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Tetraploidization Increases the Contents of Functional Metabolites in *Cnidium officinale*

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Abstract: *Cnidium officinale* is an important medicinal crop grown in Asia for its pharmacological properties. In this study, tetraploid breeding was conducted to increases the content of medicinal compound and tolerance to the environmental conditions using in vitro shoot culture of *C. officinale*. For this, we generated tetraploid *C. officinale* plants using oryzalin, a chromosome doubling agent, and compared the morphological traits, cytological characteristics, and heat stress-responsive gene expression levels between tetraploid and diploid genotypes. Chromosome doubling efficiency was the highest in plantlets treated with 4.0 mg·L^{-1} oryzalin for 2 days. Compared with diploids, the plant height of tetraploids was reduced, while the petiole diameter was increased by approximately 39%. The dry matter of tetraploid leaves was significantly higher than that of diploid leaves. Compared with diploids, tetraploids showed higher chloroplast number and stomatal complex size but lower chlorophyll and carotenoid contents. The phenolic content of tetraploid plantlets was significantly higher than that of diploid plantlets. Contents of naringin as well as salicylic acid and gentisic acid, which are strong antioxidant compounds, were dramatically increased upon tetraploidization. Interestingly, liquid chromatography–mass spectrometry (LC–MS) analyses revealed increased levels of senkyunolide F and phthalide in tetraploid roots but not in tetraploid or diploid leaves.

Keywords: heat tolerance; tetraploid; secondary metabolites; phenolic compounds

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1. Introduction

Cnidium officinale Makino (Apiaceae) is a perennial plant cultivated in East Asia [1]. The dried rhizome of *C. officinale* is widely used in oriental medicine to treat female menstrual disorders by improving blood circulation [2,3]. *C. officinale* plants are rich in phenolic compounds and phthalides, such as ligustilide, butylidenephthalide, and cnidilide, which according to previous studies, possess pharmacological properties and are therefore used for the treatment of fatty liver disease, diabetes, and inflammation [2,4–6].

Field cultivation of *C. officinale* is limited by temperatures exceeding 30 °C during summer, and recent changes in global climate are mainly responsible for the decreasing yield and cultivable area of *C. officinale* [7,8]. High temperature is a typical stressor that inhibits plant growth and production by affecting photosynthesis, thus causing leaf senescence and reducing fruit development [8]. Sustaining crop yield simply by mitigating the effect of high temperature using different technologies and management systems is difficult [9]; therefore, various breeding strategies and methods have been developed to

generate heat-tolerant cultivars, such as individual selection, polyploid induction, and transformation [9–11]. In *C. officinale*, an efficient breeding method is needed for the development of a new high-temperature tolerant cultivar that contains more functional metabolites than the existing cultivars.

Polyploid induction is a breeding method widely used in plants to enhance the desired traits [12]. Generally, polyploidy is used to increase organ size in plants, with the ultimate goal to maximize plant yield or to enhance its ornamental value [12]. Induced polyploidy has also been used to enhance the physiological characteristics of plants [11,13]. Thus, genome doubling not only increases plant biomass but also improves environmental stress resistance and bioactive compound production [12]. Tetraploids developed in various plant species show increased tolerance to extreme temperatures; for example, tetraploid *Lonicera japonica* plants exhibit high temperature tolerance [11], and tetraploid *Erianthus arundinaceus* plants display low temperature tolerance [14].

To determine the heat resistance phenotype of a plant, it is critical to analyze the expression levels of various heat stress-responsive genes [15]. Heat shock proteins (HSPs) act as chaperones under heat stress [16], and play a key role in protecting plant proteins from damage and refolding denatured proteins [17]. Catalase (CAT) exhibits antioxidant activity and removes the oxidative stress-inducing reactive oxygen species (ROS) [18]. Cysteine protease (CP) is involved in plant senescence and ubiquitination-mediated protein degradation [19]. Genes encoding these proteins serve as molecular markers of high temperature tolerance in plants [20].

In this study, to develop a new *C. officinale* cultivar with high adaptability to diverse environmental conditions, specifically high temperature, and with increased levels of functional secondary metabolites, we induced tetraploidy in this species using oryzalin. We compared the cytological characteristics of tetraploid and diploid *C. officinale* genotypes, and examined the differences in their functional metabolite contents by performing high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) analyses. Furthermore, we analyzed the expression levels of heat stress-responsive genes in tetraploid to investigate their tolerance to high temperatures.

2. Materials and Methods

2.1. Plant Material

In vitro-cultured *C. officinale* clone 5 was used for tetraploid induction. Before treatment with oryzalin, a chromosome doubling agent, *C. officinale* clone 5 was maintained on Murashige and Skoog (MS) medium [21] containing 0.1 mg·L^{-1} benzyladenine (BA) in a tissue culture room at 24 ± 1 °C under 16 h light/8 h dark photoperiod.

2.2. Oryzalin Treatment

To determine the optimal conditions for polyploid induction, in vitro cultured *C. officinale* plants were treated with various concentrations of oryzalin using different methods and for different treatment durations. First, all leaves of the in vitro cultured plants were removed, and shoots (including the apical meristem) were cut into approximately 1-cm sections, which were then immersed in liquid MS medium containing 0.1 mg·L^{-1} BA and 0 (control), 1, 2, or 4 mg·L^{-1} oryzalin (Duchefa, The Netherlands). The explants were cultured on a shaker at 100 rpm for 1 or 2 days, and then shoots were transferred to solid MS medium containing 0.1 mg·L^{-1} BA. In another treatment, 0 or 1 mg·L^{-1} oryzalin was applied directly to the shoot tip of the plant cultured on solid medium. The experiment was replicated three times, with 30 explants per treatment. The pH of the culture medium was adjusted to 5.8 before autoclaving. All treatments were subcultured on a fresh medium every 4 weeks, and then incubated at 24 ± 1 °C under 16 h light/8 h dark photoperiod. The ploidy level of plants was analyzed after the second subculture.

2.3. Flow Cytometry

Flow cytometry was performed to determine the ploidy level of in vitro-grown *C. officinale* plants. Briefly, fresh staining solution was prepared on the day of analysis by mixing the staining buffer with the PI solution, and RNase solution. Leaf discs (0.5 cm^2) were prepared from the leaves of *C. officinale* and *Nicotiana tabacum* cv. Xanthi (internal standard) plants and placed in a Petri dish containing 200 µL of extraction buffer. Nuclei were isolated from the leaf discs using the CyStain PI Absolute P Kit (Partec, Germany), according to the manufacturer's instructions. The solution was filtered through a 50-µm nylon mesh. Then, 800 µL of staining solution was added to the filtered solution to label the DNA with a fluorescent dye. The samples were incubated in the staining solution on a stationary surface in the dark for 20 min, and then analyzed by CytoFLEX (Beckman Coulter Inc., Fullerton, CA, USA). At least 5000 nuclei were analyzed per sample.

2.4. Multiplication and Acclimatization

After confirmation of the ploidy level, the plants, which had different ploidy level, were given the line number, and then the plants were proliferated on MS medium containing 0.1 $mg \cdot L^{-1}$ BA up to 30 clones in each line. All plants in each line were transfer to hormone-free MS medium for rooting, and then rooted plants were used for acclimatization for further studies.

For acclimatization, one day prior to acclimatization, the lid of the culture vessel was opened by ~20% for adaptation to the ex vitro conditions. Then, diploid (20 plants) and 3 lines of tetraploids (20 plants per each tetraploid line) were harvested from the vessel, carefully washed with distilled water, and planted in wet Growfoam (Horticubes, Smithers-Oasis, KENT, OH, USA). The plants in Growfoam were placed in a plastic container ($25 \times 25 \times 3$ cm), and water was changed every week. In all lines, 100% plants were successfully acclimatized. After 3 months of growing ex vitro, the growth of plants was investigated and the whole plants were used for HPLC analysis, and then plants were transplanted and cultivated in plastic pots (diameter 10 cm) filled with artificial soil mixture (peat moss 1:perlite 1:vermiculite 1). Potted plants were fertilized with 1.0 $g \cdot L^{-1}$ Hyponex solution (N20:P20:K20) one time per month. The 6-month-old plants were used for LC-MS analysis (Figure S1).

2.5. Growth and Cytological Characterization

2.5.1. Growth Characteristics

After 3 months of ex vitro cultivation, the effect of ploidy level on plant growth was investigated based on the evaluation of 10 clonally multiplicated plants at each ploidy level. First of all, the fresh weight of the plant was measured. After that morphological traits including leaf number per plant, leaf area, petiole length, petiole diameter, and leaf shape were measured in ex vitro-grown *C. officinale* plants from diploid and tetraploid (line 81-12). Only fully developed leaves were counted to determine the leaf number per plant. To determine the leaf area, three fully developed leaves were randomly selected from each plant and analyzed by an LI-3000C Portable Leaf Area Meter (LI-COR, Lincoln, NE, USA). Petiole diameter was measured at the midpoint of the petiole using a Vernier caliper. For the dry matter, the plants were dried in 50 °C dry oven for one week, and then weighed. Dry matter was calculated as dry weight divided by fresh weight multiplied by 100.

2.5.2. Cytological Analysis

For microscopic observation, leaves were harvested from 10 randomly selected three-month-old plants (after three-month ex vitro growing) of each ploidy level. The leaf epidermal cells were peeled off using a pair of tweezers and mounted on a slide, which was then observed under an OLYMPUS BX40 microscope (Olympus, Tokyo, Japan). Chloroplast number per cell, chloroplast area, stomata length and width, and guard cell area were measured using LAS V4.5 (Leica-microsystems, Wetzlar, Germany).

2.5.3. Pigment Quantification

To determine the chlorophyll and carotenoid contents of *C. officinale* plants, 50–100 mg of leaf tissue collected from 10 plants at each ploidy level was immersed in 6 mL of 80% acetone and incubated in the dark for 48 h. The absorbance of the resulting extract was measured at 663.2, 646.8, and 470 nm wavelengths using a spectrophotometer (Uvikon-930, Kontron Instruments, Zurich, Switzerland). The chlorophyll *a*, chlorophyll *b*, and carotenoid contents were calculated according to the following equations [22]:

$$Chl_a = (12.25 \times A_{663.2}) - (2.79 \times A_{646.8}), \quad (1)$$

$$Chl_b = (21.5 \times A_{646.8}) - (5.1 \times A_{663.2}), \quad (2)$$

$$Chl_{a\text{-}b} = (7.15 \times A_{663.2}) + (18.71 \times A_{646.8}), \quad (3)$$

$$Carotenoid = (1000 \times A_{470}) - (1.82 \times Chl_a) - (85.02 \times Chl_b)/198, \quad (4)$$

where Chl_a and Chl_b represent chlorophyll *a* and chlorophyll *b* contents, respectively, and $A_{663.2}$, $A_{646.8}$, and A_{470} represent absorbance values measured at 663.2, 646.8, and 470 nm, respectively.

2.6. Extraction and Analysis of Funtional Secondary Metabolites

2.6.1. Extraction and Determination of Phenolic Compounds by HPLC

A whole plant (3-month-old after acclimatization) from each tetraploid line (56-9, 81-12, and 81-18) and a diploid *C. officinale* were collected and freeze-dried until analyzed. For this around 20 plants were used from each line of tetraploid and a diploid, and three replicated samples were prepared. For the extraction, those samples were ground to a fine powder using a sterilized mulberry bowl. Then, 250 mg of powdered samples were sonicated (Sonicator, Mujigae, Korea) in 80% methanol for 1 h to ensure complete extraction. The extract was filtered through a filter paper (Advantec 110 mm, Japan), and the solvent was evaporated. The dried residue was dissolved in 10% methanol, fractionated twice with 10 mL of diethyl-ether/ethyl-acetate (1:1, *v*/*v*), and then evaporated under vacuum to complete dryness. Residues of both fractions were combined and dissolved in methanol before filtration through a 0.2-µm membrane filter (Whatman, England). A PDA-equipped HPLC system (2690 Separations Module, Waters Chromatography, Milford, MA, USA) was used to measure the phenolic compounds. The separation of phenolic compounds was performed using a Fortis C18 column (5 µL, 150 mm × 4.6 mm). A 20-µL aliquot of each sample was injected into the HPLC system, and the column was re-equilibrated for 10 min between injections. The sample was eluted at a flow rate of 1.0 mL min^{-1} using acetonitrile (A) and 0.1% aqueous acetic acid (*v*/*v*) (B) as the mobile phase, with the following linear gradient: 8%–10% A for 0–2 min, 10%–30% A for 2–27 min, 30%–90% A for 27–50 min, 90%–100% A for 50–51 min, 100% A for 51–60 min, and 100%–8% A for 60–70 min. Calibration plots were constructed by measuring the peak areas. Ultraviolet (UV) absorption spectra and retention time were used as the criteria for the identification of individual compounds.

2.6.2. Metabolite Profiling

The metabolite profiling of the leaf and root samples of 6-month-old diploids and tetraploids was conducted by performing liquid chromatography coupled with quadrupole time-of -flight tandem mass spectrometry (LC–QTOF-MS/MS). HPLC analysis was carried out on the Shiseido C18 column (5 µm I.D., 4.6 mm × 150 mm) using the 1260 Infinity II LC System (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), which were applied with the following gradient elution: 5% B (0–5 min) and 5–95% B (5–30 min). The flow rate was 0.6 mL/min. A UV chromatogram was recorded at 220, 254, 280, 330, and 365 nm. Agilent Technologies 6530 Accurate-Mass Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA) was connected to the HPLC system with an electrospray ionization

(ESI) interface and ionized in negative mode. Data acquisition and processing were carried out by Mass Hunter Workstation software LC/MS Data Acquisition for 6530 series QTOF (version B.05.00).

2.7. Heat Stress Treatment

To examine the effect of ploidy level on high temperature tolerance, 30 diploids and 30 tetraploid *C. officinale* plants grown ex vitro were exposed to 32.5 °C in a growth chamber for 0, 6, and 12 h. After the heat treatment, leaves were harvested, frozen in liquid nitrogen, and cryopreserved for gene expression analysis.

2.8. Gene Expression Analysis

2.8.1. RNA Isolation and cDNA Synthesis

Frozen leaf samples (100 mg), with 10 replications, were pulverized in 1 mL of Nucleo-ZOL reagent (MACHEREY-NAGEL, Düren, Germany) with a stainless ball in TissueLyserII (QIAGEN, Hilden, Germany). Then, 400 µL of sterilized water was added to the tissue suspension and shaken vigorously for approximately 15 s. The sample was allowed to rest at room temperature for 15 min and then centrifuged at 12,000× *g* at 4 °C. The supernatant was transferred to a clean tube containing 1 mL of isopropanol. The mixture was allowed to rest for another 10 min and centrifuged at 12,000× *g* for 10 min at 4 °C. The supernatant was discarded, and the pellet was washed twice with 75% ethanol and then centrifuged at 3000× *g* for 3 min. The pellet was resuspended in 50 µL of sterile water by incubation at 65 °C for 5 min. Then, cDNA was synthesized from the isolated RNA using a ReverTra Ace® qPCR Master Mix (TOYOBO, Osaka, Japan), and diluted to a concentration of 100 ng·µL^{-1} for gene expression analysis.

2.8.2. Quantitative Real-Time PCR (qRT-PCR)

The expression of heat-responsive genes, including *CoHSP*, *CoCAT*, and *CoCP*, was analyzed by qRT-PCR. First, 10 µL of SYBR Premix Ex Taq (Takara, Japan) and 2 µL of cDNA were mixed with 1 µL each of forward and reverse gene-specific primers. Then, 6 µL of sterile water was added to the sample to adjust the total volume to 20 µL. To obtain accurate results, air bubbles were removed from each sample. Then, qRT-PCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The *CoActin* gene was used as an internal reference for data normalization. Primers used for qRT-PCR were designed based on the NCBI nucleotide database and are listed in Table S1.

2.9. Statistical Analysis

One-way analysis of variance (ANOVA) was performed to determine significant differences between plants of different ploidy levels. Relative changes in growth were calculated by dividing the growth of tetraploid plants by the that of diploid plants. The statistical significance of the differences between mean values was then assessed by Duncan's multiple range test at $p < 0.05$. All statistical analyses were performed using the SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Chromosome Doubling

Oryzalin did not induce tetraploidization at a concentration of 1.0 mg·L^{-1} (Table 1). Treatment with oryzalin at 2.0 mg·L^{-1} or higher concentration produced mixoploids (Figure 1). Tetraploids appeared only in the 4.0 mg·L^{-1} oryzalin treatment (Table 1). The survival rate of *C. officinale* plants decreased sharply at concentrations above 4.0 mg·L^{-1} (data not shown). No tetraploids were induced when a drop of oryzalin was directly applied to the shoot tip of in vitro cultured plantlets. We verified the ploidy level by flow cytometry (Figure 1). A 2C peak was observed in approximately 1.5 × 10^6 cells in the

phycoerythrin-A (PE-A) fluorescence channel, and 4C and 8C peaks were observed in 3×10^6 and 6×10^6 cells, respectively.

Table 1. Effect of various concentrations of oryzalin on polyploid induction in *C. officinale*.

Oryzalin		Survival Rate (%) [1]	Rate of Mixoploid Induction (%) [1]	Rate of Polyploid Induction (%) [1]
Conc. (mg·L^{-1})	Time (Days)			
Control		100.00 ± 0.00 a	0.00 ± 0.00 b	0.00 ± 0.00 c
Addition in culture medium				
1	1	100.00 ± 0.00 a	0.00 ± 0.00 b	0.00 ± 0.00 c
	2	86.61 ± 5.85 bc	0.00 ± 0.00 b	0.00 ± 0.00 c
2	1	92.86 ± 4.12 b	3.13 ± 3.13 ab	0.00 ± 0.00 c
	2	84.38 ± 9.38 c	0.00 ± 0.00 b	0.00 ± 0.00 c
4	1	89.73 ± 6.78 bc	3.57 ± 3.57 ab	3.13 ± 3.13 b
	2	82.59 ± 6.93 c	9.82 ± 6.08 a	5.00 ± 5.00 a
Drop on in vitro shoot tip		100.00 ± 0.00 a	0.00 ± 0.00 b	0.00 ± 0.00 c

[1] Data represent mean ± standard error (SE). Different lowercase letters indicate significant differences ($p < 0.05$; Duncan's multiple range test).

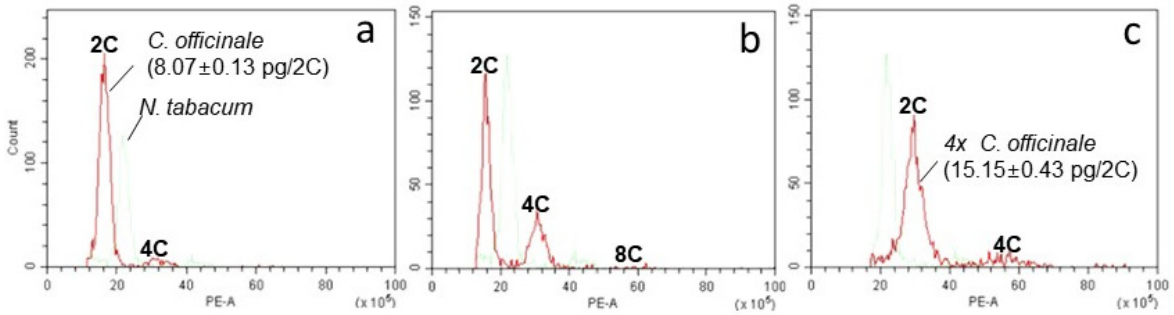

Figure 1. Analysis of the ploidy level of *C. officinale* plants using flow cytometry. (**a**–**c**) Histograms showing the DNA content of diploids (control) (**a**), mixoploids (**b**), and tetraploids (**c**). *Nicotiana tabacum* cv. Xanti (10.07 pg/2C; yellow-green peak) was used as an internal standard.

3.2. Growth and Cytological Characterization

Ex vitro-grown *C. officinale* plants showed no significant differences in leaf number, bud number, leaf area, and fresh weight (Table 2, Figure 2a,b). Compared with diploid *C. officinale* plants, the petiole length of tetraploid plants was shorter, whereas the petiole diameter was larger. Diploids and tetraploids also showed differences in leaf shape. The leaves of tetraploids were more tortuous than those of diploids, and the gap between the leaflets was less in tetraploids than in diploids (Figure 2a). In diploids, the dry matter content of leaves and roots was highly similar; however, in tetraploids, the dry matter content of leaves was significantly greater than that of roots (Figure 2b). Microscopic observation of the leaf epidermal cells revealed that the number of chloroplasts and the size of the stomatal complex increased with the increase in ploidy level (Figure 3 and Table 3). However, no significant difference was detected in the chloroplast area between diploids and tetraploids.

Table 2. Measurements of the morphological traits of diploid and tetraploid (line 81-12) ex vitro-grown *C. officinale* plants.

Ploidy Level	No. of Leaves per Plant [1]	No. of Shoots per Plant [1]	Leaf Area (cm^2) [1]	Petiole Length (mm) [1]	Petiole Diameter (mm) [1]
Diploid	7.57 ± 1.00	3.14 ± 0.86	38.69 ± 3.84	134.88 ± 4.14 a	1.93 ± 0.03 b
Tetraploid	6.50 ± 1.34	3.67 ± 0.49	37.71 ± 3.84	114.89 ± 2.19 b	2.69 ± 0.08 a
Significance	ns	ns	ns	**	*

[1] Data represent mean ± SE. Different lowercase letters indicate significant differences ($p < 0.05$; Duncan's multiple range test; * $p < 0.05$; ** $p < 0.01$). ns, non-significant.

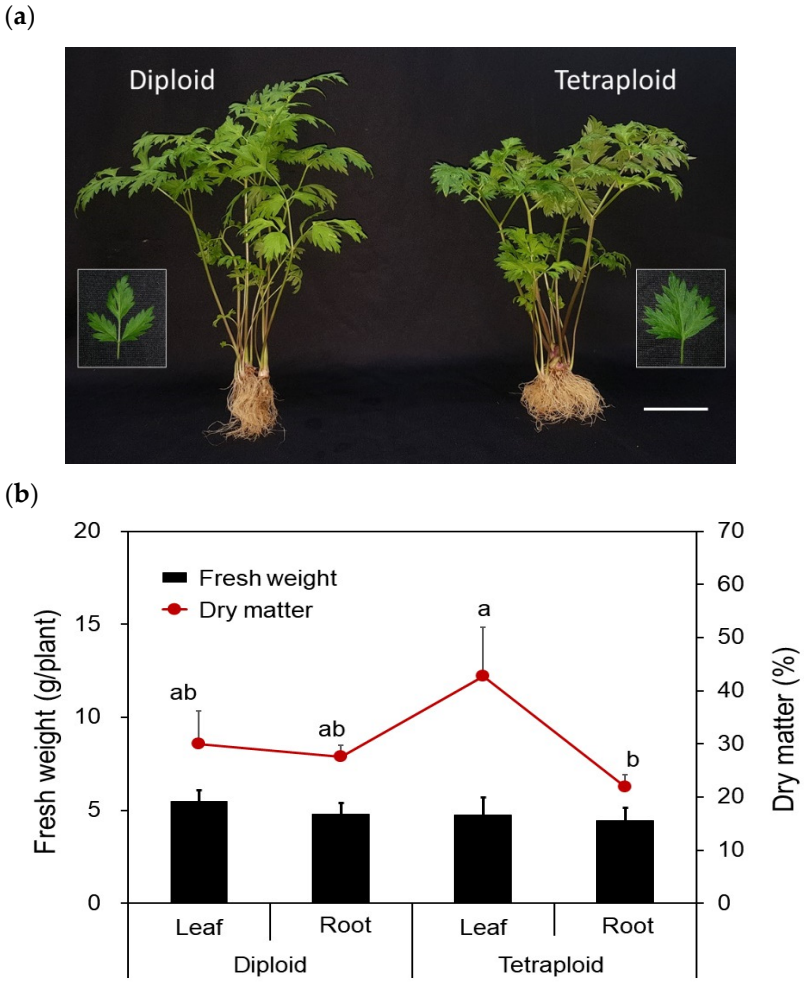

Figure 2. Phenotypic and quantitative analysis of the growth of diploid and tetraploid (line 81-12) in *C. officinale*. (**a**) Morphology of diploid and tetraploid plants. Scale bar = 5 cm. (**b**) Fresh weight and dry matter content of diploid and tetraploid plants. Data represent mean ± SE. Different lowercase letters indicate significant differences ($p < 0.05$; Duncan's multiple range test).

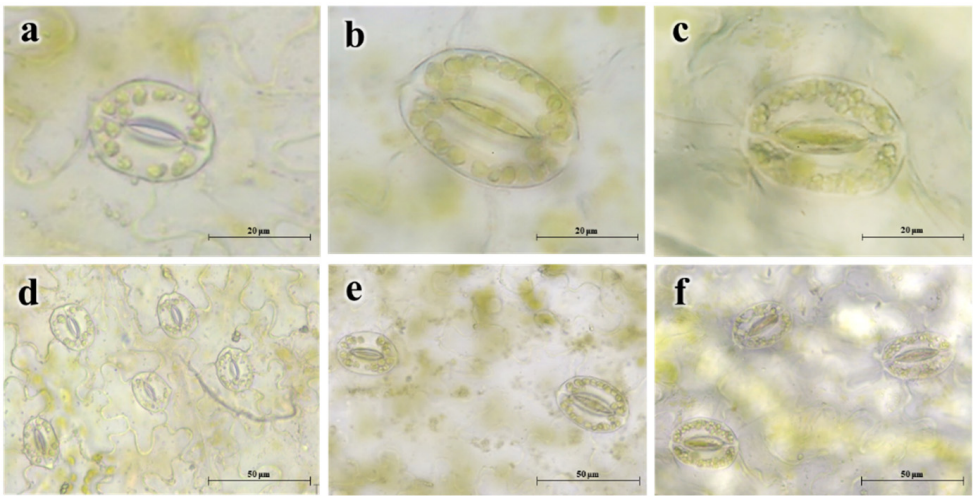

Figure 3. Microscopic analysis of the stomatal complex and chloroplasts in diploid, mixoploid, and tetraploid *C. officinale* plants. (**a**,**d**) Stoma of diploid; (**b**,**e**) stoma of mixoploid; (**c**,**f**) stoma of tetraploid. Scale bar = 20 µm (**a**–**c**), 50 µm (**d**–**f**).

Table 3. Measurements of the cytological traits of leaf epidermal cells of diploid, mixoploid, and tetraploid *C. officinale* plants.

Ploidy Level	No. of Chloroplasts per Guard Cell [1]	Chloroplast Area (μm^2) [1]	Stoma Length (μm) [1]	Stoma Width (μm) [1]	Guard Cell Area (μm^2) [1]
Diploids	10.70 ± 0.04 c	5.94 ± 0.42 ab	26.48 ± 0.38 c	20.13 ± 0.36 b	184.06 ± 3.05 b
Mixoploids	12.40 ± 0.04 b	7.79 ± 0.67 a	30.68 ± 1.56 b	23.74 ± 0.84 a	293.56 ± 13.75 a
Tetraploids	20.80 ± 0.05 a	4.17 ± 0.38 b	33.60 ± 0.91 a	25.92 ± 0.68 a	275.42 ± 21.51 a

[1] Data represent mean ± SE. Different lowercase letters indicate significant differences ($p < 0.05$; Duncan's multiple range test).

3.3. Pigment Content Analysis

The chlorophyll *a* content of tetraploid and mixoploid plants was lower than that of diploid plants; however, no difference was observed between the chlorophyll *a* content of tetraploids and mixoploids (Figure 4). Moreover, no significant difference was detected in the chlorophyll *b* content of plants at different ploidy levels. Consequently, the ratio of chlorophyll *a* to chlorophyll *b* was lower in mixoploids and tetraploids than in diploids. Additionally, the carotenoid contents in mixoploid and tetraploid plants were significantly lower than in diploid ones.

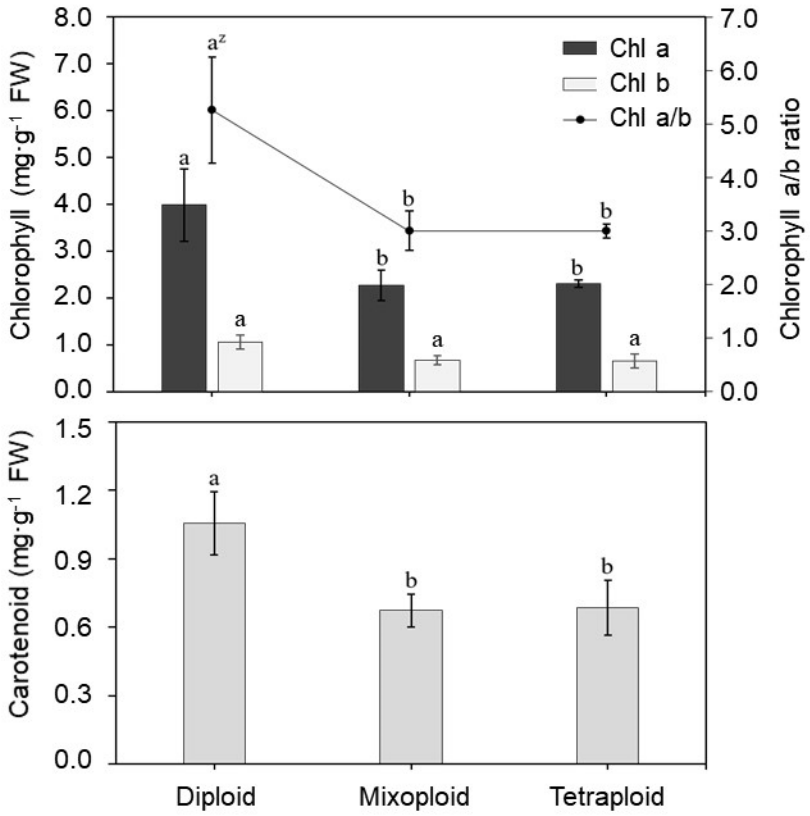

Figure 4. Chlorophyll and carotenoid contents of diploid, mixoploid, and tetraploid *C. officinale* plants. Data represent mean ± SE. Different letters indicate significant differences ($p < 0.05$; Duncan's multiple range test).

3.4. Changes in Functional Metabolites by HPLC and LC–MS

Fifteen phenolic compounds isolated from three tetraploid lines (56-9, 81-12, and 81-18) were analyzed by HPLC. The contents of gentisic acid, salicylic acid, and naringin were the highest among the 15 phenolic compounds analyzed in diploid and 3 lines of tetraploids (Figure 5). The total phenolic content of tetraploid plants was significantly

higher than that of diploid plants; however, variation in total phenolic content was found among the tetraploid individuals.

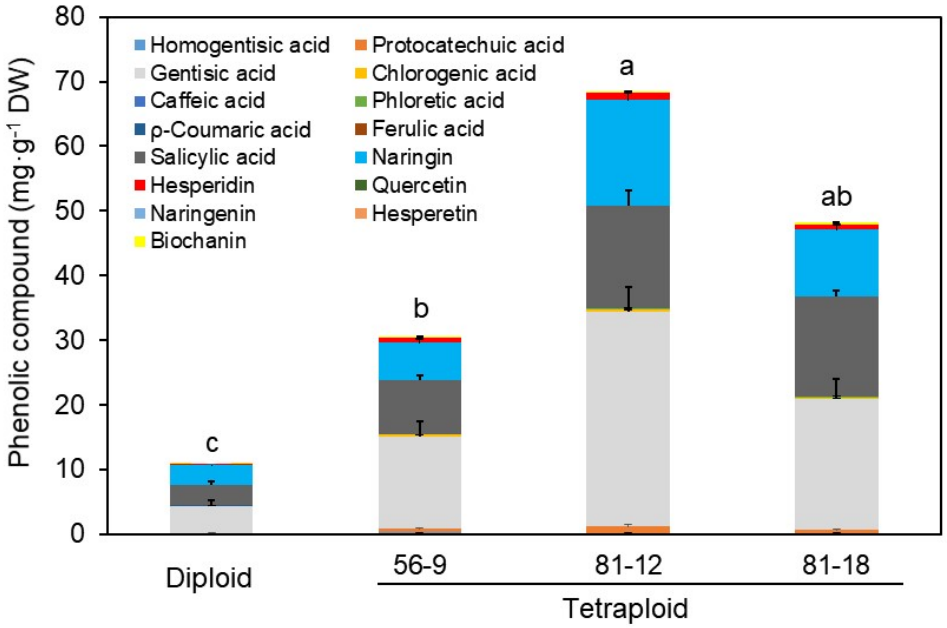

Figure 5. HPLC analysis of the contents of various phenolic compounds in whole plants of diploid and three tetraploid lines (56-9, 81-12, and 81-18) after 3 month of ex vitro cultivation in *C. officinale*. Different lowercase letters above the bars indicate significant differences ($p < 0.05$; Duncan's multiple range test).

To obtain a metabolite profile of the crude extract of diploid and tetraploid *C. officinale* plants, an analytical method based on LC–MS was developed (Figure 6). Individual components were identified by comparing their *m/z* values in the total ion count (TIC) profile with those of the selected compounds described in Table 4. The LC–MS profile highlighted the presence of a group of compounds corresponding to the protonated molecular ions of different caffeoylquinic acids (peaks 3–6). The quantity of these compounds were similar in shoots of diploid and tetraploid plants. Interestingly, unlike the results of shoots, the roots of tetraploids contained more senkyunolide F (peak 8) and phthalides (senkyunolide B and senkyunolide C; peak 9), which are specific functional metabolites in *C. officinale*.

3.5. Expression Levels of Heat Stress-Responsive Genes

We performed qRT-PCR to examine the expression levels of heat-responsive genes in diploid and tetraploid *C. officinale* plants exposed to high temperature (32.5 °C). No significant difference was detected in *CoHSP* expression among the different heat treatment durations or ploidy levels (Figure 7). Unlike *CoHSP*, the *CoCP* gene showed changes in expression between pre- and post-heat treatments. In a diploid, the expression level of this gene increased significantly under the influence of high temperature and was highest after 12 h. In contrast, in the tetraploid, a significant increase in *CoCP* expression was only visible after 12 h and the level of this expression was lower than that observed in the diploid at the same time. The expression level of *CoCAT* decreased in both diploids and tetraploids after 12 h of heat treatment, and the expression pattern of this gene was similar in both genotypes.

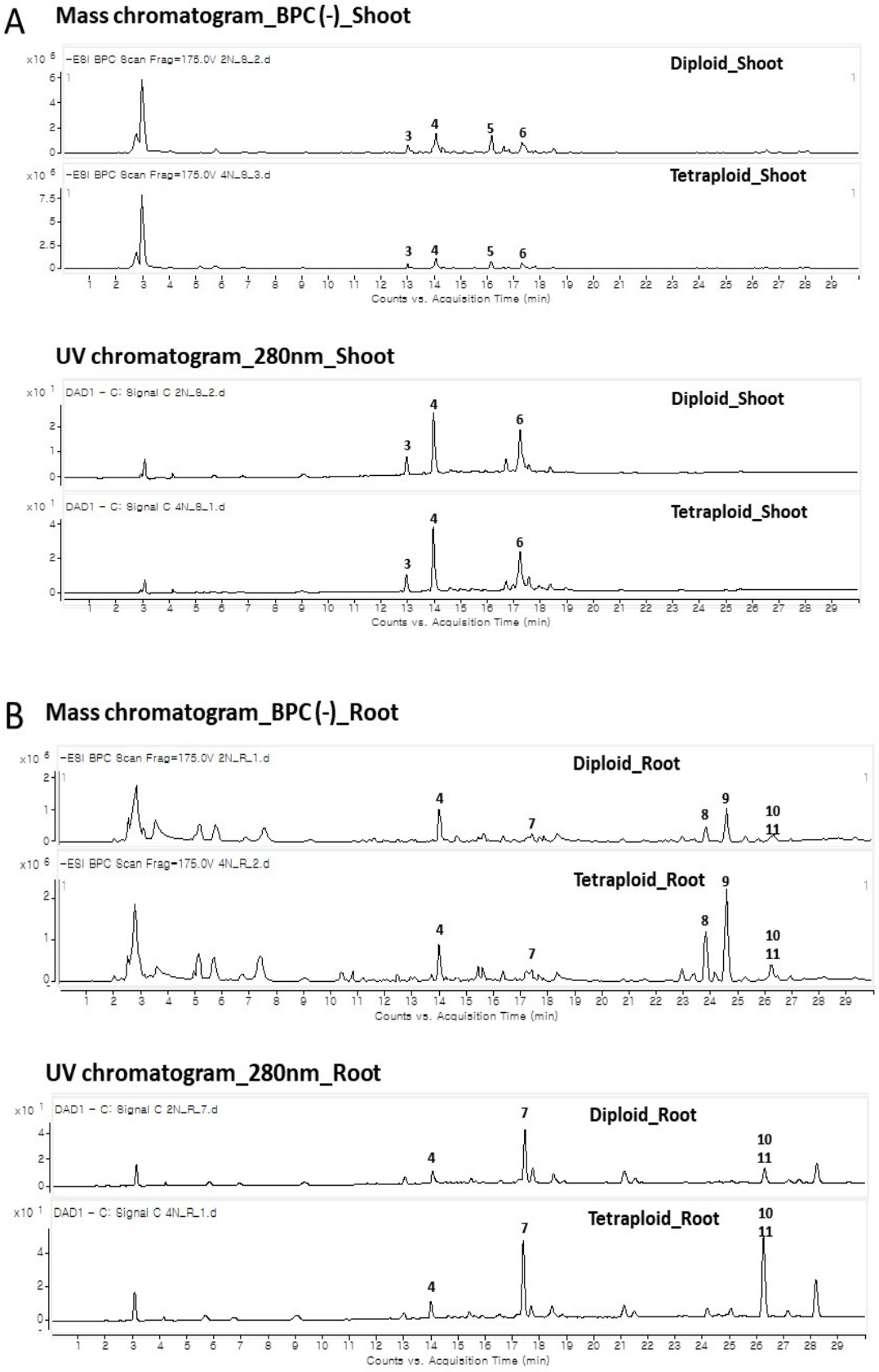

Figure 6. LC–MS profiles of the methanol extracts of the leaves (**A**) and roots (**B**) of 6-month-old diploid and tetraploid (line 81-12) *C. officinale* plants. Samples were analyzed in the negative ion mode. Peak numbers are described in Table 4.

Table 4. LC–MS profiles of individual compounds detected in the methanol extract of di- and tetraploid in *C. officinale*.

Peak No.	Expected Compounds	t_R (min)	Observed *m/z*	Calculated *m/z*	Molecular Formula $[M-H]^-$	MS/MS Fragments (*m/z*)	Collision Energy (eV)	UV (λmax, nm)
1	Unidentified	10.417	383.1557	383.1559	$C_{15}H_{27}O_{11}$	101 $[M-282-H]^-$	20	
2	Unidentified	11.663	335.0444	335.0444	$C_8H_{15}O_{14}$	172 $[M-163-H]^-$	30	272
3	Neochlorogenic acid(5-caffeoylquinic acid)	12.971	353.0864	353.0864	$C_{16}H_{17}O_9$	191 $[M-C_9H_6O_3-H]^-$	20	298, 324
4	Chlorogenic acid(3-caffeoylquinic acid)	14.033	353.0865	353.0864	$C_{16}H_{17}O_9$	191 $[M-C_9H_6O_3-H]^-$	30	300, 327
5	Quinic acid derivative	16.163	417.1748	417.1766	$C_{19}H_{29}O_9$ [a]	191 [M-180-HCOOH-H]$^-$	10	
6	Dicaffeoylquinic acid	17.288	515.1177	515.1177	$C_{25}H_{23}O_{12}$	191 $[M-C_9H_6O_3-C_9H_6O_3-H]^-$	20	328
7	Unidentified	17.471	193.0505	193.0506	$C_{10}H_{10}O_4$	134 $[M-59-H]^-$	20	295, 323
8	Senkyunolide F	23.903	205.0868	205.0870	$C_{12}H_{13}O_3$	161 $[M-CO_2-H]^-$	10	
9	Phthalide (senkyunolide B, C)	24.652	203.0710	203.0714	$C_{12}H_{11}O_3$	160 $[M-C_3H_7-H]^-$	20	
10	Unidentified	26.340	223.0970	223.0976	$C_{12}H_{15}O_4$	161 $[M-62-H]^-$	20	
11	Unidentified	26.403	311.2228	311.2228	$C_{18}H_{31}O_4$	171 $[M-140-H]^-$	20	

[a] M + HCOO.

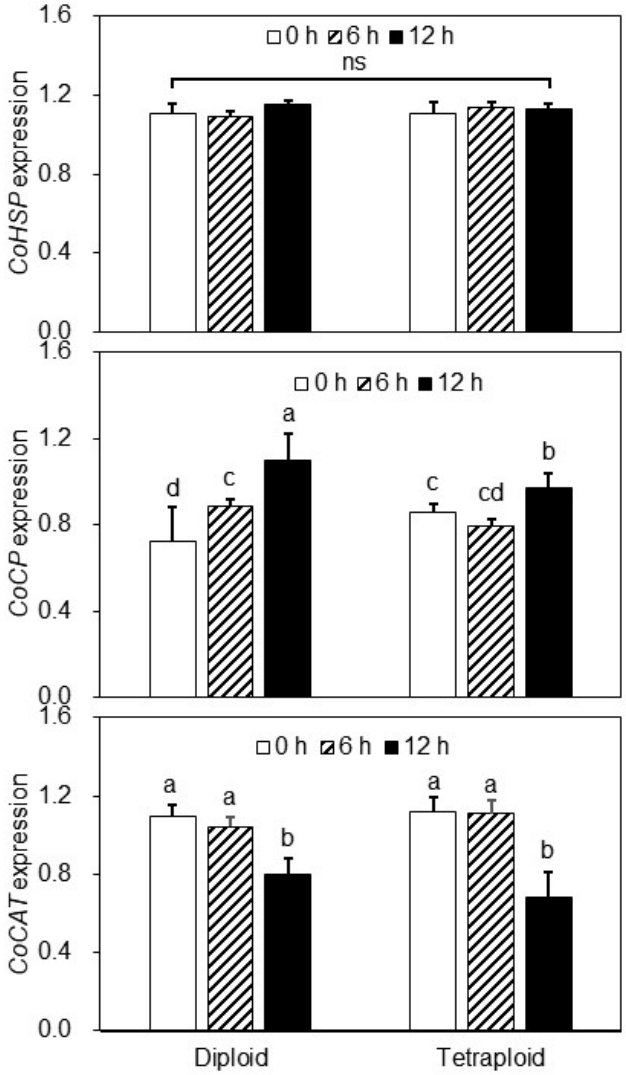

Figure 7. Effect of high temperature treatment on the expression of heat stress-responsive genes (*CoHSP*, *CoCP*, and *CoCAT*) in diploid and tetraploid (line 81-12) *C. officinale* plants. *CoActin* was used as a reference gene. Data represent mean ± SE. Different letters indicate significant differences among treatments in diploids and tetraploids ($p < 0.05$; Duncan's multiple range test). ns, non-significant.

4. Discussion

Artificial polyploid induction uses antimitotic chemicals such as oryzalin and colchicine [23]. Because colchicine is toxic to plants and humans [24–26], oryzalin and trifluralin have emerged as alternative chemicals for polyploid induction [27,28]. Oryzalin is more effective than colchicine in genome doubling of orchids and cassava [24,29]. In previous studies, polyploids were induced using oryzalin at concentrations ranging from approximately 289 μM to 15 mM [23,29]. In addition to the antimitotic concentration, also the treatment method and the treatment time also affect tetraploid induction in plants [29]. Carvalho et al. [29] reported that the growth of cassava plants was inhibited when exposed to oryzalin-containing liquid medium for 2 days. However, no such phenomenon was observed in the 2-day treatment group in the current study. Regarding the successful induction of tetraploids, prolonged exposure to oryzalin decreases the survival of explants but increases the chance of polyploid induction [29].

In the current study, oryzalin was most effective at inducing tetraploids at a concentration of approximately 4.0 $mg \cdot L^{-1}$, consistent with the results of previous studies [12,13,24,25]. Importantly, no tetraploids were induced when a drop of oryzalin was directly applied to the shoot tip of in vitro cultured *C. officinale* plants. These results indicate that 4.0 $mg \cdot L^{-1}$ oryzalin is the most effective at inducing tetraploidy in *C. officinale* in vitro, and any concentration above 4.0 $mg \cdot L^{-1}$ is toxic.

Herein, there was no significant difference in the shoot and leaf numbers and the leaf area between diploid and tetraploid plants, but there was a difference in the leaf shape and petiole size. Kaensaksiri et al. [30] reported an increase in fresh weight with the increase in ploidy level in *Centella asiatica*. In *Calanthe*, Chung et al. [31] reported an increase in leaf width following polyploid induction. Chaves et al. [32] reported no correlation between ploidy level and leaf area; however, differences were observed in leaf shape between diploids and tetraploids.

In our study, the dry matter of leaves of tetraploid *C. officinale* plants was higher than that of diploid plants. The water content of a plant is an important factor that affects adaptation to environmental stresses [8]. In wheat (*Triticum aestivum*), Sharma et al. [33] reported that the quantum yield of photosystem II (PSII), estimated as Fv/Fm, and dry matter content are higher in heat tolerant cultivars than in heat sensitive cultivars.

Here, we found that the number of chloroplasts and the size of the stomatal complex were higher in tetraploid *C. officinale* plants than in diploid plants; however, no difference was detected in chloroplast size between the two ploidy levels. This is consistent with the study of Kaensaksiri et al. [30], who also reported a higher stomata index in tetraploid *Centella asiatica* plants compared with their diploid counterparts. Chaves et al. [32] showed that the number of chloroplasts in *Cynodon dactylon* increased in proportion to the number of chromosomes, but the size of chloroplasts decreased. In *Anthurium andraeanum*, Winarto et al. [34] reported that the number of chloroplasts increased with the increase in chromosome number, but the size of chloroplasts decreased. Chlorophyll and carotenoids are two of the main pigments of plant chloroplasts. Previously, Mansouri and Bagheri [35] reported no differences in chlorophyll and carotenoid contents between diploid and tetraploid *Cannabis sativa* plants. However, in *L. japonica*, Li et al. [11] showed that the chlorophyll content of diploid plants is higher than that of tetraploids.

Previous studies have shown that chromosome doubling increases the plant biomass, bioactive compound yield, and environmental stress resistance. Ciccoritti et al. [36] reported that the total phenolic content of *Triticum* subspecies, which differ in ploidy level, can vary by up to 3-fold. Xu et al. [14] found that tetraploidization in *Echinacea purpurea* enhances not only the content of caffeic acid derivatives, alkamides, and phenylalanine but also that of chicoric acid by increasing the production of ammonia lyase and cinnamate 4-hydroxylase enzymes. However, in *Solanum bulbocastanum*, the contents of phenylpropanoids, tryptophan, tyrosine, and α-chaconine are lower in tetraploids than in diploids [37]. This indicates that chromosome doubling does not increase the production of bioactive compounds in all plant species. In this study, all tetraploid individuals

showed higher levels of phenolic compounds than diploid counterpart, suggesting that polyploidization could be used as a breeding method in *C. officinale* for increasing the abundance of secondary metabolites.

Polyploidization affects not only the level of functional metabolites but also increases the tolerance to environmental stresses in some plant species. For example, polyploidy induction increased tolerance to high temperature in *L. japonica* [11] and to low temperature in *E. arundinaceus* [14]. Tolerance to extreme temperatures can be easily detected by analyzing the expression levels of specific genes under critical temperatures. In creeping bentgrass, Jespersen et al. [20] reported that the expression level of *HSP70* was higher in heat-susceptible individuals, whereas that of *HSP26* was higher in heat-resistant individuals. These differences arise from the slightly different functions of HSP family members under stress conditions. CoHSP belongs to a small HSPs (sHSPs), which exhibit a molecular weight of 15–40 kDa, and a structurally diverse group of heat-induced proteins [17]. Recent study indicated that sHSPs act in preventing thermal aggregation of other proteins, However, the function was also different depending on the type of sHSPs [17]. In our study, *CoHPS* gene expression was not altered neither in diploid nor tetraploid plants under the heat treatment.

Thus our results suggest that *CoHSP* cannot be used as a molecular marker to distinguish between heat-susceptible and -resistant individuals in *C. officinale*.

CP is involved in leaf senescence. Consistently, Jespersen et al. [20] reported a lower level of *CP* expression in heat-tolerant bentgrass genotypes than in heat-sensitive genotypes under heat stress. In our study, the expression level of *CoCP* in diploids increased significantly as the high-temperature treatment time increased, whereas in tetraploids, the dynamics of the rise of this gene expression was smaller. CP is involved in the degradation of cell membrane proteins in plants during aging or under adverse conditions [19]. In many plant species, including bentgrass, the *CP* gene is used as a molecular marker of high temperature sensitivity [20]. This suggests that the *CoCP* gene could be used as one of the molecular marker candidates for the selection of heat resistant genotypes in *C. officinale*. CAT acts as a primary ROS scavenger in plants under stress conditions [18,38]. Therefore, CAT activity is generally positively correlated with the degree of stress [39]. Because the expression level of *CAT* decreases after ROS eradication [38], it is possible that the *CAT* gene is upregulated to eliminate ROS. However, in this study, the *CoCAT* expression pattern did not show a significant difference between diploid and tetraploid.

Compared with diploid *C. officinale* plants, tetraploid plants showed no clear morphological changes in response to high temperatures (data not shown). This suggests that a more severe stress treatment is required to enable the visualization of high temperature-induced damage in ex vitro-grown *C. officinale* plants.

In conclusion, our results showed that immersion in 4.0 $mg \cdot L^{-1}$ oryzalin for 2 days is the most effective treatment for the induction of polyploidy in *C. officinale*. Tetraploid *C. officinale* plants had shorter leaf petiole than diploid plants; however larger petiole diameter than diploids. The dry matter content of tetraploid leaves was slightly higher than that of diploid leaves, but this difference was not significant. The chloroplast number and stomata complex size increased with the increase in ploidy level. Among the functional metabolites, phenolic compounds were much more abundant in tetraploids than in diploids, and the levels of senkyunolide F and phthalide were also higher in the roots of tetraploids than in those of diploids. Among the heat stress-responsive genes, *CoCP* was downregulated upon tetraploidization, implying that tetraploid plants can be more resistant to high temperatures. We found that the phenolic content of *C. officinale* plants increased following the induction of tetraploidy with oryzalin. It is considered the obtained tetraploids can be used in the breeding of new cultivars with an increased content of functional metabolites.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11081561/s1, Figure S1: Acclimatized plants of *Cnidium officinale*. Table S1: List of primers used for the expression analysis of heat stress-responsive genes in *C. officinale*.

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