The Electrical Conductivity of Nutrient Solution Influenced the Growth, Centellosides Content and Gene Expression of *Centella asiatica* in a Hydroponic System

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Abstract: *Centella asiatica* is a herbaceous plant containing medicinal and cosmetic properties: antibacterial, anti-aging, memory enhancing and wound healing. The lack of information impedes the development of suitable growth conditions for *C. asiatica* in the hydroponic system. Maintaining proper electrical conductivity (EC) of a nutrient solution is considered crucial for plant growth and the accumulation of bioactive compounds in a plant grown in hydroponics. This study aimed to investigate an optimal EC that enhances the growth of *C. asiatica* and its centellosides content. Seedlings were grown in commercial nutrient solution and treated with four different strengths of EC (0.6, 1.2, 1.8 and 2.4 indicated T1, T2, T3 and T4, respectively) under controlled environment conditions. Our results demonstrate that the number of leaves, leaf area, number of runners, shoot fresh weight and shoot dry weight were significantly increased in T2 among the treatments. However, these growth parameters were lowest in T4. Furthermore, the content of asiaticoside, madecassoside, asiatic acid and madecassic acid was the highest in plants that were treated with T2. The expression of centelloside biosynthesis-related genes is also affected by the strength of the nutrient solution. A positive correlation was observed between the number of leaves, leaf area and centellosides content. This study provides valuable background on optimal EC content in the nutrient solution in a hydroponic system with enhanced centellosides content to leverage the *C. asiatica* production.

Keywords: bioactive compounds; controlled environment; growth characteristics; nutrient film technique; real-time qPCR

1. Introduction

*Centella asiatica* (L.) is a perennial leafy plant belonging to the Apiaceae family and is widely used in traditional medicine in many countries [1]. Centellosides are the main bioactive compounds of this plant and are primarily found in their leaves [2,3]. These have therapeutic potential against numerous diseases, such as skin wounds, depression, stress, heart disease and cancer [4,5]. Recently, the popularity of *C. asiatica* is rising as a raw product in the pharmaceutical and cosmetic industries. However, these plants usually grow in forests and wetland and their commercial cultivation technique is yet unexplored. As a consequence, the maximum proportion of industrial demand is filled by noncommercial farmers and wild-grown centella. Generally, *C. asiatica* harvested from the wild is contaminated with excess dirt, heavy metals and microbes, including yeasts and molds, resulting in the poor quality of the final product after processing, based on its purity.
and bioactive compounds [6]. For this reason, clean and high-quality *C. asiatica* is required in the pharmaceutical industry.

Nowadays, the hydroponic system is an updated technology and is advantageous for cleanly cultivating some herbaceous plants without using soil as a growing medium. The nutrient solution is a significant part of this system and acts as a source of nutrients and water. Notably, the electrical conductivity (EC) of a nutrient solution in a hydroponic system is an important factor for plant growth and development [7,8]. Excessively high EC causes plant ion toxicity and hinders nutrient uptake by increasing osmotic pressure [7]. On the other hand, excessively low EC leads to nutrient deficiency and severely affects plant growth and yield [8]. Thus, maintaining an optimal level of EC is essential in water system cultivation and it is plant-specific [9]. Contrarily, to the best of our knowledge, research on *C. asiatica* in the hydroponic system using the nutrient film technique is still absent.

Four major triterpenoids, namely, asiaticoside, asiatic acid, madecassoside and madecassic acid, are also known as centellosides [2,3]. Madecassoside remarkably facilitates skin-related burn wound healing due to its competency to intensify collagen synthesis and protect lipid peroxidation and expression while stimulating angiogenesis. Asiaticoside has been shown to attenuate burn injury and neuronal damage. Its acute response to burn injury is due to the coordinated influx of macrophages and leukocytes [10]. In addition, asiaticoside showed anxiolytic effects during acute and chronic stress [11]. Madecassic acid exhibited anti-inflammatory and anti-oxidative activities in kidneys and hearts and provided lipid-lowering effects in the liver. In particular, it has the capability for cytotoxicity and cell selectivity in several human tumor cell lines [12]. Likewise, asiatic acid is an antibacterial agent and helps to prevent food contamination from foodborne bacterial pathogens [13].

Two different pathways mevalonic acid (MVA) pathway and methylerythritol 4-phosphate (MEP) pathway are responsible for centellosides biosynthesis [14]. Several genes are involved in these pathways and their role is crucial. Their expression level is correlated with centellosides accumulation, and the growing environment of a plant also affects these expression levels [15]. Furthermore, gene expression has many applications in plant biotechnology and physiology [16]. Such applications control the production of novel phytochemicals in plants for pharmaceutical or industrial uses [17]. Moreover, it helps to assess the adaptive potentiality of plants in their growing environment. To examine gene expression, the RNA sequencing method is convenient [18].

Along with clean cultivation, the hydroponic system can control different growing factors that are capable of regulating the physiological activities of plants including related gene expression levels. Therefore, in the present study, we investigated centellosides content and their relevant gene expression of *C. asiatica* in different strengths of nutrient solutions, which is important to understand the putative benefits of consumption and ensure clean production with the desired quality of this plant.

### 2. Materials and Methods

#### 2.1. Plant Materials

*C. asiatica* (L.) Urban used in this study was obtained from Hapcheon, Gyeongnam, Republic of Korea and clones through stolon multiplication were established in the seedling tray under controlled environment conditions. This experiment was conducted in a room facilitated by controlled environment conditions located at Kangwon National University, Department of Smart Farm and Agricultural Industry, Chuncheon, Republic of Korea (latitude, 38°06′ N; longitude, 128°17′ E) from 5 October 2021 to 3 November 2021. The nutrient film technique (NFT) was used as a means of hydroponic methods.

#### 2.2. Growing Conditions

*C. asiatica* plantlets were transplanted into the small tank for acclimatization with a hydroponic solution (EC 0.2) for 7 days. After acclimatization, uniform-sized *C. asiatica* plantlets
(height with 4–5 leaves/plantlet) were transplanted into a tank (46 cm × 32 cm × 24 cm; KSF-1000, Gahwa Tech, Hwaseong, Republic of Korea) for growth. The tank was separated into upper and bottom sections. The upper section holds plants and provides nutrients for roots through the solution, and the bottom is for the nutrient solution (Figure 1A). The total amount of solution per treatment was 15 L, and it circulated from the bottom to the upper section by an underwater motor. Furthermore, the water was drained from the upper section of the tank to the bottom section. The temperature of the room was maintained at 25 ± 3 °C and relative humidity was 65 ± 5%. White fluorescent light was used, and the intensity was set at 150 µmol m²s⁻¹ for plants. From the beginning of the experiment, the light:dark cycle was 16:8.

Irrigation circulation included a 16 h light period with irrigation maintained every 15 min at 30 min intervals and an 8 h dark period maintaining 30 min irrigation at 4 h intervals.

Figure 1. Schematic diagram of the tank where plants grow (A). Effect of nutrient strength on plant morphology (B–F). T1, T2, T3 and T4 indicate that the electrical conductivities of the nutrient solution are 0.6, 1.2, 1.8 and 2.4, respectively.
Figure 1. Schematic diagram of the tank where plants grow (A). Effect of nutrient strength on plant morphology (B–F). T1, T2, T3 and T4 indicate that the electrical conductivities of the nutrient solution are 0.6, 1.2, 1.8 and 2.4, respectively.

2.3. Treatment

Plants were treated with four different strengths of nutrient solution EC 0.6, 1.2, 1.8 and 2.4, which were indicated by T1, T2, T3 and T4, respectively. The same nutrients and ratios were used in every treatment. The formula provided by the University of Seoul was followed to prepare this nutrient solution. Firstly, two sets of nutrients A and B were prepared and then mixed in the same amount in the required volume of water. A contained Ca(NO$_3$)$_2$.H$_2$O, KNO$_3$ and Fe-EDTA (12.5%) at 35.4, 13.4 and 1.6 gL$^{-1}$, respectively. Whereas, B contained KNO$_3$, MgSO$_4$.7H$_2$O, NH$_4$H$_2$PO$_4$, H$_3$BO$_3$, MnSO$_4$.H$_2$O, ZnSO$_4$.7H$_2$O, CuSO$_4$.5H$_2$O, Na$_2$MoO$_4$.2H$_2$O at 27, 24.6, 7.6, 0.12, 0.063, 0.009, 0.004 and 0.0013 gL$^{-1}$, respectively. The EC and pH levels of each treatment were checked at 2-day intervals with a multipurpose water quality meter (HI9813-6, Hanna Instruments Inc., Salaj county, Romania). The EC level of the treatment was regulated by adding nutrients or water. If the EC level was lower than the set EC, nutrients were added. Conversely, when the EC level was higher than the set EC, water was added. The pH level was maintained at 5.6–6.1 and when it decreased to 5.6 or less, the pH was balanced in the solution by adding 5 mL 1 N KOH.

2.4. Analysis of Growth

After 4 weeks of treatment, plants were picked up from the tank and analyzed for different parameters. The petiole length of treated *C. asiatica* was measured from the growing point to the tip of the plant using a ruler. The number of leaves (except those less than 1 cm) was counted manually. Fully expanded leaves of a plant were selected to measure leaf width and leaf length using a ruler. A leaf area meter (Li-3100, Li-COR Inc., NE, USA) was used to measure the leaf area of a plant. The root length of a plant was measured by a ruler. An electronic balance (PAG4102C, Ohaus Corporation, NJ, USA) was used for measuring the fresh weight of the shoot and root. Dry weights of the shoots and roots of plants were calculated after drying in a conventional dryer (JEIO TECH OF-22GW, Daejeon, Republic of Korea) at 60 °C for 48 h. An electronic balance also measured the shoot and root dry weight.

2.5. High-Performance Liquid Chromatography (HPLC) Analysis

For centelloidoses extraction, 1 g freeze-dried sample of leaf powder of plants grown in different treatments was taken and mixed with 100 mL 80% methanol in a glass conical
beaker (200 mL). The mixture was shaken for 24 h in a shaker. After shaking, the extract was filtrated by filter paper. To obtain crude extracts, the extracts were filtered through Advantech 5B filter paper (Tokyo Roshi Kaisha Ltd., Saitama, Japan) and dehydrated using a vacuum rotatory evaporator (EYLA N-1000, Tokyo, Japan) at 40 °C. To prepare a 10 mg mL⁻¹ stock solution, concentrated crude extracts were diluted with 80% ethanol and stored at −20 °C for further analysis. This solution was used for qualitative analysis using the HPLC system for major centellosides, i.e., asiaticoside, madecassoside, asiatic acid and madecassic acid. A modified formula provided by Biswas et al. and Prasad et al. [3,19] was followed. Briefly, the analysis was carried out on a Shimadzu 20AD (Shimadzu, Canby, OR, USA) fitted with a Shimadzu SIL-20AC autosampler and a Shimadzu CTO-20AC column temperature oven with a C18 column (250 mm × 4.6 mm i.d.; 5 µm). A known amount of sample (10 µL) was injected and eluted using a gradient elution protocol consisting of solvent system-A containing acetonitrile and solvent system-B containing water. A linear gradient programming was executed at 30 °C with the initial composition of 100% A, changing to 22% A at 15 min, 40% A at 32 min, 46% A at 48 min, 70% A at 50 min and 22% A at 55 min, while maintaining a constant flow rate of 1 mL/min. Total runtime was 65 min. Detection of centellosides was performed at 205 nm using UV-Vis. Reference standards of four centellosides were purchased from Fluka Analytical, QFC, France, Sigma–Aldrich, MO, USA and ChromaDex Ltd., CA, USA.

2.6. Gene Expression Analysis

Freeze-dried leaves were ground to powder using a pestle and mortar and stored at −20 °C until further use. Total RNAs were extracted by following the RNA extraction protocol using Trizol™ reagent (Invitrogen, ThermoFisher Scientific, Seoul, Republic of Korea). Total RNAs were quantified by electrophoresis and Multiskan sky nanodrop (Thermo Fisher Scientific, Republic of Korea). The cDNA synthesis was carried out using 1.0 µg of total RNA by following the protocol of PrimeScript one-step RT-PCR kit (Takara Korea, Seoul, Republic of Korea). The synthesized cDNA was diluted with 100 µL of nuclease-free water, of which 3 µL was used as template cDNA for PCR analysis. Primers were designed from coding sequences of seven genes (Table 1) retrieved from the NCBI nucleotide database using the Primer 3 online tool (webpage). Each primer was confirmed for PCR amplification. For real-time PCR (qPCR), the Thermo Scientific SureTect PCR system (Thermo Scientific Korea) and TOPreal™ SYBR Green qPCR PreMIX (Enzymatics Inc., MA, USA) were used for PCR amplification. qPCR was carried out in 10 µL reactions containing 5 µL of TOPreal™ SYBR Green premix, 2 µL of cDNA and 2 µL of 10 µM gene-specific primers, 1 µL of ddH₂O under the following conditions: an initial denaturation step at 95 °C for 7 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s, polymerization at 72 °C for 20 s and a final extension at 72 °C for 30 s.

Table 1. Sequences of the primers used for qPCR analysis of genes involved in centelloside biosynthesis in C. asiatica.

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<th>Accession ID *</th>
<th>Functions</th>
<th>Primer ID</th>
<th>Primer Seq.</th>
<th>Tm</th>
<th>Size (bp)</th>
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<td>JK517508.1</td>
<td>Beta-actin (standard)</td>
<td>CaACT-F</td>
<td>AATGGTGAGGCTGGTTTG</td>
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<td>GTGGTGCCGCTGATGAAGA</td>
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<td>AY787627.1</td>
<td>Farnesyl diphosphate synthase (FPS)</td>
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<th>Primer Seq.</th>
<th>Tm</th>
<th>Size (bp)</th>
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<td>AY520818.1</td>
<td>Beta-amyrin synthase (CaABS)</td>
<td>CaAY520818.1-F</td>
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<td>255</td>
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<td></td>
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<td>CaAY520818.1-R</td>
<td>TTTGCTGCTATGGAATGG</td>
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<td>KT004520.1</td>
<td>Cytochrome P450 C-23 oxidase-like (CYP-450)</td>
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<td>CaKT004520.1-R</td>
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<tr>
<td>KP195716.1</td>
<td>UDP-glucosyltransferase (UGT-1)</td>
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<td>UDP-glucosyltransferase 73AH1 (UGT73AH1)</td>
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* NCBI accession.

2.7. Statistical Analysis

The experiment was arranged in a completely randomized design with three replications and 14 plants in each replication. Statistical analysis was performed using the IBM SPSS for Windows, version 24 (IBM Corp., Armonk, NY, USA). The analysis of variance (ANOVA) procedure was followed by Duncan’s Multiple Range Test (DMRT) at \( p \leq 0.05 \) to determine differences between treatment means. Principal component analysis (PCA) was carried out by OriginLab (Version: 10.0, software 176, Northampton, MA, USA).

3. Results and Discussions

3.1. Plant Growth Parameters

The petiole length of \( T_2 \)-treated \( C. asiatica \) was significantly 59% and 39% higher than in \( T_1 \)- and \( T_3 \)-treated plants, respectively (Table 2). There was no significant difference between \( T_2 \) and \( T_4 \) treatments. The smallest petiole length (5.6 cm) was observed in the \( T_1 \) treatment. Notably, research on \( C. asiatica \) based on the strength of electrical conductivity (EC) in hydroponic systems is still lacking. Research on other cultivation systems showed that under favorable conditions, its petiole length increased. Siddiqui et al. [20] noted that the petiole length of \( C. asiatica \) was 60% and 23% longest in well-fertilized soil (compost and inorganic fertilizer 1:1) compared to those grown in control and 50%-compost mixed soil, respectively. Song et al. [21] reported that the petiole length was enlarged in their preferred growing conditions (based on light intensity) of \( C. asiatica \).

Among the treatments, the number of leaves per plant was the highest (32) in the \( T_2 \) treatment (Table 2). It is 38% higher than \( T_1 \)-treated plants. Moreover, \( T_1 \)-treated plants showed a 35% significantly higher number of leaves than those grown in \( T_3 \). On the other hand, the lowest number of leaves (10.4) was observed in the \( T_4 \) treatment. Leaf length was not significantly different between \( T_1 \)- and \( T_2 \)-treated plants (Table 2). In addition, plants that were grown in \( T_3 \) and \( T_4 \) treatments have no significant difference in their leaf length. However, the leaf length of \( C. asiatica \) was significantly around 15% higher in \( T_1 \) and \( T_2 \) compared to those grown in \( T_3 \) and \( T_4 \). Furthermore, the leaf width of \( C. asiatica \) was not statistically different in \( T_1 \), \( T_2 \) and \( T_4 \) treatments. In spite of that, it was significantly 12% higher in \( T_1 \) than in those treated by \( T_3 \).

\( T_2 \)-treated plants showed the largest leaf area (Table 2). The smallest leaf area was observed in \( T_4 \)-treated plants. The leaf area of \( T_2 \)-treated plants was 5 times larger compared to \( T_4 \)-treated plants. In addition, it was significantly 2 times and 3 times larger than those that were grown in \( T_1 \) and \( T_3 \) treatments, respectively. Although, leaf length and leaf width were statistically similar between \( T_1 \) and \( T_2 \)-treated plants, their cumulative effect significantly increased leaf area in \( T_2 \)-treated plants than in \( T_1 \)-treated plants (Table 1). Siddiqui et al. [20] noted that soil conditions (based on nutrient availability) influenced the
leaf length and leaf width of *C. asiatica*. They also showed that the leaf area of *C. asiatica* was 4 times and 1.5 times larger in well-fertilized soil (compost and inorganic fertilizer 1:1) compared to those grown in control and 50%-compost mixed soil, respectively.

**Table 2.** Effect of nutrient strength on plant growth characteristics of *C. asiatica* at 4 weeks after treatment. T1, T2, T3 and T4 indicate that the electrical conductivities of the nutrient solution are 0.6, 1.2, 1.8 and 2.4, respectively.

<table>
<thead>
<tr>
<th>Growth Parameter</th>
<th>Treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
</tr>
<tr>
<td>Petiole length (cm)</td>
<td>5.60 ± 1.17 a</td>
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<tr>
<td>Leaf number (ea)</td>
<td>23.20 ± 0.84 b</td>
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<tr>
<td>Leaf length (cm)</td>
<td>2.62 ± 0.20 a</td>
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<tr>
<td>Leaf width (cm)</td>
<td>2.94 ± 0.17 ab</td>
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<tr>
<td>Leaf area (cm²)</td>
<td>47.15 ± 3.92 b</td>
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<tr>
<td>Node number (ea)</td>
<td>3.20 ± 1.10 ab</td>
</tr>
<tr>
<td>Node length (cm)</td>
<td>6.04 ± 0.90 ab</td>
</tr>
<tr>
<td>Runner number (ea)</td>
<td>1.00 ± 0.00 b</td>
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<tr>
<td>Runner length (cm)</td>
<td>26.50 ± 9.66 a</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>15.48 ± 2.04 a</td>
</tr>
<tr>
<td>SPAD (value)</td>
<td>40.78 ± 5.61 c</td>
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<tr>
<td>Shoot FW (g)</td>
<td>2.93 ± 0.30 b</td>
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<tr>
<td>Root FW (g)</td>
<td>1.25 ± 0.21 b</td>
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<td>Shoot DW (g)</td>
<td>0.37 ± 0.04 b</td>
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<tr>
<td>Root DW (g)</td>
<td>0.18 ± 0.03 ab</td>
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</table>

* Each value represents the means ± standard deviation (n = 10). Y Means within columns sharing the same letter are not significantly different based on Duncan’s multiple range test at p ≤ 0.05.

The number of nodes of *C. asiatica* in T2-treated plants was significantly 3 times and 5 times higher than those grown in T3 and T4, respectively (Table 2). There was no significant difference observed in plants grown between T1 and T2, as well as T3 and T4 treatments. Furthermore, the longest (7.04 cm) and shortest (4.38 cm) node lengths were observed in T2- and T4-treated plants, respectively. In addition, the node length of T2-treated plants was 1.5 times longer than those grown in T3 treatment. However, there was no significant difference observed between the node length of T1- and T2-treated plants.

In the T2 treatment, the number of runners in *C. asiatica* was significantly 80% higher than those that were grown in other treatments (Table 2). Nevertheless, there was no significant difference found among the treatments T1, T3 and T4. Also, the shortest runner length of *C. asiatica* was observed in the T4 treatment. It was 85% lower than T2-treated plants and 80% lower than T3-treated plants. There was no significant difference between the runner’s length of *C. asiatica* that was grown in T1 and T2 treatments.

Roots always play a crucial role in plant growth by ensuring water and nutrients supply for photosynthesis. Generally, the root length of a plant in soil depends on the soil’s water availability [22,23]. In this study, plants were grown using a recycling deep flow hydroponic system in a small tank, which ensures nutrient solutions available in the same range for all treated plants. As a result, after 4 weeks’ duration, the root lengths of *C. asiatica* are similar in all treatments (Table 2). Single-photon avalanche diode (SPAD) is a useful tool for the diagnosis of leaf chlorophyll content and crop nitrogen nutrition [24]. In this study, the SPAD value was significantly highest in T4-treated plants and it was higher than those grown in the T2 treatment.

Among the treatments, T2-treated plants showed the highest shoot fresh weight and root fresh weight. The second largest shoot fresh weight was observed in the T1-treated plants and was 47% lower than those grown in the T2 treatment. Contrarily, there was no significant difference in root fresh weight of T1-, T3- and T4-treated *C. asiatica*. As a consequence, shoot dry weight was the largest in T2-treated plants, and it was 70% higher than in T1-treated plants (Table 2). In addition, shoot dry weight in the T1 treatment was significantly higher than those grown in the T3 and T4 treatments. There was no significant
difference between the treatments T₃ and T₄. Root dry weight was significantly 53% higher in T₂-treated C. asiatica than those grown in T₄. However, there was no significant difference between T₁, T₃ and T₄-treated C. asiatica.

3.2. Centelloside Content

In this study, centellosides content was analyzed using HPLC chromatography (Figure 2A). We observed madecassoside content (6.15 mg/g DW) was significantly higher in plants that were grown in T₂ treatment (Figure 2B). It was around 10% higher than plants grown in T₁ and T₃-treated C. asiatica. Plants treated by T₄ treatment showed significantly lowest madecassoside than others. It is 14% lower than T₂-treated C. asiatica. A similar range (5–6.5 mg/g DW) of madecassoside contents was observed in wild-grown C. asiatica harvested from different regions of India and Thailand [25,26]. These results indicate that our hydroponically cultivated C. asiatica contains a similar concentration of madecassoside compared to wild-grown plants.

Figure 2. High-performance liquid chromatography (HPLC) chromatogram of centellosides (A). Effect of different EC on madecassoside (B), asiaticoside (C), madecassic acid (D) and asiatic acid (E) content of C. asiatica. The standard deviation of the mean (n = 10) is indicated by the lines above the bar. The same letters between the bars are not significantly different by Duncan’s multiple range test (DMRT) at p ≤ 0.05. T₁, T₂, T₃ and T₄ indicate that the electrical conductivities of the nutrient solution are 0.6, 1.2, 1.8 and 2.4, respectively.
Figure 2C illustrated that compared to all treatments, asiaticoside content was highest in T2-treated C. asiatica. Plants that were treated by T2 also showed asiaticoside content was significantly 6% higher than T3 and 15% higher than those treated by T1 and T4. In spite of that, there was no significant difference in asiaticoside content in T1- and T4-treated plants. Other researchers also found 7–20 mg/g DW of asiaticoside content in cell-cultured C. asiatica using different elicitors [27–30].

Figure 2D showed that madecassic acid content was significantly higher in T1- and T2-treated plants than those grown in T3 and T4 treatments. There was no significant difference between T1- and T2-treated plants. Among the treatments, the lowest madecassic acid content (1.14 mg/g) was observed in T4-treated plants. It was significantly 55% and 19% lower than T2- and T3-treated plants, respectively. Alqahtani et al. [31] observed that the concentration of madecassic acid in the aerial parts of wild-grown C. asiatica fluctuated (0.33–5.12 mg/g DW) across seasons and regions in Australia.

Asiatic acid content was the highest in T2-treated plants (Figure 2E). It was significantly 27% higher than those grown in the T3 treatment. Furthermore, there was no significant difference in asiatic acid content between T1- and T4-treated plants. However, plants grown in the T3 treatment showed 4% and 5% significantly higher asiatic acid content than those grown in T1 and T4 treatments, respectively. Prasad et al. [6] found 4–6 mg/g asiatic acid in 3 to 6 weeks of hydroponically grown C. asiatica. Although this research used a hydroponic system, they only used a 2 L-sized growing jar without a motor for water circulation. Whereas, in this study, plants were grown in a 15 L nutrient solution with good circulation. For these reasons, plants treated with different EC levels showed a higher content of asiatic acid compared to plants in [6]. Borhan et al. [32] also found 7 mg/g asiatic acid content in wild-grown C. asiatica.

3.3. Correlation

The principal component analysis (PCA) was applied to expose the correlation between the centellosides and plant growth characteristics of C. asiatica with the electrical conductivity of nutrients (Figure 3). It is observed from Figure 3 that PC1 indicates 73.24% variability and PC2 indicates 18.65% variability. The graph indicates that the number of leaves and the number of nodes are positively correlated. The number of leaves also shows a positive relation with petiole length. Devokota and Jha [33] showed that there is a positive correlation between the number of leaves, nodes and runners of C. asiatica. Other researchers noted that the number of leaves and plant height are positively correlated in plants that were grown in controlled conditions [34,35]. Leaf length, leaf width and centellosides (asiaticoside, madecassoside, asiatic acid and madecassic acid) content also showed a positive correlation, and their response is closer to the T2 treatments (Figure 3). Furthermore, the biplot also showed that the SPAD value is closer to T3 and T4 treatments and it has a negative correlation with the runner length of C. asiatica.

3.4. Expression of Genes Involved in Centelloside Biosynthetic Pathway

Two different pathways mevalonic acid (MVA) pathway and methylerythritol 4-phosphate (MEP) pathway are responsible for centellosides biosynthesis (Figure 4A). To know how various ECs of nutrient solutions in a hydroponic culture affect the expression of genes, real-time qPCR was carried out for seven genes that are potentially involved from geranyl pyrophosphate (GPP) to madecassoside and asiaticoside synthesis in MVA pathway (Table 1). The expression of three upstream genes of the biosynthesis, *Farnesyl diposphate synthase* (FPS), *Squalene synthase* (SQS) and *Squalene epoxidase* (SQE), was examined. FPS encodes farnesyl diposphate synthase that isomerizes isopentyl diphosphate (IPP) and dimethylallyl diposphate (DMAPP) to farnesyl diposphate (FPP). SQS catalyzes squalene to generate 2,3-oxidosqualene. The relative fold enhancement of the aforementioned genes in their transcript levels in T1, T2, T3 and T4 treatments was analyzed (Figure 4B–H). The most upstream gene, FPS, tested in this study was upregulated in response to T1 and T4.
treatments than T2 and T3. After 4 weeks, the SQS transcript level increased by more than 1 fold in all treated plants except T2. SQE was a more over-expressed gene in T2 and T3 treatments and it was around 3 fold. Furthermore, in T1 and T4 treatments, it was 1.7 and 2.5 fold, respectively. A similar range (in folds) of expression was observed in these three genes FPS, SQS and SQE in 4 weeks of in vitro cultured C. asiatica treated with AgNO3 [36].

![Figure 3](image_url)

**Figure 3.** Principal component analysis (PCA) of C. asiatica at 4 weeks after treatment in a controlled condition. T1, T2, T3 and T4 indicate that the electrical conductivities of the nutrient solution are 0.6, 1.2, 1.8 and 2.4, respectively. The lines starting from the central point of the biplots display the negative or positive associations of the different variables, and their proximity specifies the degree of correlation with specific treatment. PL, petiole length; LN, number of leaves; LL, leaf length; LW, leaf width; LA, leaf area; RN, number of runners; RNL, runner length; NN, number of nodes; NL, node length; RL, root length; SPAD, SPAD value; SFW, shoot fresh weight; RFW, root fresh weight; SDW, shoot dry weight; RDW, root dry weight; MS, madecassoside; AS, asiaticoside; MA, madecassic acid; AA, asiatic acid.

In the last three steps of the centelloside biosynthesis, α- and β-amyrin, asiatic acid and madecassic acid and asiaticoside and madessoside are produced by α- and β-amyrin synthase (CaABS), Cytochrome P450 (CYP) and UDP-glucosyltransferase (UGT), respectively [14]. The expression of CaABS was reduced in T2, T3 and T4 treatments compared to T1 treatment (Figure 4E). CYP450 was upregulated by ~1.5 fold under T1 and T4 treatments (Figure 4F). Mangas et al. [37] showed that the SQS and CaABS genes are involved in centelloside biosynthesis.
Figure 4. (A) Recommended biosynthetic pathway of centellosides in *C. asiatica* by Prasad et al. [19].

DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesy1 pyrophosphate; FPS, farnesyl diphosphate synthase; βAS, beta amyrin synthase; αAS, alpha amyrin synthase; SQS, squalene synthase; SQE, squalene epoxidase; CYP450, cytochrome P450; UGT, glycosyltransferase; MVA, mevalonate; MEP, methylerythritol 4-phosphate. Relative fold change of genes involved in the production of centelloside in hydroponically cultured *C. asiatica* after 4 weeks of different EC treatments: FPS (B), SQS (C), SQE (D), CaABS (E), CYP450 (F), UGT-1 (G) and UGT-2 (H). T1, T2, T3 and T4 indicate that the electrical conductivities of the nutrient solution are 0.6, 1.2, 1.8 and 2.4, respectively.
The last step of the biosynthesis was regulated by two UGT genes, KP195716 and MF471454, which were examined for their expression in response to the treatments (Figure 4G,H). UGTs encode glycosyltransferases that induce the generation of glycosylated triterpenes, such as madecassoside from madecassic acid and asiaticoside from asiatic acid, by adding a composed structure of glucose–glucose–rhamnose [38]. They are considered tailoring enzymes since they are capable of modifying the triterpene scaffolds by adding sugar chains [39]. The transcript level of gene KP195716 was increased with the strength of EC in the nutrient solution. On the other hand, UGT-2 expression was down-regulated by T2 and T3 treatments compared to T1 and T4, and all are below 0.6 fold at 4 weeks after treatment (Figure 4H). Other research also observed less than 0.4-fold transcript level of the UGT gene in C. asiatica after 4 weeks of treatment [36]. As far as we know, this is the first work that represents the expression of essential genes participating in the centellosides production in hydroponically cultured C. asiatica. It will help the commercial manufacturing of these compounds to meet their rising demand in pharmaceutical industries.

4. Conclusions

The present results indicate that T2 treatment (EC 1.2) is preferable for the growth of C. asiatica and centellosides (asiaticoside, madecassoside, asiatic acid and madecassic acid) biosynthesis. A higher strength of nutrient solution (EC 2.4) has a detrimental effect on both plant growth and their centellosides accumulation. Morphological features and bioactive compounds have a positive correlation. Expressions of centellose biosynthesis-related genes varied with different strengths of nutrient solutions. As an initiative, these findings will be helpful for future research on potential biotechnological applications and ensuring large-scale clean cultivation of C. asiatica with their required phytochemicals through hydroponic systems. In addition, investigations into other physiological activities of C. asiatica in hydroponic systems are needed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13122236/s1, Figure S1: Melt curve of the targeted genes CaAY787627.1 (FPS) and CaAY787628.1 (SQS) (A), CaMF480551.1 (SQE) and CaKT004520.1 (CYP-450) (B), CaAY520818.1 (CaABS) and CaKP195716.1 (UGT-1) (C), CaMF471454 (UGT-2) (D) and standard CaACT-F (Beta Actin) (A–D).

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