings from studies on the micropropagation of members of the genus *Agave* suggest that SNP enhances the multiplication rate and overall plant size by exploring nitric oxide donors like SNP, for the development of innovative plant micropropagation protocols aimed to commercial applications (Rico-Lemus and Rodríguez-Garay, 2014). In our pilot investigation in *H. perforatum* subsp. *veronense*, low concentrations of SNP (1 µM) notably increased shoot numbers by nearly three-fold.

Conversely, higher concentrations exhibited adverse effects, leading to pale-green leaves (100  $\mu\text{M}$ ) and explant necrosis (200  $\mu\text{M}$ ).

PVP was also used for the first time in the proliferation of members of the genus *Hypericum*. The outcomes of previous studies in other plant species have underscored that PVP may possess capabilities beyond mere antioxidant action (inhibitory effects on phenolic compound accumulation) leading to broader development of *in vitro* cultures of various species, and thus suggesting potential implications for organogenesis and microcallus formation (Reustle and Natter, 1994). Our results highlighted the positive impact of PVP on shoot numbers of *H. perforatum* subsp. *veronense*, thus indicating its suitability for shoot propagation, albeit not in terms of enhancing shoot elongation or inducing root formation.

Previous findings were collectively highlighted TRIA's influence on physiological growth parameters of several plant species, signifying its efficacy as an influential growth regulator in *in vitro* cultivation settings (Obul *et al.*, 2002; Verma *et al.*, 2011).

Presently, no literature is available to document TRIA's application in *H. perforatum*. Thus, this study marks the pivotal inclusion of TRIA into species-specific cultivation substrates inducing rhizogenesis. Our experiment demonstrated that TRIA supplemented with BA improved shoot induction but adversely impacted shoot elongation. Although rooting was observed across all treatments, TRIA concentrations ranging from 1 to 10  $\mu\text{g L}^{-1}$  compressed root induction, thus indicating its unsuitability for promoting rooting in *H. perforatum* as effective in shoot production.

DNA barcoding has become a powerful tool to identify and validate medicinal plants globally. Therefore, DNA sequences serve as valuable DNA-based tools in developing species-specific identification techniques of commercialized plant material. *Hypericum perforatum* products in the market showed that only 68% of the samples were authentic (Raclariu *et al.*, 2017). Therefore, there was a need to develop authentication DNA techniques to protect the genuine *H. perforatum* herbal products targeting the ITS region (Howard *et al.*, 2019), the ITS1 and *matK* loci (Costa *et al.*, 2019), *matK* and *trnH-psbA* as applied to Greek specimens (Pyrka *et al.*, 2021), *rbcL* and ITS2 regions using next generation sequencing (Ivanova *et al.*, 2016); in parallel, Raclariu *et al.* (2017) used the DNA barcoding tools to authenticate 16 herbal products from 79 species of *Hypericum perforatum*. Six complete chloroplast genomes of *Hypericum* are currently available in GenBank for *H. perforatum*, *H. ascyron* L., *H. hookerianum* Wight & Arn., *H. breviflorum* Wall. ex Dyer, *H. uralum* Buch.-Ham. ex D. Don, and *H. monogynum* L. showing distinct DNA sequence differences among these six *Hypericum* species (Liu *et al.*, 2023). In the frame of this study, four new sequences (i.e., *petB/petD*, *rbcL*, *trnL/trnF* and *rpoC1*) were generated and deposited in GenBank for *H. perforatum* subsp. *veronense* GR-1-BBGK-06,3755, obtaining unique accession numbers and creating a genetic fingerprinting for *H. perforatum* subsp. *veronense*. Two of them (i.e., *rbcL*; OR866208 and *rpoC1*; OR866210) are reported for the first time, whereas sequence data for *petB/petD* and *trnL/trnF* are also provided by Nürk *et al.* (2015).

The phylogenetic analysis confirmed the identity of the specimen and indicated its phylogenetic position compared to other *Hypericum* taxa. As it was anticipated, *H. perforatum* subsp. *veronense* GR-1-BBGK-06,3755 showed high similarity to *H. perforatum*, *H. perforatum* subsp. *veronense* and *H. perforatum* subsp. *perforatum*, yet being distinguishable from *H. perforatum*, as also mentioned in Zeliou *et al.* (2020) for the ITS nuclear molecular marker.

The current study provides important information for a wild-growing St. John's wort subspecies in Greece, offering the chance to create genetic databases for local plants of medicinal interest. Although there is a lot of DNA sequence information in the GenBank, any DNA study that focuses on new DNA datasets of species and subspecies of *Hypericum* is still valuable, due to both the multifaceted value of these phylogenetic resources and the significance of *Hypericum* products in the global industry and market.

## CONCLUSION

The establishment of *in vitro* cultures of *Hypericum perforatum* required meticulous disinfection procedures using an optimal concentration of NaOCl, supplemented by fungicide applications and fertilizer treatments for mother plants. The success in obtaining sterilized apical shoots provided a robust starting point for subsequent experiments. Shoot proliferation experiments revealed the significant role of betaine in promoting shoot and root development, while BA concentrations influenced shoot quality, with lower concentrations showing better outcomes. The combination of SNP and IAA significantly affected shoot proliferation, albeit certain concentrations resulted in adverse effects, highlighting the importance of dosage control. Conversely, while the combination of PVP and IBA affected shoot proliferation, the control treatment exhibited superior shoot and root characteristics. Additionally, the use of triacontanol in combination with IBA for rooting displayed varying effects, with some concentrations promoting shoot numbers but exhibiting shorter roots compared to the control. Successful acclimatization of micro-plants was achieved under greenhouse conditions, paving the way for further mass propagation and successful cultivation across diverse geographical areas of Greece. DNA analysis of *Hypericum perforatum* subsp. *veronense* GR-1-BBGK-06,3755 provides distinct genetic documentation for this *Hypericum* subspecies, thus allowing identification and authentication of plant materials in the market.

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