Article

Effects of Preharvest Methyl Jasmonate Elicitation and Electrical Stimulation on Camptothecin Production by *In Vitro* Plants of *Ophiorrhiza ridleiana* Craib

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Abstract: To enhance plant camptothecin (CPT) production *in vitro*, 5-month-old *Ophiorrhiza ridleiana* Craib plant cultures were treated with solutions of methyl jasmonate (MeJA) dissolved in ethanol, which were applied to the surface of the solid culture medium. It was demonstrated that the maximum CPT content in the tissue-cultured plants was achieved after 12 h elicitation with 50 μ M MeJA. The mean CPT contents in roots and stems were 50.8 and 67.0 μ g/g DW, respectively, which were approximately 1.8- and 2.6-fold higher, respectively, than those of the control. However, MeJA elicitation showed no significant effect on CPT accumulation in *O. ridleiana* leaves. Moreover, it was found that direct electric current (DC) stimulation also significantly increased CPT accumulation in *O. ridleiana*. The treatment with DC at 20 mA for 3 min of stimulation enhanced 3-fold the CPT content in roots, stems, and leaves to 41.9, 36.0 and 19.6 μ g/g DW, respectively, which were approximately 1.5-, 1.7- and 1.4-fold higher, respectively, as compared to those of the control. The results demonstrate that preharvest treatment by MeJA elicitation and electrical stimulation can be beneficial for secondary metabolite production of CPT in tissue-culture plants of *O. ridleiana*.

Keywords: camptothecin; *Ophiorrhiza ridleiana*; *in vitro* plants; elicitation; methyl jasmonate; electrical stimulation

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1. Introduction

Camptothecin (CPT) is a pentacyclic monoterpene indole alkaloid with anticancer properties, originally identified from the bark extracts of the Chinese tree *Camptotheca acuminata* (Nyssaceae) by Wall et al. in 1966 [1–3]. Camptothecin showed low solubility in water and toxicity during clinical trials [4,5], therefore the compound has not been used directly in practice owing to its side effects. However, semi-synthetic derivatives of CPT—topotecan and irinotecan—have been approved by the U.S. Food and Drug Administration for use as anticancer drugs and are widely prescribed in the treatment of ovarian, colorectal and small-cell lung cancer. The anticancer activity of CPT and its derivatives is due to the property of reversible binding to the topoisomerase I-DNA complex in cancer cells, thereby inhibiting DNA replication and triggering cancer cell death [6,7]. Other medicinal properties of CPT and its related derivatives include antiviral properties, pesticide activity, and antiparasitic activity [8].

Given the desirable pharmacological activities of CPT derivatives, the global market for CPT analogs from natural sources has increased rapidly, which has caused a decline in wild plant populations. This has led to the search for alternative sources and *in vitro* culture methods for CPT production [9,10]. A biotechnological method, employing plant cell

culture technology, is a promising alternative strategy [11]. The production of secondary metabolites by genetically engineered plant cell culture has become an important topic because of a shortage of natural resources and subsequent environmental concerns [1]. Recently, tissue-culture CPT-producing *Ophiorrhiza* (Rubiaceae) species have attracted attention as alternative sources for CPT production [12]. Camptothecin is synthesized by a number of *Ophiorrhiza* species, e.g., *O. liukuensis* and *O. kuroiwai* [4], *O. prostrata* [13], *O. alata* [10], *O. pumila* [14], and *O. mungos* [5].

One approach to promote plant tissue metabolism is by using elicitors [15]. The treatment of plant cells with biotic elicitors, such as bacterial and fungal cell walls, yeast extract and microbial homogenate, or abiotic elicitors, such as jasmonic acid and methyl jasmonate (MeJA), is a useful strategy to enhance production in cell/organ cultures of secondary metabolites, such as terpenoids, flavonoids, phenolic, alkaloids, and phytoalexins [16–18]. Methyl jasmonate and its derivatives are important signal transduction molecules widely used as elicitor compounds for induction of secondary metabolites in many plant species and in plant cell and callus cultures [19,20]. Enhanced accumulation of secondary metabolites in *in vitro* culture systems using MeJA has been frequently reported. Methyl jasmonate acts as a signal transducer to promote *in vitro* production of CPT from multiple shoots of *O. mungos* var. *angustifolia* in liquid culture medium [21]. The *in vitro* production of the secondary metabolite plumbagin in roots and shoots of *Drosera burmanii* in half-strength Murashige and Skoog ($1/2$ MS) liquid medium was enhanced after elicitation for 6 d with 50 μ M MeJA [22]. In addition, preharvest MeJA application to 2-year-old raspberry and blackcurrant plants as a foliar spray significantly increased the synthesis of flavonoids, particularly, ellagic acid, quercetin and myricetin [23].

Accumulation of secondary plant compounds can also be induced by physiology—electrical stimulation. Electrical stimulation has shown to have effects on living cells, including plant cells. Pulsed electric field treatment is a promising tool to induce various biological effects for cellular compound stimulation [24]. However, research on the effects of electricity on plant response is quite limited. Electric current is a flow of electric charge carried by moving electrons through conductors or semiconductors or by ion in an electrolyte, which is measured in ampere (A) [25]. Electricity is found to have an influence on growth and the secondary metabolism in plants. It was demonstrated that pulsed electric field stimulated plant secondary metabolism in suspension cultures of *Taxus chinensis* [26]. Direct-electric-current (DC) intensities between 100 and 500 mA with applications of 15 to 60 min enhanced the accumulation of carotenoids, phenolic compounds in tomatoes [27]. The application of DC with a voltage of 8 and 16 V, 10 h/day for 12 days could promote growth and bioactive compounds of African nightshade (*Solanum scabrum* Mill.) plants, in which applying DC was found to increase leaf fresh and dry weight and enhance biosynthesis of chlorophylls and carotenoids [28]. Pulsed electric fields pre-treatment on grapes enhanced the release of the major anthocyanin found in Pinot Noir, i.e., malvidin-3-O-glucoside and thus promoted the accumulation of total phenolic and vitamin C as well as the improvement in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and bioprotective capacity [29]. However until now, no study has been done for the effect of electrical stimulation on CPT production/accumulation in plants or plant tissue cultures.

In the present study, we developed elicitation techniques to stimulate CPT production *in vitro* in 5-month-old plants of *O. ridleyana* cultured in solid medium. The effects of MeJA elicitation and electrical stimulation by DC current on CPT production in roots, stems and leaves of treated *O. ridleyana* were investigated in order to develop practical methods to generate a higher yielding source of CPT from tissue-culture plants.

2. Materials and Methods

2.1. Plant Materials

Ophiorrhiza ridleyana plants with seeds were collected from Chang Island, Trat Province, Thailand. All voucher specimens collected from plants for research were preserved at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn

University, Thailand. Plants were identified by an experienced botanist, Associate Professor Thatree Phadungcharoen at Chulalongkorn University.

2.2. Culture of Aseptic Plants

Ophiorrhiza ridleyana seeds were soaked with running tap water for 1 h, surface-sterilized in 70% ethanol for 1 min and then in 1% sodium hypochlorite (Clorox®) with the addition of two drops of Tween-20 and shaken 15 min. The sterilized seeds were washed three times with sterile distilled water for 5 min each. The seeds were germinated on $\frac{1}{2}$ MS solid medium supplemented with 2% sucrose and 0.8% agar. The medium pH was adjusted to 5.7 prior to autoclaving at 121 °C and 1.2 kg cm⁻² pressure for 20 min. All aseptic plants were cultured in the prepared solid medium at 25 °C under a 16 h/8 h (light/dark) photoperiod. Plantlets were used as material for shoot induction.

2.3. Shoot Induction

Stem segments from *in vitro* plantlets were cultured in $\frac{1}{2}$ MS solid medium for shoot multiplication. The seeds germinated after 4 weeks. The plantlets were subcultured on fresh medium every four weeks for shoot elongation. Eight weeks after germination, the seedlings had developed a single shoot with 2–4 true leaves and roots. Shoots approximately 1 cm in length were excised and used as shoot explant. The explants were subcultured on the same solid medium in a 30 mL culture bottle for elicitor treatment.

2.4. Elicitation Treatment

Aseptic plants of *O. ridleyana* were subcultured by transferring the shoot explants to $\frac{1}{2}$ MS solid medium, which were cultured under a photoperiod of 16 h/8 h (light/dark). The explants were cultured for 5 months (Figure 1) so as to develop sufficient roots for chemical analysis. *In vitro* plants of 5-month-old *O. ridleyana* were treated with MeJA solution. The MeJA solution was gently and thoroughly poured onto the surface of the solid medium in each culture bottle using sterile pipette tip. Four concentrations of MeJA (0, 20, 50 and 100 µM) in 0.2 mL ethanol were used for the elicitor treatment. The culture bottles were incubated with the elicitor for 12 and 24 h at 25 °C. Controls were cultured under the same condition but without addition of an elicitor to the culture bottle. The elicitor treatment with 0 µM MeJA used pure ethanol as an elicitor. Each experiment was performed in triplicate.

Figure 1. Tissue culture plants of *O. ridleyana* in half strength MS medium after cultivation for 1–5 months.

2.5. Direct-Electric-Current Treatment

The 5 month-old tissue culture plants of *O. ridleyana* in a 30 mL culture bottle were treated with four different DC currents (10, 20, 30 and 40 mA) by electrical stimulator (Wanptek KPS1203D Switching DC Power Supply, Overwin Electronic Source Company Limited, Bangkok, Thailand) as shown in Figure 2. The electric current flowed through the solid medium by passing through a solid stainless bar of thickness 0.031 cm, width 1.40 cm and length 11.50 cm. Total treatment time is 15 min, which was divided into 3 cycles. Each cycle took 5 min, performed by supplying electricity for 3 min and cutting off the electricity for 2 min. After the DC treatment, the treated culture bottles were incubated for 24 h at 25 °C, before the tissue culture plants were taken for the analysis of CPT concentration. Controls were the tissue cultured plants cultured under the same condition but without the electrical stimulation. Each experiment was performed in triplicate.

Figure 2. Experimental study for electrical stimulation on 5-month-old tissue culture plants of *O. ridleyana*.

2.6. Camptothecin (CPT) Analysis

The quantitative analysis of CPT in the leaves, stems and roots *in vitro* culture was performed according to a previously reported procedure with minor modifications [10,13] by using Agilent HPLC 1260 Infinity II LC System (Santa Clara, CA, USA). For CPT extraction, plant samples were dissected into leaves, stems and roots. The samples were freeze-dried at −90 °C for 72 h. The freeze-dried samples were ground into powder for further analysis. A sample of powder (15 mg) was placed in a 1.5 mL tube to which 0.5 mL methanol was added. The mixture was placed in an ultrasonic bath for 30 min at room temperature (30 °C) and incubated at 4 °C overnight. The homogenates were centrifuged at 12,000× *g* for 5 min. The supernatants were filtered through a 0.45 µm syringe filter for high-performance liquid chromatography (HPLC) analysis. For quantification, a calibration curve for standard CPT was prepared. The standard CPT (Sigma Chemical Co., St Louis, MO, USA) was dissolved in dimethyl sulfoxide and diluted to generate a concentration series of 5–50 µg/mL. For separation by HPLC, a Zorbax Eclipse Plus C18 column (4.6 × 250 mm, 5 µm) (Agilent Technologies) with a mobile phase of acetonitrile:water (3:7) was used. The flow rate was 1 mL/min and the injection volume was 20 µL. The CPT detection wavelength was set at 254 nm. The peak identification was performed by comparing the peak's retention time with that of injected reference standard of CPT in the same chromatographic system. The quantity of CPT in the samples was estimated based on standard curve quantitation by a CPT standard, using area under the curve. Data analysis was performed by using Agilent ChemStation software.

2.7. Statistical Analysis

All the experiments were repeated in triplicate (three culture bottles) under the same conditions and values were expressed as the mean ± standard deviation (SD). The data were analyzed by variance (ANOVA) followed by Duncan post hoc test to compare means ($p \leq 0.05$) using the SPSS Statistics 17 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Accumulation of CPT in 5-Month-Old *O. ridleyana* Plant Cultures

The biomass of fresh and dry weights in different parts of 5-month-old tissue culture plants of *O. ridleyana* in a 30 mL culture bottle is shown in Table 1. The accumulation of CPT in the roots, stems, and leaves of 5-month-old tissue-culture plants of *O. ridleyana* without elicitor treatment is shown in Figure 3 and Table 2. The highest CPT content was recorded in the roots (0.0028% dry weight (DW)), followed by the stems (0.0025% DW) and the leaves (0.0013% DW). It should be noted that, the amount of CPT produced by leaf was significantly lower than that produced by root and stem, however the biomass of leaf was considerably higher as compared to root and stem.

Table 1. Biomass of fresh and dry weights in different parts of 5-month-old tissue culture plants of *O. ridleyana* in a 30 mL culture bottle.

Plant Part	Biomass	
	Average Fresh Weight (mg)	Average Dry Weight (mg)
Root	178.7 ± 81.0	54.2 ± 48.7
Stem	786.7 ± 186.1	67.0 ± 8.3
Leaf	1023.0 ± 182.0	191.9 ± 9.2

Figure 3. Distribution of CPT accumulation in roots, stems and leaves of 5-month-old tissue culture plants of *O. ridleyana*. The values are expressed as mean ± SD (n = 3).

Table 2. Average CPT concentrations in different parts of 5-month-old tissue culture plants of *O. ridleyana* treated with MeJA in ethanol (0–100 µM).

Treatment	Incubation Time (h)	CPT Content (µg/g DW)		
		Root	Stem	Leaf
Control	-	28.1 ± 4.0	25.4 ± 3.0	12.7 ± 3.3
Ethanol	12	31.4 ± 3.0	24.4 ± 2.1	12.7 ± 2.6
	24	26.5 ± 2.8	21.2 ± 2.8	13.4 ± 2.7
MeJA 20 µM	12	35.5 ± 3.2 ^a	24.9 ± 4.3	14.3 ± 1.7
	24	34.2 ± 2.6 ^a	29.0 ± 3.3	14.0 ± 2.9
MeJA 50 µM	12	50.8 ± 4.3 ^a	67.0 ± 5.8 ^a	15.9 ± 2.8
	24	33.8 ± 3.9	48.9 ± 2.6 ^a	13.9 ± 2.0
MeJA 100 µM	12	31.3 ± 1.0	25.4 ± 4.0	11.8 ± 2.2

^a: significant at $p < 0.05$ vs. control.

3.2. Effect of MeJA Elicitation on CPT Accumulation in *O. ridleyana*

3.2.1. Roots

As compared to the control, accumulation of CPT in the roots to some extent increased after 12–24 h exposure to 20–100 μM MeJA (Figure 4). Higher CPT contents were observed in response to MeJA elicitation for 12 h compared with elicitation to 24 h, except for 100 μM MeJA treatment. The optimal elicitor concentration was 50 μM MeJA. No significant increase was observed by pure ethanol (0 μM MeJA) as an elicitor. The HPLC chromatograms obtained from extracts of roots of *O. ridleyana* treated with 50 μM MeJA for 12 h showed a much larger peak area than those of the untreated controls (Supplementary Materials), in which the maximum CPT content represented approximately 1.8-fold increase compared with that of the controls. However, only a slight increase in CPT content was observed in response to elicitation with a higher MeJA concentration at 100 μM .

Figure 4. Effects of MeJA elicitation on CPT concentration in roots by elicitation with ethanol and MeJA (20, 50 and 100 μM) for 12 h (□) and 24 h (■) before harvesting. The values are expressed as mean $\pm$ SD ($n = 3$); a: significant at $p < 0.05$ vs. control.

3.2.2. Stems

Following 50 μM MeJA elicitation for 12 and 24 h, the CPT content in the stems of *O. ridleyana* increased significantly (Figure 5). The maximum CPT content in the stems in response to MeJA elicitation was increased by 2.6-fold (66.98 $\mu\text{g/g}$ DW) and 1.9-fold (48.85 $\mu\text{g/g}$ DW) after MeJA treatment for 12 and 24 h, respectively. No significant effect of elicitation with ethanol, 20 and 100 μM MeJA on CPT content in *O. ridleyana* stems was observed.

Figure 5. Effects of MeJA elicitation on CPT concentration in stems by elicitation with ethanol and MeJA (20, 50 and 100 μM) for 12 h (□) and 24 h (■) before harvesting. The values are expressed as mean $\pm$ SD ($n = 3$); a: significant at $p < 0.05$ vs. control.

3.2.3. Leaves

Overall, the CPT content in *O. ridleyana* leaves was lower than those in the roots and stems. No significant effect of MeJA and ethanol elicitation on CPT accumulation in *O. ridleyana* leaves was detected. Under the 20 and 50 μM MeJA treatments, only slight increases in CPT content in the leaves was observed (Figure 6).

Figure 6. Effects of MeJA elicitation on CPT concentration in leaves by elicitation with ethanol and MeJA (20, 50 and 100 μM) for 12 h (□) and 24 h (■) before harvesting. The values are expressed as mean $\pm$ SD ($n = 3$).

3.2.4. Total CPT Accumulation in Tissue Culture Plants

The fresh weight ratio of root:stem:leaf was about 1:4.4:5.7 and the corresponding DW ratio was 1:1.2:3.5. The moisture contents of the root, stem and leaf of 5-month-old tissue-culture plants were 69.67%, 91.48%, and 81.24%, respectively. The CPT concentrations in the root, stem, and leaf of 5-month-old *O. ridleyana* after elicitation treatment for 12 and 24 h was analyzed by HPLC (Table 2). In this study, no significant difference in CPT accumulation in the root, stem, and leaf was observed in response to elicitation with ethanol. However, significant increases in CPT content in the root and stem were detected following MeJA elicitations, particularly with 50 μM MeJA. However, no significant difference in CPT content in the leaves was observed after elicitation with MeJA. After 50 μM MeJA elicitation for 12 h, the highest contents of CPT observed in stems, roots, and leaves were 66.98, 50.79 and 15.85 $\mu\text{g/g}$ DW, respectively. Based on the average biomass (Table 1) and average CPT contents (Table 2), the CPT accumulation of 5-month-old tissue-culture plants of *O. ridleyana* treated with MeJA of 0–100 μM in ethanol was calculated (Table 3). The CPT content in the root and stem elicited with 50 μM MeJA for 12 h was 1.8- and 2.6-fold higher than that of the controls, respectively.

3.3. Effect of Electrical Stimulation on CPT Accumulation in *O. ridleyana*

3.3.1. Roots

The present study indicated an influence of DC intensity on CPT accumulation in plant cultures of *O. ridleyana*. Roots of 5-month-old tissue culture plants of *O. ridleyana* had higher CPT contents compared with stems and leaves. Average CPT concentrations in different parts of *O. ridleyana* treated with DC current at 10–40 mA are shown in Table 4. The DC stimulation resulted in significant increases in CPT content in all parts of the tissue culture plants (Table 4 and Figures 7–9). The highest CPT content in roots at 41.9 $\mu\text{g/g}$ DW was obtained from the stimulation by the DC current at 20 mA, followed by the stimulation at 30 mA, 40 mA and 10 mA, respectively.

Table 3. The estimated CPT content per 30 mL culture bottle of 5-month-old tissue culture plants of *O. ridleyana* treated with MeJA in ethanol (0–100 μ M) compared to the control.

Treatment	Incubation Time (h)	CPT ^a Content (μ g/ Culture Bottle)			
		Root	Stem	Leaf	Total
Control	-	1.5	1.7	2.4	5.7
Ethanol	12	1.7	1.6	2.4	5.8
	24	1.4	1.4	2.6	5.4
MeJA 20 μ M	12	1.9	1.7	2.7	6.3
	24	1.9	1.9	2.7	6.5
MeJA 50 μ M	12	2.8	4.5	3.0	10.3
	24	1.8	3.3	2.7	7.8
MeJA 100 μ M	12	1.7	1.7	2.3	5.7
	24	1.9	1.6	2.2	5.7

^a: calculation by using the average dry weight in Table 1.

Table 4. Average CPT concentrations in different parts of 5-month-old tissue culture plants of *O. ridleyana* treated with DC current at 10–40 mA and the estimated CPT content per 30 mL culture bottle.

Treatment	CPT Content (μ g/g DW)			CPT Content ^b (μ g/ Culture Bottle)			
	Root	Stem	Leaf	Root	Stem	Leaf	Total
Control	27.3 $\pm$ 1.3	21.3 $\pm$ 3.0	13.8 $\pm$ 2.7	1.5	1.4	2.6	5.5
DC 10 mA	33.5 $\pm$ 3.8 ^a	24.9 $\pm$ 3.5	15.9 $\pm$ 2.9	1.8	1.7	3.1	6.5
DC 20 mA	41.9 $\pm$ 2.7 ^a	36.0 $\pm$ 2.7 ^a	19.6 $\pm$ 2.1 ^a	2.3	2.4	3.8	8.4
DC 30 mA	36.4 $\pm$ 4.8 ^a	31.8 $\pm$ 2.4 ^a	16.7 $\pm$ 3.0	2.0	2.1	3.2	7.3
DC 40 mA	34.7 $\pm$ 3.3 ^a	26.1 $\pm$ 1.8	16.0 $\pm$ 3.4	1.9	1.8	3.1	6.7

^a: significant at $p < 0.05$ vs. control, ^b: calculation by using the average dry weight in Table 1.

Figure 7. Effects of DC intensities (10, 20, 30 and 40 mA) on CPT accumulation in *O. ridleyana* roots. The values are expressed as mean $\pm$ SD ($n = 3$); a: significant at $p < 0.05$ vs. control. **Figure 8.** Effects of DC intensities (10, 20, 30 and 40 mA) on CPT accumulation in *O. ridleyana* stems. The values are expressed as mean $\pm$ SD ($n = 3$); a: significant at $p < 0.05$ vs. control.

Figure 9. Effects of DC intensities (10, 20, 30 and 40 mA) on CPT accumulation in *O. ridleyana* leaves. The values are expressed as mean $\pm$ SD ($n = 3$); a: significant at $p < 0.05$ vs. control.

The HPLC chromatograms obtained from *O. ridleyana* roots of those treated with DC 20 mA showed a much larger peak area than those of the untreated controls (Supplementary Materials). It was also demonstrated that CPT concentrations in roots of all treated plants by DC stimulation were significantly higher in comparison to that of the untreated plant, which contained CPT at 27.3 $\mu\text{g/g}$ DW.

3.3.2. Stems

Compared to the control, by the DC stimulation, the CPT concentrations in *O. ridleyana* stems of all treated plants were relatively higher (Figure 8, Table 4). Significant higher CPT concentrations were obtained by DC stimulation at 20 mA and 30 mA, which were 36.0 and 31.8 $\mu\text{g/g}$ DW, respectively, whereas that of untreated plant was 21.3 $\mu\text{g/g}$ DW. The results, therefore, indicate that the electrical treatment is a promising tool to induce secondary metabolites in plant cells.

3.3.3. Leaves

The CPT concentrations in *O. ridleyana* leaves of all treated plants were also relatively higher than that of the control plants (Figure 9 and Table 4). The DC stimulation at 20 mA resulted in a significant increase in CPT content (19.6 $\mu\text{g/g}$ DW) compared to the non-treated plants (13.8 $\mu\text{g/g}$ DW). In this study, the electrical stimulation could effectively promote CPT accumulation in all parts of the treated tissue culture plants (roots, stems and leaves) of tissue culture plants of *O. ridleyana* Craib, but MeJA elicitation was found to promote CPT accumulation only in roots and stems.

Overall, by means of the DC stimulation at 20 mA, the total CPT accumulation of 5-month-old tissue culture plants of *O. ridleyana* in solid medium was increased to 8.4 $\mu\text{g/bottle}$, which was by approximately 1.5-fold increase as compared to that of the control.

4. Discussion

Tissue-culture plants can be exploited as an ecofriendly approach to produce secondary metabolites *in vitro* and protect endangered plants from extinction. The present results demonstrated the development of plant science technologies to enhance the productivity of CPT from *O. ridleyana* plants *in vitro*. Without the elicitation, the accumulation of CPT of 5-month-old tissue-culture plants of *O. ridleyana* was higher in roots, followed by stems and the leaves, respectively. Previously, Roja [12] reported CPT contents in plants of *O. rugosa* at 0.002% DW in roots, trace quantities in stems, and 0.0002% DW in leaves. Significant improvement in CPT production was attained by simple pouring of MeJA solution of 50 μM onto the surface of the solid medium and incubation for 12 h before harvesting, in which the CPT contents in roots and stems were at approximately 1.8- and 2.6- fold increase, respectively compared with that of the controls. The higher CPT content in roots and stems can be explained from the role of MeJA as a signal compound responsible for the induction of signal transduction, the expression of many defense genes followed by the

accumulation of secondary metabolites in plants. MeJA could act as a signal transducer in the enhancement of alkaloid biosynthesis. Previously, upon elicitation of hairy roots in a liquid medium, MeJA significantly enhances alkaloid production, particularly CPT in *O. liukuensis* hairy roots; however, this effect is not observed in *O. kuroiwai* and *O. pumila* [4]. On the other hand, elicitation by salicylic acid and yeast extract showed a repressive effect on alkaloid production [4]. The present results are also compatible with previous research by Xu et al. [30], who observed an increase in triterpenoids production in shake flask cultures of *Inonotus obliquus* in response to addition of MeJA as an exogenous elicitor and fatty acids as a stimulatory agent in liquid medium; the optimal elicitor concentration was 50 μ M MeJA. After elicitation for 6 d, the production of total triterpenoids was increased by 66% compared with that of the control. The kinetics of CPT accumulation in suspension cultures of *Camptotheca acuminata* in response to elicitation with jasmonic acid showed that CPT accumulation peaked at 4 d after treatment and thereafter, the CPT concentration rapidly decreased [11]. Previously, increased CPT production in multiple shoot cultures of *O. mungos* elicited with 150 μ M MeJA in 1/2 MS liquid medium for 72 h was also reported [21]. In the present study, the maximum increase in CPT content in the roots and stems of tissue-cultured plants of *O. ridleyana* in 1/2 MS solid medium was observed after the elicitation with 50 μ M MeJA for 12 h. However, no significant difference was noticed from the elicitation with pure ethanol. Ethanol has been reported as a small-molecule elicitor, which could alter the expression of secondary metabolic genes in tissue cultures of certain plant species. It was previously reported that addition of absolute ethanol (up to 5% v/v) to the suspension culture of *Fusarium solani* results in enhanced CPT production compared with that of the control [7]. However, in the current study, no significant effect of ethanol on CPT production by tissue-cultured plants of *O. ridleyana* was observed *in vitro*. These different responses may reflect differences in plant species, age of plants, cultivation methods, and elicitation procedures.

Under the 20 and 50 μ M MeJA treatments, only slight increases in CPT content in the leaves was observed. Accumulation of secondary plant metabolites, triterpenoids in *Centella asiatica* leaves might be triggered by postharvest application of MeJA [31]. However, in a study of the distribution of strictosidine synthase (STR), a crucial enzyme for CPT biosynthesis in different tissues of *O. pumila*, STR activity was detected in stem, root, and hairy root extracts, but no activity was detected in leaf and callus extracts [1]. It has been suggested that roots and stems are the predominant tissues in which CPT biosynthesis occurs [1]. The distribution of STR activity is correlated with the accumulation of mRNA and CPT production in *O. pumila* tissues. Presence of MeJA as a signaling molecule on the plasma membrane could initiate a signal transduction network inside the cells, leading to the expression of biosynthetic genes involved in the production of plant secondary metabolites of CPT in *Ophiorrhiza* [21,32]. In the present study, following elicitation with MeJA (50 μ M) for 12 h, the total CPT accumulation of whole plants of 5-month-old *O. ridleyana* cultured *in vitro* in solid medium was increased by approximately 1.8-fold as compared with that of the control (Table 3). In comparison with plant tissue culturing in liquid medium, plant culture on solid medium is simpler, requires less expensive equipment, consumes less energy, and is easier for large-scale production.

Furthermore, the use of electrical stimulation for CPT accumulation in tissue culture plants of *O. ridleyana* was investigated. The DC current at 10–40 mA with the total treatment time of 15 min was applied to 5-month-old *O. ridleyana* plants *in vitro*. Effects of electrical stimulation on some secondary plant metabolisms have been previously reported. Enhanced lycopene, β -carotene, total phenols contents and antioxidant activity were attained with a DC treatment of 500 mA with an application time of 15–30 min on *Solanum Lycopersicon* L. after an adaptation time of 2–24 h [27]. Electrical stimulation was also found to increase anthocyanin and resveratrol contents in berries of grapevines relative to those of control plants and electrode-treated plants [33]. Previously, the treatment by DC current demonstrated effects on the accumulation of some bioactive compounds in leaves. Applied DC induced alteration of cell membrane permeability due to DC could

have improved physiological processes, leading to enhanced accumulation of bioactive compounds such as chlorophylls carotenoids in African nightshade leaves [28]. Biosynthesis of secondary plant compounds is usually associated with plant defense response. Plant defense response can be induced by an elicitor and/or physiological stimulation such as changes in membrane potential and ion fluxes to trigger oxidative burst, resulting in the activation of defense-related genes for synthesis of secondary compounds [26,34]. It was suggested that DC influences the voltage-gated ion channels and increases the membrane permeability for calcium cations (Ca^{2+}) at the cellular level, followed by a rapid influx of Ca^{2+} through cation channels. Voltage-gated calcium (Ca^{2+}) channels are key transducers of membrane potential changes into intracellular Ca^{2+} transients that initiate many physiological events. Ca^{2+} plays an important role in signal transduction and in the absorption of nutrients across the cell membranes [35,36] and helps in the upregulation of respective genes for biosynthesis of secondary metabolites in plants such as polyphenols, flavonoid [37]. Therefore, electrical stimulation could be considered as physiological stimulation to induce plant secondary metabolism. The additional potential induced from external electric field in plants was reported to raise pressure on the membrane due to attraction between opposite charges on both sides of the membrane, whereby a faster transport of ions was induced simultaneously and this could cause an accumulation of minerals and bioactive compounds [28]. In the present study, the treatment by applied DC current at 20 mA could enhance the CPT content in roots, stems and leaves at 1.5-, 1.7- and 1.4-fold higher than that of the controls, respectively. The results indicate that electrical stimulation is also a promising tool to induce secondary metabolites in plant cells. The modified techniques used in this work are also of interest for potential application to other plant secondary metabolite production. The proposed procedures should be beneficial for the preharvest treatment of tissue-cultured plants to stimulate biosynthesis of secondary metabolic products. However, the detailed mechanism for electrical stimulation of plant tissue cultures to enhance the production of bioactive secondary metabolites is still unclear and needs further study.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11104555/s1>, Figure S1: HPLC chromatograms of the extracts of *O. ridleyana* on CPT content detected at 254 nm: (A) extracts from roots of the untreated controls, and (B) extracts from roots of the treated tissue-cultured plants with 50 μM MeJA and incubation for 12 h, Figure S2: HPLC chromatograms of the extracts of *O. ridleyana* on CPT content detected at 254 nm: (A) extracts from roots of the untreated controls, and (B) extracts from roots of those treated by electrical stimulation with DC 20 mA.

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