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High Frequency Direct Organogenesis, Genetic Homogeneity, Chemical Characterization and Leaf Ultra-Structural Study of Regenerants in *Diplocyclos palmatus* (L.) C. Jeffrey

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Abstract: *Diplocyclos palmatus* (L.) C. Jeffrey, commonly referred to as "Shivalingi" or "Lollipop climber" is a valuable medicinal plant with a climbing growth habit used in traditional medicine. It is reputed to have antiarthritic, anti-diabetic properties and to be useful in various skin and reproductive problems. Overexploitation of wild plants and low seed germination have resulted in the decline of the species in the wild. Thus, the present investigation was aimed to establish an effective in vitro propagation procedure for its large-scale production and conservation. Nodal explants, obtained from an established mother plant were grown on MS basal medium augmented with various cytokinins, alone or in combination with auxins, to study the morphogenic response. A maximum of 8.3 shoots/explants with an average shoot length of 7.2 cm were produced after six weeks on MS containing benzylaminopurine 5.0 µM + 1-naphthaleneacetic acid 2.0 µM. After 4 weeks of transfer, microshoots rooted well on a low nutrient medium of $\frac{1}{2}$ MS + 1.0 µM indole-3-butyric acid, with a maximum of 11.0 roots/microshoot and an average root length of 7.4 cm. With an 80% survival rate, the regenerated plantlets were effectively acclimatized to natural conditions. DNA-based molecular markers were used to investigate the genetic uniformity. Scanning Electron Microscopic examination of leaves indicated the adaptation of the plantlets to natural, as evidenced by the formation of normal stomata. Gas chromatography-mass spectrometry analyses of mother and micropropagated plants were performed to identify essential secondary metabolites. The results obtained show that the in vitro propagation system can be adopted for preservation, large-scale production and secondary metabolites' production in *D. palmatus*.

Keywords: micropropagation; genetic fidelity; scanning electron microscopy; gas chromatography-mass spectrometry

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1. Introduction

Diplocyclos palmatus (L.) C. Jeffrey belonging to the family Cucurbitaceae, is an annual climber [1–3] and reported to synthesize compounds with medicinal properties [4,5]. Commonly, it is known as Shivalingi or Lollipop climber and is distributed throughout India. *Diplocyclos* is a small genus of four species [6] and in India it is represented by *D. palmatus* which grows on bushes, trees and hedges [7]. In traditional medicine, the whole plant has been used to treat several diseases, such as fever [8], asthma [9], inflammations [10,11], and various skin conditions [12–14]. The fruits of this plant are mostly used in reproductive

medicine, especially to cure female infertility, leucorrhoea, and as an aphrodisiac and tonic too [15]. It is also used to enhance ovulation, as well as to improve sperm count by different tribal communities of the Umarkhed region of Maharashtra and the Wayanad region of Kerala [16,17]. The whole plant in the form of juice is also taken for the treatment of cough [18,19], while the leaf paste is used to treat joint discomfort and rheumatism. [20]. Ethanolic and methanolic extracts of seeds were reported to possess anti-arthritic and anti-diabetic activity, respectively [21,22]. The main active constituents of the plants are bryonin [23,24], punicic acid [25], non-ionic glucomannan [26] and goniothalamin [27].

Due to its medicinal use, the overexploitation of the wild fruits has threatened the plant population. The conventional way of propagating *D. palmatus* through seeds is ineffective due to its low viability and germination rate [28]. Advanced in vitro techniques in biotechnology have enabled us to multiply, preserve and propagate several rare/endangered plant species of medicinal value [29]. The micropropagation of medicinal plants through in vitro techniques has a tremendous potential for producing superior quality planting materials, isolating useful variants in well-adapted high yielding genotypes with enhanced secondary metabolites of therapeutic potential, and has a number of advantages over traditional methods of propagation such as seed, cutting, grafting, and air-layering, etc. Vijayashalini, et al. [30], as well as Rethinam and Jeyachandran [28], were the first to report their findings on in vitro propagation of *D. palmatus*. However, mediocre results were reported in terms of shoots and root production. Considering this background, it is imperative to develop better methods for high frequency in vitro clonal multiplication of *D. palmatus*. Analysis of morphological, molecular, and biochemical attributes in micropropagated plants assure the long-term stability of the acclimatization procedure. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR), both of which are DNA-based molecular markers, are highly repeatable marking systems that have been widely used to preserve the genetic integrity of in vitro-propagated plants [31–37]. The present investigation was carried out to develop a micropropagation protocol with improved results in terms of shoot formation, rooting and acclimatization. Moreover, an ultramicroscopic study of the leaf of both mother and in vitro-propagated plants was also conducted. The study is also coupled with both genetic and chemical profiling of the plant, which, to our knowledge, have not been published before.

2. Materials and Methods

2.1. Collection of Explants and Culture Establishment

Nodal segment (NS) explants were excised from an eight-week-old seed-derived mother plant established at the botany department of Aligarh Muslim University, Aligarh, India and used to study the direct shoot regeneration efficiency. Before transfer to solid media, the NS were thoroughly rinsed in tap water over half an hour, followed by a 20-minute treatment with a 1% (*w*/*v*) Bavistin solution (carbendazim powder, BASF India Ltd., Mumbai, India). Following treatment with the Bavistin solution, the NS were washed for about 15 min with 5% (*v*/*v*) Teepol—a mild liquid detergent—before being surface sterilized for 3 min with 0.1% (*w*/*v*) mercuric chloride ($HgCl_2$, Qualigens, Worli, Mumbai, Maharashtra, India,) solution freshly prepared in sterile water. To remove the traces of $HgCl_2$, NS were rinsed in autoclaved double distilled water under a laminar flow hood for 4–5 times. After sterilization, single NS explants were transferred in glass vials containing 20–25 mL semisolid MS basal medium [38] supplemented with different cytokinins (benzylaminopurin-BA, kinetin-Kn, and thidiazuron-TDZ) at varying doses (0.5, 2.5, 5.0, 7.5, and 10.0 µM), or without cytokinins as a control for induction of multiple shoots. The optimal concentration of cytokinin was examined in combination with auxins including indole-3-butyric acid-IBA, indoleacetic acid-IAA, and 1-naphthaleneacetic acid-NAA in the concentration range of 1.0 to 3.0 µM for continued growth and proliferation.

All tests were carried out in the MS basal medium, which included 3% (*w*/*v*) sucrose (Qualigens Fine Chemicals, Mumbai, India) and 0.8% (*w*/*v*) agar (Bacteriological grade, Hi media, Mumbai, India). All the cultures were set up in 25 × 150 mm glass tubes (Borosil, Mumbai, India) and in 100 cm^3 Erlenmeyer flasks (Borosil, Mumbai, India). The medium's pH was then adjusted to 5.8 with 1N HCl and 1 N NaOH before being autoclaved at 121 °C and 15 psi for 15 min. The cultures were grown under typical conditions, which included a temperature of 25 ± 2 °C, a relative humidity of 55%, and a photoperiod of 16/8 h with a PPFD (Photosynthetic photon flux density) of 50 µmol m^{-2} s^{-1} provided by cool fluorescent lamps (40 W, Philips, Kolkata, India).

2.2. Root Induction and Acclimation

For in vitro root induction, regenerated healthy microshoots were separated from cultures and moved to the rooting media composed of a half-strength MS medium supplemented with various auxins, namely IAA, IBA or NAA (0.5, 1.0, 1.5 and 2.0 µM) solidified with 0.25% phytagel. The plantlets were gently cleaned with tap water after being removed from the culture vessels. Following that, they were planted in thermocol containers containing three different planting materials: garden soil + manure (3:1), vermicompost, and sterilized soilrite. The plantlets were completely covered with clear polybags and transferred into a growth room at 25 ± 2 °C under 16 h photoperiod with 40–50 µmol m^{-2} s^{-1} irradiance provided by white LED tubes (Wipro High Lumen 2 × 22-Watt). The polybags were gradually removed after 2 weeks to minimize shock caused by variations in humidity, followed by significant exposure to fluorescent light in the growth room, and then remained in the greenhouse for another 2 weeks under natural light with day/night ventilation temperature setpoints of 25/22 °C. Finally, hardened plants were transplanted into garden soil-filled pots and placed in the outdoors, where they were exposed to the natural environment.

2.3. Genetic Analysis

To assess genetic integrity, nine acclimatized plants were chosen at random, along with the mother plant, for molecular analysis. Genomic DNA was extracted from fresh leaf tissues of *D. palmatus* using the cetyltrimethyl ammonium bromide (CTAB) method, as described by Doyle and Doyle [39]. On a UV-vis spectrophotometer, the extracted DNA was checked for purity (A260/280 ratio). On a thermocycler, PCR analysis was performed using a set of ten random amplified polymorphic DNA-RAPD (OPL Kit; Operon Technologies Inc., Alameda, CA, USA) and ten inter simple sequence repeat-ISSR (UBC; Vancouver, BC, Canada) primers (Biometra, T Gradient, Thermoblock, Germany). The preparation of reaction mixtures, setting of PCR amplification and separation of amplicons were carried out as described by Ahmad et al. [40].

2.4. Scanning Electron Microscope (SEM)

Leaf samples from in vitro conditions (prior to transplanting) and 4-week-old acclimatized plants were collected for SEM examination. Following a fifteen-minute gradual dehydration with an increasing alcohol series (30, 50, 70, 90, and 100%), the leaves were fixed in 2% (*v*/*v*) glutaraldehyde (Merck, Merck Specialities Pvt. Ltd., Mumbai, India) and left at room temperature for two hours. Further, the fixed tissues were subsequently dried to critical point, and the dorsal surface of the leaves was coated with gold particles. The analysis was made by mounting the samples over aluminium stubs with the help of double-sided 3M scotch tape and examining them under SEM (JSM-6510, JEOL Ltd., Tokyo, Japan) which was operated at 15 kV. The pictures of the leaf surface were all digitally processed.

2.5. Gas Chromatography and Mass Spectrometry (GC-MS)

For the GC-MS analysis, fully expanded healthy leaves were harvested from a 8-week-old ex vitro acclimated plant as well as the mother plant, which was both growing in the same growth environment at the time of sampling. The harvested leaves were cleaned

and air dried for 2–3 days before being crushed with mortar and pestle to produce a fine powder. An amount of 1 g of powder was diluted in 50 mL of methanol (70% *v*/*v*) and left for 24 h for the complete extraction of phytochemicals. The methanolic extract of leaves was centrifuged at 5000 rpm for 5 min before being filtered through a syringe filter (0.22 µm) to remove residues. Finally, with the addition of solvent, a total volume of 10 mL of extract was obtained, which was then utilized for phytochemical's profiling. One µL of extract was manually injected into an RTX-5 column of GC-MS (QP-2010, Shimadzu Corporation, Kyoto, Japan) running at 1000 eV ionization energy, with helium as carrier gas, and 173 kPa as the inlet pressure. The phytochemicals were identified using the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) databases and online Wiley Library (John Wiley & Sons, Inc., New York, NY, USA) for mass spectra. In the test sample, the name of the phytochemicals and their molecular weight (MW) together with their structure were determined.

2.6. Data Analysis

Data for shoots per explant and shoot length were collected after six weeks of culture to the assess regeneration percentage, whereas rooting data were collected after four weeks of culture. In three repeated experiments, one explant was used per replication, with a total of 20 replicates for each treatment and the results were analyzed using a one-way ANOVA in SPSS Version 16 (IBM-SPSS, Chicago, IL, USA) to determine which treatment was the most effective. The significance of difference between means was determined using Duncan's multiple range test (DMRT) at $p = 0.05$, and the findings were presented as mean ± SE. A Sigma Plot v. 10.0 (SYSTAT Software, Inc., San Jose, CA, USA) was used to present the data graphically.

3. Results and Discussion

3.1. Shoot Induction and Plant Regeneration

An efficient in vitro propagation procedure was developed for *D. palmatus*, a medicinally important plant species. The NS harvested from an established plant of *D. palmatus* were used for shoot regeneration throughout the experiment (Figure 1a). Plant growth regulators (PGRs) of various kinds and concentrations have a substantial impact on shoot and root development, hence, the process of micropropagation. In this study, NS were transplanted in MS nutrient media without PGRs (control) or with PGRs (supplementation). Three types of cytokinins viz. BA, Kn, and TDZ were used at different concentrations, namely 0.5, 2.5, 5.0, 7.5 and 10.0 µM. The NS on the control medium did not show any response and, therefore, it was clear that the media needed the addition of PGRs. Among the three cytokinins used, the optimal and most appropriate response of shoot initiation and regeneration was recorded on BA-supplemented media. Initially, the explants swelled within one week of inoculation and then green protuberances emerged which subsequently developed directly into shoot buds. Among all the BA treatments, 5.0 µM BA was found as the best concentration. On this medium, a mean of 6.6 shoots per NS with an averaged length 2.4 cm of shoot was recorded in 95.0% of the cultures after six weeks of growth (Figure 1b, Table 1). The response was significantly affected by reducing or increasing the concentration of BA: on the media supplemented with a lower concentration (0.5 µM), the shoots number decreased to 1.6 shoots/explant and with a higher concentration (10.0 µM) it was 2.3 shoots/explant (Table 1) which clearly showed how the growth of regenerated shoots was more prone to increase or decrease with BA concentrations beyond the optimal one (5 µM). The BA, a first-generation synthetic cytokinin, was shown to be efficient in bud breaking; hence, it aids in the production of numerous shoots due to its improved permeability across the plasma membrane and high cell absorption [41].

Figure 1. Regeneration of *D. palmatus* (**a**) NS cultured on MS + 5.0 µM BA (Bar = 0.43 cm); (**b**) Multiple shoot initiation and regeneration on MS + 5.0 µM BA (Bar = 0.40 cm); (**c**) Proliferation of shoots on MS + 5.0 µM BA + 2.0 µM NAA (Bar = 1.03 cm); (**d**) Rooting on $\frac{1}{2}$ MS + 1.0 µM IBA (Bar = 1.25 cm); (**e**) Exposed view of a micropropagated plant (Bar = 1.64 cm); (**f**) The regenerated plantlet hardened in Soilrite (Bar = 1.67 cm); (**g**) Successfully acclimatized plantlet in garden soil (Bar = 6.41 cm).

Table 1. Effect of various cytokinins on regeneration of *D. palmatus* after six weeks.

Cytokinins (µM)			Explant Response (%)	Number of Shoots per Explant	Shoot Length (cm)
BA	Kn	TDZ			
0.0	0.0	0.0	00.00 ± 0.00 ^{k}	0.00 ± 0.00 ^{h}	0.00 ± 0.00 ^{h}
0.5	-	-	33.33 ± 1.67 ^{i}	1.67 ± 0.33 ^{fg}	0.50 ± 0.10 ^{fg}
2.5	-	-	68.33 ± 1.67 ^{cd}	4.33 ± 0.67 ^{bcd}	1.23 ± 0.07 ^{de}
5.0	-	-	95.00 ± 2.89 ^{a}	6.67 ± 0.33 ^{a}	2.40 ± 0.12 ^{a}
7.5	-	-	73.33 ± 1.67 ^{c}	5.33 ± 0.67 ^{ab}	1.43 ± 0.18 ^{cd}
10.0	-	-	43.33 ± 3.33 ^{h}	2.33 ± 0.33 ^{efg}	0.73 ± 0.03 ^{f}
-	0.5	-	23.33 ± 1.67 ^{j}	1.33 ± 0.33 ^{g}	0.53 ± 0.03 ^{fg}
-	2.5	-	56.67 ± 3.33 ^{ef}	3.00 ± 0.00 ^{def}	1.17 ± 0.07 ^{de}
-	5.0	-	81.67 ± 1.67 ^{b}	4.67 ± 0.88 ^{bc}	1.80 ± 0.06 ^{b}
-	7.5	-	73.33 ± 1.67 ^{c}	3.67 ± 0.33 ^{cde}	1.37 ± 0.07 ^{d}
-	10.0	-	46.67 ± 1.67 ^{gh}	1.67 ± 0.33 ^{fg}	0.50 ± 0.58 ^{fg}
-	-	0.5	21.67 ± 1.67 ^{j}	2.33 ± 0.33 ^{efg}	0.43 ± 0.33 ^{g}
-	-	2.5	55.00 ± 2.89 ^{f}	3.33 ± 0.67 ^{cde}	1.07 ± 0.33 ^{e}
-	-	5.0	73.33 ± 3.33 ^{c}	4.33 ± 0.33 ^{bcd}	1.67 ± 0.12 ^{bc}
-	-	7.5	63.33 ± 3.33 ^{de}	2.67 ± 0.33 ^{efg}	1.33 ± 0.09 ^{d}
-	-	10.0	51.67 ± 1.67 ^{fg}	1.33 ± 0.33 ^{g}	0.47 ± 0.03 ^{fg}

The data indicates the Mean ± SE of three repeated experiments with a total of 20 replicates. Using Duncan's multiple range test, values denoted by the same letter within a column are not statistically different at $p = 0.05$.

The treatment of Kn and TDZ could not improve the regeneration efficiency in comparison to BA, as only 4.6 shoots/NS in 81.6% of the cultures and 4.3 shoots/NS in 73.3% of the cultures were produced on MS + 5.0 µM Kn and MS + 5.0 µM TDZ, respectively. Higher doses of all the PGRs examined in this study were shown to induce callus at the basal end, resulting in a low number of shoot induction. The advantageous effect of BA on direct shoot buds differentiation has also been reported in other medicinal plants, such as *Trichosanthus dioica* [42], *Decalepis arayalpathra* [43], and *Rauwolfia serpentina* [44].

To improve the regeneration efficiency of the NS, combination treatments of cytokinin and auxin were evaluated. The optimal level of 5.0 µM—BA was used with different auxins, namely IAA, IBA and NAA at different doses viz. 1.0, 2.0 and 3.0 µM (Table 2). A 5.0 µM BA + 2.0 µM NAA was determined to be the most efficient cytokinin-auxin combination tested, producing a maximum of 8.3 shoots per NS with a maximum shoot length of 7.2 cm in 86.6% of the cultures after six weeks of growth (Figure 1c). The combination of cytokinin and auxin was shown to be more efficient in the control of apical dominance and morphogenesis [45]. The presence of endogenous PGRs, PGRs present in the growth media and their interaction might be another reason for the successful regeneration of explants [46]. A similar synergism was also observed in other medicinal plants, such as *Salacia chinensis* [47] and *Decalepis salicifolia* [40]. While BA and NAA were found to be the most effective combination in our study, the other two auxins, IAA and IBA, in combination with cytokinin also showed enhanced shoot growth, but were less efficient in comparison to NAA, with only 4.3 and 5.6 shoots/NS being overserved after 6 weeks in 55.0% and 61.6% of the cultures, respectively. (Table 2). Several medicinal plant species, including *Withania somnifera* [48], *Artemisia abrotanum* [49], *Daphne mezereum* [50] and *Rauvolfia tetraphylla* [51] exhibited a significant synergistic effect on overall shoot multiplication and growth when BA and NAA were used in combination. An increase in the number and length of *Tecoma stans* shoots was recently observed by [52] by adding NAA at concentrations between 0.1–2.0 µM with an optimum concentration of BA.

Table 2. Effect of auxin with BA (5 µM) on shoot multiplication of *D. palmatus* after six weeks.

Plant Growth Regulators (µM)				Explant Response (%)	Number of Shoots per Explant	Shoot length (cm)
BA	IAA	IBA	NAA			
5.0	0.0	0.0	0.0	95.00 ± 2.89 ^{a}	6.67 ± 0.33 ^{a}	2.40 ± 0.12 ^{a}
5.0	1.0	-	-	13.33 ± 3.33 ^{h}	1.67 ± 0.67 ^{e}	3.47 ± 0.18 ^{g}
5.0	2.0	-	-	55.00 ± 2.89 ^{de}	4.33 ± 1.20 ^{cd}	5.20 ± 0.06 ^{d}
5.0	3.0	-	-	46.67 ± 3.33 ^{f}	2.67 ± 0.88 ^{de}	4.30 ± 0.15 ^{f}
5.0	-	1.0	-	16.67 ± 3.33 ^{h}	2.33 ± 0.88 ^{de}	3.73 ± 0.12 ^{g}
5.0	-	2.0	-	61.67 ± 1.67 ^{d}	5.67 ± 0.33 ^{bc}	5.80 ± 0.05 ^{c}
5.0	-	3.0	-	51.67 ± 1.67 ^{ef}	3.33 ± 0.33 ^{de}	4.50 ± 0.10 ^{ef}
5.0	-	-	1.0	35.00 ± 2.89 ^{g}	3.67 ± 0.89 ^{cde}	4.77 ± 0.03 ^{e}
5.0	-	-	2.0	86.67 ± 1.67 ^{b}	8.33 ± 0.33 ^{a}	7.20 ± 0.11 ^{a}
5.0	-	-	3.0	78.33 ± 1.67 ^{c}	6.67 ± 0.31 ^{ab}	6.53 ± 0.20 ^{b}

The data indicates the Mean ± SE of three repeated experiments with a total of 20 replicates. Using Duncan's multiple range test, values denoted by the same letter within a column are not statistically different at $p = 0.05$.

3.2. Rooting and Acclimatization

Adventitious rooting can be achieved by transferring the elongated microshoots on the rooting medium. The highest rooting was found on the half-strength MS medium supplemented with 1.0 µM IBA with the induction of 11.0 roots per microshoot with a mean root length of 7.4 cm in 91.6% cultures after four weeks (Table 3, Figure 1d,e). The roots produced on IBA were healthier, thicker, and more branched, whereas IAA and NAA-supplemented produced short, brittle, fibrous roots with less branching. Similarly, Shibli et al. [53] also used IBA for *Artemisia* rooting.

Table 3. Effect of various auxins on root induction in *D. palmatus* on phytagel solidified half-strength MS medium after four weeks.

Auxins (μM)			Explant Response (%)	Number of Roots per Shoot	Root Length (cm)
IAA	IBA	NAA			
0.0	0.0	0.0	00.00 ± 0.00 ^{i}	0.00 ± 0.00 ^{i}	0.00 ± 0.00 ^{h}
0.5	-	-	35.00 ± 2.89 ^{fg}	2.33 ± 0.33 ^{h}	4.03 ± 0.14 ^{ef}
1.0	-	-	53.33 ± 1.67 ^{d}	4.67 ± 0.67 ^{defg}	6.03 ± 0.17 ^{c}
1.5	-	-	36.67 ± 1.67 ^{efg}	3.33 ± 0.88 ^{fgh}	5.27 ± 0.17 ^{d}
2.0	-	-	23.33 ± 3.33 ^{h}	1.67 ± 0.33 ^{h}	2.47 ± 0.20 ^{g}
-	0.5	-	51.67 ± 1.67 ^{d}	8.33 ± 0.67 ^{b}	5.87 ± 0.18 ^{c}
-	1.0	-	91.67 ± 1.67 ^{a}	11.00 ± 0.58 ^{a}	7.40 ± 0.23 ^{a}
-	1.5	-	70.00 ± 2.89 ^{b}	6.67 ± 0.88 ^{bcd}	6.63 ± 0.12 ^{b}
-	2.0	-	41.67 ± 1.67 ^{ef}	3.67 ± 0.88 ^{efgh}	5.07 ± 0.17 ^{d}
-	-	0.5	43.33 ± 1.67 ^{e}	5.33 ± 0.33 ^{def}	4.43 ± 0.18 ^{e}
-	-	1.0	63.33 ± 3.33 ^{c}	7.67 ± 0.88 ^{bc}	6.87 ± 0.09 ^{b}
-	-	1.5	56.67 ± 1.67 ^{d}	5.67 ± 0.67 ^{cde}	5.23 ± 0.12 ^{d}
-	-	2.0	31.67 ± 1.67 ^{g}	3.00 ± 0.58 ^{gh}	3.53 ± 0.22 ^{f}

The data indicates the Mean ± SE of three repeated experiments with a total of 20 replicates. Using Duncan's multiple range test, values denoted by the same letter within a column are not statistically different at $p = 0.05$.

In our study, the optimum concentration of IAA and NAA gave only 4.6 and 7.6 roots/microshoot with a mean root length of 6.0 cm and 6.8 cm in 53.3% and 63.3% of the cultures, respectively. The suitability of IBA for optimal rooting in the half-strength MS medium has already been observed in other medicinal plant species, such as *Azalea* [54], *Decalepis salicifolia* [40] and *Salvia hispanica* [55].

The transition of regenerants from an artificial to a natural environment is the most essential and vital stage in tissue culture. Plantlets were hardened in thermocol cups with three planting materials, namely garden soil + manure (3:1), soilrite, and vermicompost, with fully extended leaves and a well-developed root system. Soilrite proved to be the best planting substrate for acclimatization of regenerated plantlets which showed 93.3% survival (Figure 1f), while garden soil + manure showed 65.0% and vermicompost 71.6% survival (Figure 2). Our findings are in agreement with those of Perveen et al. [56] and Naaz et al. [57], who found that in vitro regenerated plantlets of *Euphorbia cotinifolia* and *Syzygium cumini*, respectively, had the best survival rates on Soilrite. After acclimatization, the regenerated plants were moved to garden soil which showed ca. 80% survival under green-house condition. After four weeks, the plants showed normal growth in the natural environment (Figure 1g).

3.3. Genetic Fidelity

For clonal mass multiplication to be successful, it is necessary to compare the genetic uniformity of tissue culture plants to that of the mother plant (field grown plant). There are various advantages of micropropagation, but somaclonal variation among the regenerants is one of the disadvantages encountered. It is thus necessary to check the genetic fidelity of them to infer somaclonal variation propagules. The RAPD and ISSR DNA-based molecular markers were used to assess the genetic integrity of the regenerated plantlets. The mother plant and nine in vitro-grown plantlets chosen at random from a pool of healthy ones were molecularly analyzed, the amplified DNA bands were studied. Nine of the 10 primers used for RAPD analysis yielded distinct, clear, and repeatable bands (Table 4). The primer OPL-8 produced the most monomorphic bands, with a maximum of four and the amplified bands ranged from 100 to 1000 bp (Figure 3a). The regenerated plantlets' genetic profiles were tested with 10 UBC primers for ISSR markers, nine of which yielded distinct and clear bands (Table 5). The primer UBC-818 produced a total of eight monomorphic bands. In ISSR analysis, compared to RAPD, more bands were observed, ranging from 100 to 1500 bp (Figure 3b). The monomorphic amplified-DNA profile obtained from both the markers clearly showed genetic integrity of the regenerated plants as compared to the mother *D.*

palmatus plant. Similar results were obtained in *Inula roylena* [58], *Decalypis salicifolia* [40] and *Prunus cerasifera* [59] for which RAPD and ISSR markers were used to confirm genetic homogenity. Thus, the results obtained validate the suitability of the micropropagation protocol of *D. palmatus*.

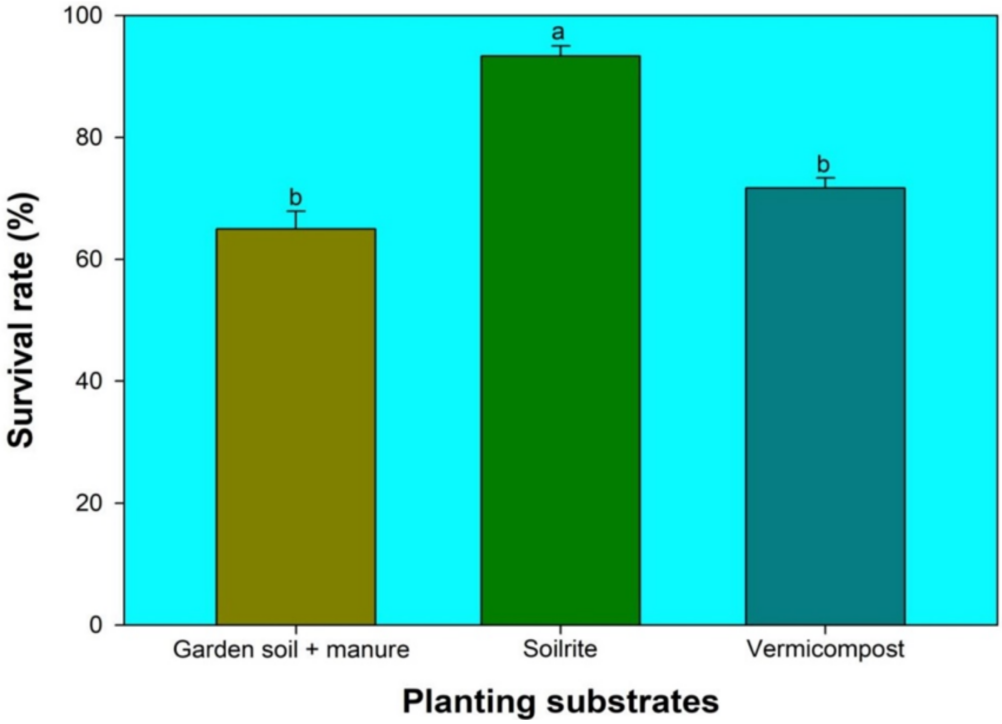

Figure 2. Effect of planting materials on the survival rate (%) of *D. plamatus* plantlets during acclimation. Bars denoted by the same letter are not statistically different at $p = 0.05$.

Table 4. Amplified-DNA bands generated from random amplified polymorphic DNA primers in the mother plant and in vitro-propagated plants of *D. palmatus*.

Name of Primers	Primer Sequence (5′-3′)	No. of Bands
OPL—01	GGCATGACCT	2
OPL—02	TGGGCGTCAA	3
OPL—03	CCAGCAGCTT	1
OPL—04	GACTGCACAC	3
OPL—05	ACGCAGGCAC	2
OPL—06	GAGGGAAGAG	0
OPL—07	AGGCGGGAAC	3
OPL—08	AGCAGGTGGA	4
OPL—09	TGCGAGAGTC	2
OPL—10	TGGGAGATGG	1

3.4. Ultra-Structural Difference between In Vitro and Acclimatized Leaves

The leaf texture and stomatal morphology of regenerated plantlets changed dramatically when transferred from in vitro to ex vitro settings, reflecting the acclimatization process. The SEM was used to compare the anatomy of in vitro and acclimatized *D. palmatus* leaves. The results show the adaptation of plantlets to high light irradiance, as evidenced from the cuticle thickness and sclerenchyma. The low irradiance of light, gaseous exchange, and nutrition in culture containers all result in aberrant phenotypes under in vitro growth conditions. The in vitro growth conditions result with abnormal phenotypes associated to the low irradiance of light, gaseous exchange and nutrition in culture containers. Electron microscopy of the lateral side of in vitro plant leaves revealed a severely constricted surface

with few stomata that were mainly closed and deep seated (Figure 4a1), as well as guard cells that were not fully functioning and stomatal apertures that were irregular (Figure 4b1). The regenerated plantlets gradually stabilized the leaf tissue structure during acclimation, allowing normal growth. At this stage, leaf morphology is a useful indication of plant development. The abaxial leaf surface of ex vitro-acclimatized plantlets revealed a relaxed surfaces with many well-defined stomata (Figure 4a2), with a homogenous aperture, and functional guard cells with open and closed stomata (Figure 4b2). Similar results were obtained in *Ceratonia siliqua* [60] and *Leucospermum cultivars* [61].

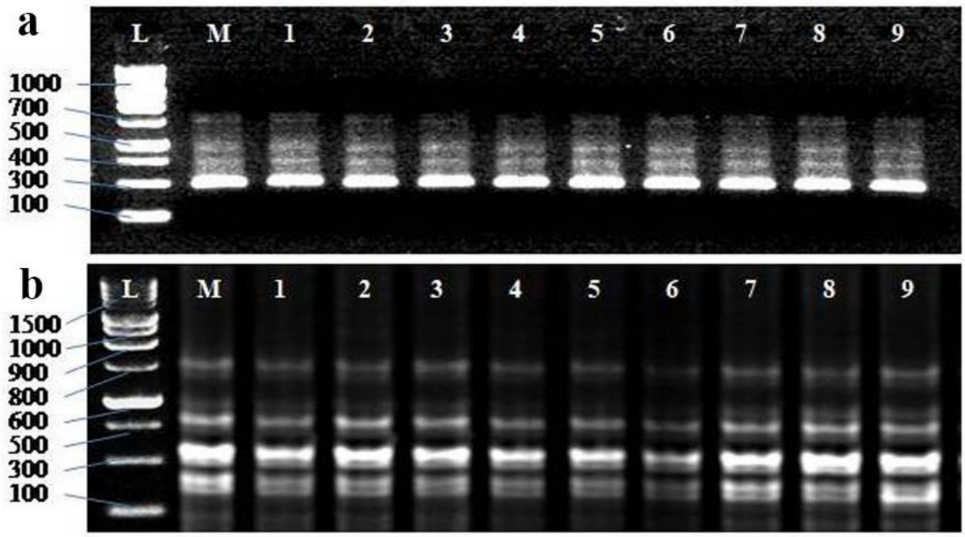

Figure 3. Amplified-DNA profile of the mother plant (Lane M) and in vitro plants of *D. palmatus* (Lane 1-9) obtained through RAPD primer (OPL-8; panel (**a**)) and ISSR primer (UBC-818; panel (**b**)) showing the monomorphic banding pattern. L—DNA ladder.

Table 5. Amplified-DNA bands generated from inter simple sequence repeat primers in the mother plant and in vitro-propagated plants of *D. palmatus*.

Name of Primers	Primer Sequence (5′-3′)	No. of Bands
UBC—812	$(GA)_8A$	5
UBC—814	$(CT)_8A$	3
UBC—818	$(CA)_8G$	8
UBC—825	$(AC)_8T$	7
UBC—827	$(AC)_8G$	6
UBC—836	$(AG)_8YA$	2
UBC—848	$(CA)_8RG$	7
UBC—855	$(AC)_8YT$	0
UBC—868	$(GAA)_6$	1
UBC—880	$(GGGGT)_3G$	3

3.5. GC-MS Analysis

The GC-MS analysis of mother and micropropagated plants was performed for the identification of medicinally important secondary metabolites. Several compounds in minor and major concentration were identified (Tables 6 and 7, Figures 5 and 6). When GC-MS was used to analyze both mother and micropropagated plants, more than fifty compounds were detected. For the extraction procedure, methanol was determined to be a suitable solvent. Tables 6 and 7 provide the names of the compounds identified, as well as their retention time (Rt), concentration (area and area percent), formula, and molecular weight (MW). Some important components, such as octadecanoic acid, octadecadienoic acid, octadecatrienoic acid, hexadecanoic acid, methyl stearate, and gamma-tocopherol

were identified in the mother plant. The in vitro produced *D. palmatus* clones, on the other hand, have greater levels of 1,3-propanediol, phytol, hexadecanoic acid, and octadecanoic acid. It is widely recognized that in vitro culturing of plant cells and tissues, carried out under strictly controlled conditions, offers a sound technological basis for the effective synthesis of plant natural products in a short period of time for commercial usage [62]. The use of phytohormones in culture medium may influence the up- or down-regulation of genes involved in the biosynthetic pathway of secondary metabolites, which may be one of the reasons contributing to the effectiveness of micropropagation in the synthesis of bioactive compounds [63,64]. The types of cytokinins employed in in vitro cultivation of medicinal plants, as well as the concentrations used, have an influence on the level of secondary metabolites produced by the plants. For example, in the *Aloe arborescens* species, media containing cytokinin alone or in combination with auxin substantially enhanced the quantity of total phenolics, flavonoids and condensed tannins compared to plant growth regulator-free media during in vitro propagation [65,66]. For the screening of metabolites in various medicinal plants, a combination of chromatography-mass spectrometry has been frequently utilized. Indeed, a similar approach was used for other medicinal plant species such as *Cassia angustifolia* [67], *Decalepis arayalpathra* [68], *Zhumeria majdae* [69], *Hemidesmus indicus* [70] and *Hildegardia populifolia* [71].

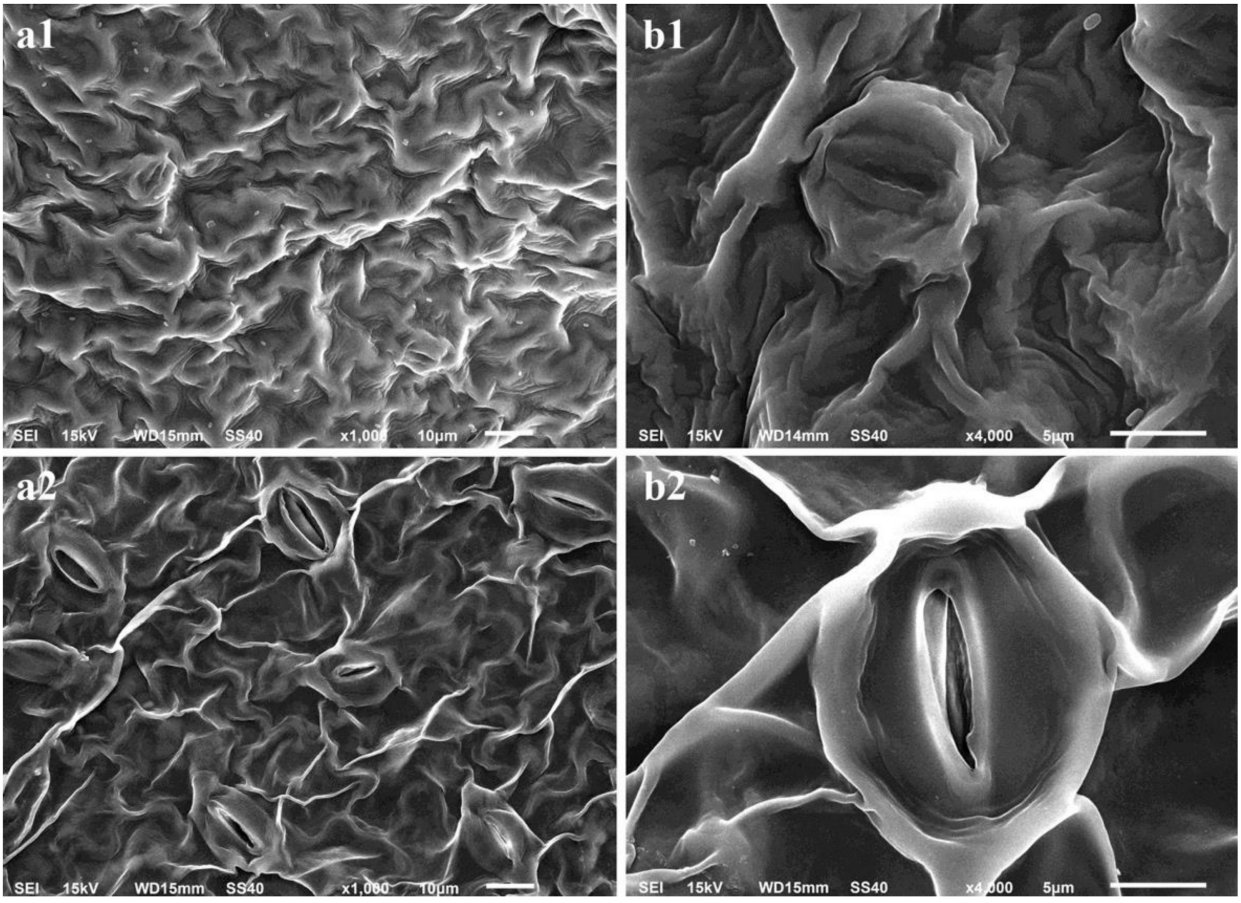

Figure 4. (1) Scanning electron microscopic examination of leaves from in vitro-propagated *D. palmatus*; (**a1**) abaxial leaf surface showing deep seated closed stomata; (**b1**) not fully functional guard cells showing irregular stomatal opening and abnormal stomata. (2) SEM examination of a leaf taken from acclimatized regenerated plantlets of *D. palmatus*; (**a2**) abaxial leaf surface showing well-developed stomata; (**b2**) open stomata with clear opening.

Table 6. Phytoconstituents detected in methanol leaf extract of the mother *D. palmatus* plant.

Peak	Rt	Area	Area %	Molecular Weight	Molecular Formula	Name of Compound
1	4.507	908,320	1.41	182	$C_6H_{14}O_6$	Hexitol
2	6.070	277,121	0.43	126	$C_3H_6N_6$	1,3,5-Triazine-2,4,6-triamine
3	6.210	181,003	0.28	156	$C_{11}H_{24}$	Undecane
4	6.312	305,048	0.47	100	$C_6H_{12}O$	Oxetane
5	6.997	146,388	0.23	102	$C_4H_{10}N_2O$	2-Propanamine, N-methyl-N-nitroso
6	7.091	732,652	1.14	144	$C_6H_8O_4$	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one
7	10.733	273,923	0.43	206	$C_{13}H_{18}O_2$	1-(3,6,6-Trimethyl-1,6,7,7A-Tetrahydro-Cyclopenta[C]Pyran-1-yl)-Ethanone
8	11.350	4,066,940	6.32	134	$C_6H_{14}O_3$	1,3-Propanediol, 2-ethyl-2-(hdroxymethyl)
9	12.173	377,158	0.59	206	$C_{14}H_{22}O$	Phenol, 2,4-bis(1,1-dimethylethyl)
10	12.342	755,352	1.17	194	$C_{11}H_{14}O_3$	Benzoic acid, 4-ethoxy-, ethyl ester
11	13.450	1,963,722	3.05	208	$C_{12}H_{16}O_3$	Benzene, 1,2,4-trimethoxy-5-(1-propenyl)-, (Z)-
12	13.660	652,689	1.01	194	$C_7H_{14}O_6$	Methyl. beta.-d-galactopyranoside
13	13.964	427,432	0.66	148	$C_{10}H_{12}O$	2,3-Dihydro-1H-inden-2-ylmethanol
14	14.486	143,730	0.22	270	$C_{16}H_{30}O_3$	*cis*-11,12-Epoxytetradecen-1-ol
15	15.028	289,126	0.45	228	$C_{14}H_{28}O_2$	Tetradecanoic acid
16	15.454	268,447	0.42	242	$C_{16}H_{34}O$	3-Hexadecanol
17	15.653	432,185	0.67	270	$C_{17}H_{34}O_2$	Isopropyl myristate
18	15.819	158,020	0.25	296	$C_{20}H_{40}O$	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl
19	15.896	446,768	0.69	268	$C_{18}H_{36}O$	2-Pentadecanone
20	16.706	1,067,836	1.66	270	$C_{17}H_{34}O_2$	Hexadecanoic acid
21	17.021	258,429	0.40	218	$C_{12}H_{10}O_2S$	Benzene, 1,1′-Sulfonylbis
22	17.105	4,860,713	7.55	242	$C_{15}H_{30}O_2$	Pentadecanoic acid
23	18.383	101,568	0.16	294	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z,Z)
24	18.456	707,024	1.10	292	$C_{19}H_{32}O_2$	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)
25	18.571	284,881	0.44	296	$C_{20}H_{40}O$	Phytol
26	18.650	283,100	0.44	298	$C_{19}H_{38}O_2$	Methyl stearate
27	18.857	2,412,690	3.75	234	$C_{16}H_{26}O$	*cis*,*cis*,*cis*-7,10,13-Hexadecatrienal
28	19.013	761,049	1.18	284	$C_{18}H_{36}O_2$	Octadecanoic acid
29	20.447	258,635	0.40	212	$C_{14}H_{28}O$	Tetradecanal
30	20.754	1,272,831	1.98	324	$C_{21}H_{40}O_2$	4,8,12,16-Tetramethylheptadecan-4-olide
31	20.978	286,398	0.45	262	$C_{18}H_{30}O$	Farnesyl acetone A
32	21.297	465,407	0.72	240	$C_{16}H_{32}O$	Hexadecanal
33	21.692	188,587	0.29	175	$C_{10}H_9NO_2$	1H-Indole-3-acetic acid
34	22.114	348,238	0.54	240	$C_{16}H_{32}O$	Palmitaldehyde
35	22.311	280,283	0.44	390	$C_{24}H_{38}O_4$	1,2-Benzenedicarboxylic acid
36	22.974	337,947	0.53	268	$C_{18}H_{36}O$	Octadecanal
37	23.410	81,469	0.13	190	$C_{10}H_{10}N_2S$	4-(O-Tolyl)-2-thiazolamine
38	23.536	207,065	0.32	338	$C_{24}H_{50}$	Tetracosane
39	23.956	162,244	0.25	268	$C_{18}H_{36}O$	Stearaldehyde
40	25.048	3,749,650	5.83	410	$C_{30}H_{50}$	Squalene
41	25.515	722,327	1.12	420	$C_{30}H_{60}$	8-Hexadecene, 8,9-diheptyl
42	25.838	1,751,204	2.72	618	$C_{20}H_{23}F_{17}O_2$	Heptadecafluorononanoic acid, undecyl ester
43	26.502	363,324	0.56	290	$C_{20}H_{34}O$	Neryl linalool isomer
44	26.870	1,076,776	1.67	402	$C_{27}H_{46}O_2$	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)
45	28.166	333,738	0.52	240	$C_{16}H_{32}O$	1-Hexadecanal
46	28.427	2,761,657	4.29	416	$C_{28}H_{48}O_2$	beta-Tocopherol
47	28.703	721,217	1.12	416	$C_{28}H_{48}O_2$	gamma-Tocopherol
48	29.404	1,148,973	1.79	396	$C_{27}H_{56}O$	1-Heptacosanol
49	30.413	17,190,992	26.72	430	$C_{29}H_{50}O_2$	Vitamin E
50	32.947	2,332,294	3.62	400	$C_{28}H_{48}O$	Ergost-5-en-3-ol
51	35.603	4,213,930	6.55	414	$C_{29}H_{50}O$	Stigmast-5-en-3-ol, (3.beta.)
52	36.745	363,153	0.56	486	$C_{31}H_{50}O_4$	Methyl commate C

Rt—retention time; Unit of Area—CPSeV, where CPS is counts per second.

Table 7. Phytoconstituents detected in methanol leaf extract of in vitro-propagated *D. palmatus* plants.

Peak	Rt	Area	Area %	Molecular Weight	Molecular Formula	Name of Compound
1	5.570	7,571,988	7.53	92	$C_3H_8O_3$	Glycerin
2	11.942	350,149	0.35	206	$C_{13}H_{18}O_2$	1-(3,6,6-Trimethyl-1,6,7,7A-Tetrahydro-Cyclopenta[C]Pyran-1-yl)-Ethanone
3	12.950	10,875,138	10.82	151	$C_4H_9NO_5$	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro
4	13.778	128,200	0.13	180	$C_{11}H_{16}O_2$	2(4H)-Benzofuranone
5	14.370	284,394	0.28	102	$C_{12}H_{14}O_4$	1,2-Benzenedicarboxylic acid
6	14.864	257,084	0.26	190	$C_{13}H_{18}O$	Megastigmatrienone
7	15.816	188,288	0.19	198	$C_{13}H_{26}O$	Tridecanal
8	15.926	197,429	0.20	228	$C_{15}H_{32}O$	1-Dodecanol
9	16.245	154,416	0.15	196	$C_{11}H_{16}O_3$	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one
10	16.562	282,146	0.28	196	$C_{11}H_{14}O_3$	Loliolide
11	16.717	416,109	0.41	222	$C_{13}H_{18}O_3$	2-Cyclohexan-1-one
12	17.104	1,420,117	1.41	278	$C_{20}H_{38}$	Neophytadiene
13	17.161	3,605,787	3.59	268	$C_{18}H_{36}O$	2-Pentadecanone
14	17.360	484,916	0.48	278	$C_{20}H_{38}$	7,11,15-Trimethyl-3-methylenehexadec-1-ene
15	17.552	661,569	0.66	278	$C_{20}H_{38}$	1-Hexadecene
16	17.901	576,424	0.57	268	$C_{18}H_{36}O$	Hexahydrofarnesyl acetone
17	18.005	1,173,450	1.17	270	$C_{17}H_{34}O_2$	Hexadecanoic acid
18	18.412	1,040,440	1.04	256	$C_{16}H_{32}O_2$	n-Hexadecanoic acid

Table 7. *Cont.*

Peak	Rt	Area	Area %	Molecular Weight	Molecular Formula	Name of Compound
19	18.833	306,615	0.31	710	$C_{36}H_{54}O_{14}$	Card-20(22)-enolide
20	19.643	1,765,763	1.76	294	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z,Z)
21	19.702	12,045,599	1.20	296	$C_{19}H_{36}O_2$	9-Octadecenoic acid (Z)
22	19.760	86,695	0.09	214	$C_{13}H_{26}O_2$	Undecanoic acid
23	19.805	3,641,993	3.62	296	$C_{20}H_{40}O$	Phytol
24	19.938	649,305	0.65	298	$C_{19}H_{38}O_2$	Methyl stearate
25	20.092	528,316	0.53	338	$C_{22}H_{42}O_2$	Palmitaldehyde
26	20.930	1,037,694	1.03	292	$C_{19}H_{32}O_2$	Methyl 9.cis.,11.trans.t,13.trans.-octadecatrienoate
27	21.157	1,817,402	1.81	288	$C_{21}H_{36}$	14-.beta.-H-pregna
28	21.473	338,187	0.34	312	$C_{19}H_{36}O_3$	Glycidyl palmitate
29	22.027	3,294,808	3.28	324	$C_{21}H_{40}O_2$	4,8,12,16-Tetramethylheptadecan-4-olide
30	22.165	510,086	0.51	281	$C_{18}H_{35}NO$	9-Octadecenamide
31	23.361	409,322	0.41	234	$C_{17}H_{30}$	1,8,11-Heptadecatriene, (Z,Z)
32	23.842	4,685,553	4.66	330	$C_{19}H_{38}O_4$	Hexadecanoic acid
33	24.025	487,501	0.49	530	$C_{34}H_{58}O_4$	Bis(tridecyl) phthalate
34	25.463	8,280,491	8.24	354	$C_{12}H_{38}O_4$	9,12-Octadecadienoic acid (Z,Z)
35	25.715	2,235,784	2.22	358	$C_{21}H_{42}O_4$	Octadecanoic acid
36	26.287	2,761,319	2.75	281	$C_{18}H_{35}NO$	9-Octadecenamide
37	26.594	363,564	0.36	410	$C_{30}H_{50}$	Squalene
38	26.930	1,539,925	1.53	462	$C_{29}H_{50}O_4$	alpha-Tocospiro A
39	27.160	2,179,600	2.17	462	$C_{29}H_{54}O_4$	alpha-Tocospiro B
40	28.157	1,497,815	1.49	402	$C_{27}H_{46}O_2$	delta-Tocopherol
41	29.391	1,639,399	1.63	416	$C_{28}H_{48}O_2$	beta-Tocopherol
42	29.651	1,160,593	1.15	416	$C_{28}H_{48}O_2$	gamma-Tocopherol
43	30.043	849,521	0.85	454	$C_{31}H_{50}O_2$	Stigmasta-5,22-dien-3-ol
44	30.963	1,459,626	1.45	430	$C_{29}H_{50}O_2$	Vitamine E
45	32.935	5,768,733	5.74	400	$C_{28}H_{48}O$	Ergost-5-en-3-ol
46	33.518	859,469	0.86	412	$C_{29}H_{48}O$	Stigmasterol
47	34.993	12,512,685	12.45	414	$C_{29}H_{50}O$	gamma-Sitosterol
48	36.013	1,188,777	1.18	486	$C_{31}H_{50}O_4$	Methyl Commate D
49	36.516	1,156,282	1.15	442	$C_{30}H_{50}O_2$	Betulin
50	37.350	1,493,558	1.49	470	$C_{31}H_{50}O_3$	Methyl Commate B
51	38.804	1,970,415	1.96	430	$C_{29}H_{50}O_2$	Emipherol
52	40.146	1,151,813	1.15	440	$C_{30}H_{48}O_2$	Betulinaldehyde

Rt—retention time; Unit of Area—CPSeV, where CPS is counts per second.

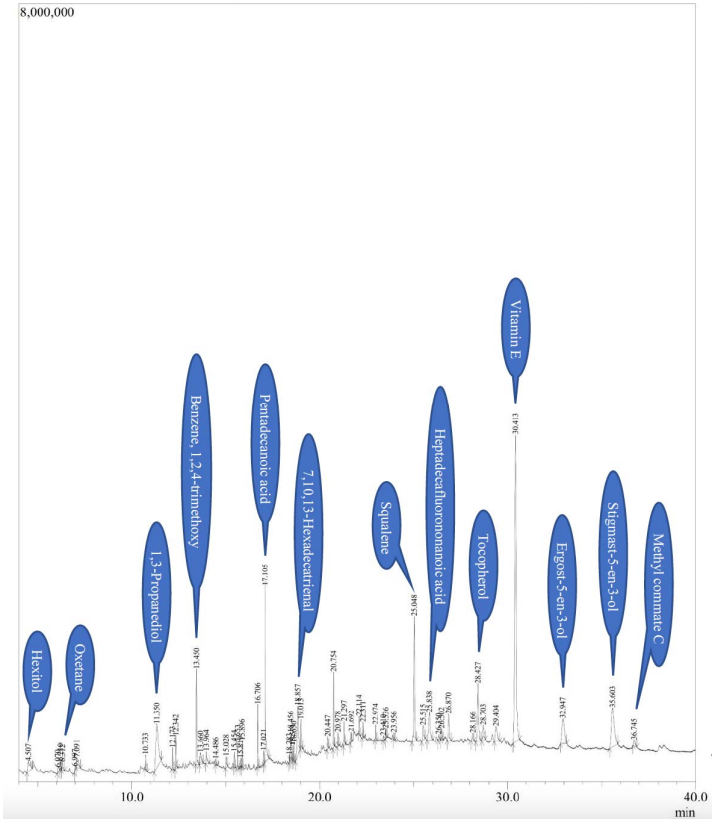

Figure 5. Phytoconstituents detected in the methanol leaf extract of the mother plant of *D. palmatus* using GC-MS.

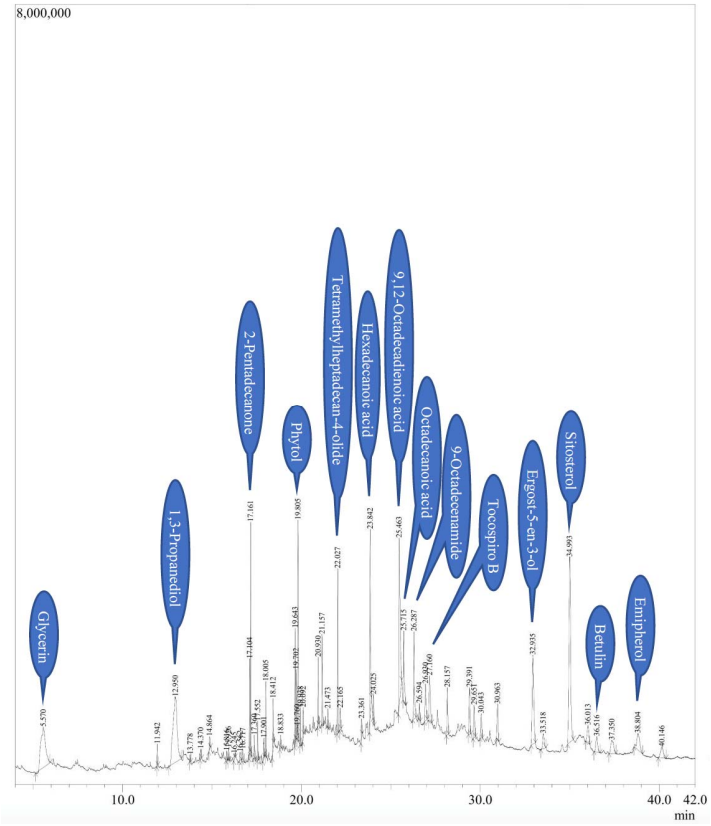

Figure 6. Phytoconstituents detected in the methanol leaf extract of a four-week-old in vitro-propagated plantlet of *D. palmatus* using GC-MS.

4. Conclusions

The present study provides a protocol for successful micropropagation of the valuable medicinal plant *D. palmatus* that has potential to lead to commercial exploitation, ex situ conservation and application of other in vitro-based biotechnological tools. The micropropagated plants were verified to be true-to-type using two different DNA molecular markers. Considering the importance of acclimatization, different potting substrates were also studied and a suitable substrate (Soilrite) was selected. The SEM analysis performed to investigate the leaf anatomy of acclimatized micropropagated plants grown under natural environmental conditions demonstrated that the function of the stomatal apparatus is restored during the acclimatization. The presence of pharmacologically significant metabolites by GC-MS analysis, confirmed the suitability of micropropagated plants in traditional and modern medicine. In conclusion, the proposed study may facilitate the large-scale *D. palmatus* production and will help to preserve the plant population.

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